CELLULOLYTIC AND XYLANOLYTIC GUT ENZYME ACTIVITY PATTERNS IN MAJOR SUBTERRANEAN TERMITE PESTS

By

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To my family, friends and teachers, without whom this degree would not have been possible

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Cellulolytic and xylanolytic termite gut carbohydrolases were assayed for two major subterranean termite pest species; *Coptotermes formosanus* (Shiraki) and *Reticulitermes flavipes* (Kollar). Carbohydrolase assays were optimized for buffer and pH. This led to the selection of a 0.1 M pH 5.5 sodium acetate buffer for endoglucanase and xylanase assays and a 0.1M pH 6.5 sodium phosphate buffer for exoglucanase, beta-glucosidase, and beta-xylosidase assays.

Endoglucanase activity was found to be mainly localized in the foregut and hindgut of both species, reflecting both endogenous and symbiont enzyme origins. Beta-glucosidase activity was found throughout the gut in both species, but was most prevalent in the midgut or hindgut. Beta-xylosidase activity was mostly restricted to the hindgut and was lowest in the foregut in both species. Exoglucanase and xylanase activities were almost entirely confined to the hindgut in both species.

C. formosanus workers were found to have a more active array of gut carbohydrolases, particularly exoglucanase and beta-glucosidase, than *R. flavipes*. This was consistent with increased metabolic demands from more aggressive foraging and a larger soldier ratio within *C. formosanus* colonies. Soldier carbohydrolase activities in both of these species were generally

lower than worker carbohydrolase activities, consistent with a caste incapable of feeding itself. *C. formosanus* soldier carbohydrolase activities were especially low, due to a reduced gut size to make room in the soldier abdomen for an enlarged frontal gland.

Cellulolytic enzyme activity levels were found to significantly change on differing diets for both *C. formosanus* and *R. flavipes* workers. Xylanolytic enzyme activities were found to change in a manner consistent with dietary xylan content in both species, being increased on diets containing more xylan. This indicates some adaptability to dietary xylan content in both species. The presence of significant xylanolytic enzyme activities in termites kept on diets without xylan indicates constitutive production of xylanases by symbionts capable of subsisting wholly on cellulose. Alternatively, it is possible that the xylanolytic enzymes seen on xylan-free diets are bifunctional enzymes that also have cellulolytic activities.

CHAPTER 1
INTRODUCTION

Two of the most economically significant pest termites in North America are the eastern subterranean termite, *Reticulitermes flavipes* (Kollar), and the Formosan subterranean termite, *Coptotermes formosanus* (Shiraki). One thing that distinguishes termites from the majority of other insect pests is their ability to digest wood, and their consequent ability to cause significant structural damage to most types of buildings. This capacity for wood digestion is based upon a complex array of enzymes, mainly carbohydrolases, which allow termites to digest cellulose and hemicelluloses such as xylan. In all lower termites, including subterranean termites, some of these enzymes are endogenously produced, while others are produced by symbiotic flagellates found in the physiologically specialized, expanded hindgut.

Perhaps more important than their status as structural pests is the enormous impact that termites have on terrestrial ecological processes. Termites are among the major terrestrial recyclers of cellulose, one of the most abundant substances in nature. Finding a way to disrupt or harness the enzymatic mechanisms that allow subterranean termites to digest cellulose and other polysaccharides may be a key to discovering new approaches in termite control or industrial methods to generate alternative fuels from cellulosic materials. As such, an understanding of the termite digestive system has significant applications.

The first objective in this dissertation is the optimization of the chosen carbohydrolase assays, which will be dealt with in Chapter 3. The next objectives concern the characterization of the activity levels of the various cellulolytic and xylanolytic gut enzymes, which will be dealt with in Chapter 4. The patterns of activity for each carbohydrolase along the gut must be determined, as well as the differences seen between the worker and soldier castes for *R. flavipes* and *C. formosanus*. This will provide insight into the mechanisms of cellulose and xylan

digestion for these two species of termites, allowing a better understanding of the interaction between the termites and their symbionts, as well as the interaction between the termite workers and soldiers. This understanding may facilitate the development of successful termite control strategies, or the discovery of novel enzymes for industrial polysaccharide degradation.

Once these basic patterns of activity have been determined, the next main objective is the exploration of how these patterns are affected by changes in termite diet, and whether the termites can adapt to their diets. Changes in patterns of *R. flavipes* worker cellulolytic and xylanolytic enzyme activities will be addressed in Chapter 5 and Chapter 6, respectively. Changes in patterns of *C. formosanus* worker cellulolytic and xylanolytic enzyme activities will be addressed in Chapter 7 and Chapter 8, respectively. In particular, the changes in xylanolytic enzyme activities across diets of varying xylan content will provide insight into the adaptive abilities of these termites with respect to diet. In turn, this will provide some idea of how termites may adapt to attempts to inhibit their digestive processes. Furthermore, any understanding of termite adaptation to diet would be useful if whole, living termites were to be incorporated into industrial processes involving cellulose and xylan degradation.

The final aim of this dissertation is to facilitate an understanding of lower termite digestive processes, both in terms of enzyme activity patterns and adaptation to different diets. This understanding may be used for development of control strategies, or for the harnessing of these termites and their enzymes for the removal of cellulosic waste and the generation of alternative fuels.

CHAPTER 2
LITERATURE REVIEW

Wood Molecular Structure

The majority of wood consists of polysaccharides and lignin. The dominant polysaccharide is cellulose, which exists within the plant cell walls as microfibers roughly 5 nm in diameter (Astley et al. 1997). Each fiber consists of multiple chains of cellulose, which are largely aligned in tight, crystalline configurations. These configurations make this molecule particularly difficult to digest. There are amorphous regions as well, where the configuration is disrupted. It is generally thought that these amorphous regions are caused by interactions with other molecules in the cell walls. Each cell wall has several layers, called lamellae, roughly one microfiber thick. The microfibers in each layer are roughly parallel, but their orientation changes between layers.

The surrounding matrix of the cell wall contains other polysaccharides, predominantly hemicelluloses, and lignin, a non-repeating aromatic polymer (Whistler and Chen 1991). Unlike cellulose, which is made up of beta-glucose units, hemicelluloses contain a variety of sugar subunits. In addition, hemicellulases are much shorter, only a few hundred units long, with short side chains (Timell 1964). These traits mean that they do not exist as microfibers, but instead form part of the matrix surrounding the cellulose microfibers. In some models, the ends of the hemicellulose chains are aligned with the cellulose microfibers while the bulk of the chains form cross-bridges between the microfibers (Whistler and Chen 1991). The majority of hemicelluloses in hardwoods consist of modified polymers of xylose, commonly called xylans. Softwoods contain other hemicelluloses in addition to xylans, which occur in roughly comparable amounts. These are known as glucomannans and galactoglucomannans, and they are mainly polymers of mannose and glucose.

Lignin is a highly complex, cross-linked polymer. It is fundamentally different from the polysaccharides, as it is a polymer of various hydroxycinnamyl alcohols. In addition, the overall structure is far more amorphous and less predictable than that of polysaccharides. A limited degree of degradation of aromatic monomers has been demonstrated in the hindgut of lower termites (Brune et al. 1995). However, it has been difficult to obtain evidence of degradation of polymerized lignin in termites. Based on the high lignin content of fecal material and nest carton in multiple termite genera (Mishra and Sen-Sarma 1979), it may be assumed that lignin degradation is limited in most termites, and certainly in lower termites. As a result, the relevance of lignin to termite digestion is fairly limited and this paper will focus on the degradation of cellulose and hemicelluloses.

Termite Taxonomy

The order Isoptera is generally divided into six families. These are the Hodotermitidae, Termopsidae, Mastotermitidae, Kalotermitidae, Rhinotermitidae and Termitidae. Termitidae contains the so-called "higher" termites, and none of the termites in this family have symbiotic flagellates in their hindguts. The remaining five families of termites are commonly called "lower" termites, and they are all symbiotically associated with flagellates in their hindguts in addition to the more ubiquitous bacteria and fungi found in termite guts.

The Hodotermitidae and Termopsidae are most often found in the tropics and are often regarded as relatively primitive termites (Eggleton 2001). The Mastotermitidae, generally considered to be the basal group, consists of the Australian species *Mastotermes darwiniensis*.

Kalotermitidae and Rhinotermitidae are the two termite families most commonly encountered in the temperate zones. The Kalotermitidae include the dampwood termites and the drywood termites. They typically live in close association with their food source, often nesting

within the wood they consume (Noirot and Darlington 2002). They are also considered to be more primitive in their behavior and morphology than the Rhinotermitidae.

Rhinotermitidae are commonly called subterranean termites. They are aptly named, as most species live within the soil and seek out dead wood and other similar food sources. These termites are typically the worst structural pests in buildings (Potter 2004). A large number of studies on termite digestion and termite symbiosis have been carried out on genera from this family. The two genera of particular interest are *Coptotermes* and *Reticulitermes*.

Termite Digestive System Anatomy

As with other insects, the termite gut may be divided into three major regions: the foregut, the midgut and the hindgut. The foregut is composed of the salivary glands, the crop and the proventriculus. The crop and proventriculus are lined with cuticle, which is shed at each molt along with the exoskeleton. The crop in termites is relatively small, but the proventriculus is fairly well-muscled and armed with scraping teeth and ridges on its inner surface (Noirot and Noirot-Timothee 1969).

The second region is the midgut. This structure is not lined with cuticle, but instead has epithelial cells on the inner surface responsible for enzyme secretion and nutrient absorption. As with many insects, there is a peritrophic membrane present in the midgut, though it does not persist into the hindgut. The midgut of termites is fairly small, often without gastric caecae. This is mainly due to the degree of digestion that takes place in the hindgut.

At the junction between the midgut and the hindgut, there are several malpighian tubules which serve to excrete nitrogenous waste. It is thought by some that this waste may be recycled based on the presence of uricolytic bacteria (Potrikus and Breznak 1980).

Symbiont Taxonomy and Anatomy

The cellulolytic system of lower termites has been extensively studied since Cleveland's early work on the hindgut symbionts (Cleveland 1924). The hindgut contains both protozoa and bacteria. These protozoa are basal flagellates. They lack mitochondria and so are anaerobic organisms, but some possess hydrogenosomes (Cavalier-Smith 1993). Many have vacuoles for engulfing the wood fragments that enter the hindgut (Cleveland 1925). Cellulolytic bacteria have been documented in *Zootermopsis angusticolis* (Wenzel et al. 2002). However, the bulk of the evidence obtained thus far suggests that the protozoa are the major sources of cellulolytic enzymes. Removal of protozoa from *Reticulitermes speratus* has resulted in a significant loss of both cellulolytic and xylanolytic activity (Inoue et al. 1997).

The taxonomy of these flagellates is continually being revised as more powerful molecular techniques become available. As it is beyond the scope of this paper to provide a detailed phylogenetic analysis, only a basic overview of current groupings will be provided.

The symbiotic flagellates may be broadly divided into three major classes. The first, class Anaeromonadea, consists of organisms often referred to as Oxymonads. Most of these protozoa have some form of structure which allows them to attach to surfaces, most often the wall of the termite hindgut (Moriya et al. 2003). They tend to have a long, thin morphology and few flagellae. They are sometimes grouped within the Kingdom Archezoa, and possess neither mitochondria nor hydrogenosomes (Cavalier-Smith 1993).

The second and third major classes may be grouped within the phylum Parabasalia, a group which possesses hydrogenosomes, but not mitochondria. The first of these two classes is the class Trichomonadea. This is a very diverse group, containing families which include Monocercomonadidae, Trichomonadidae, Devescovinidae and Calonymphidae. The Monocercomonadidae and Trichomonadidae are generally small flagellates, and it is doubtful

that they participate in cellulose digestion. The two families can be separated by the presence of a recumbent flagellum in the Trichomonadidae.

The Devescovinidae and Calonymphidae appear to sort out as one group in recent phylogenetic studies (Delgado-Viscogliosi et al. 2000, Ohkuma et al. 2000). The flagellates in these two families tend to be larger, often with many flagella, and in the case of Calonymphidae, many nuclei as well.

In addition to these families, the class Trichomonadea also contains the orders Lophomonadida and the Spirotrichonymphida. In terms of overall structure, the Spirotrichonymphida are fairly similar to the flagellates in the third class, and have been grouped with them in some earlier taxonomies.

The third class is the class Trichonymphea. These flagellates are typically large and complex, with many flagella. They are widely distributed among the termites, with *Trichonympha* being one of the more well-known genera.

Symbiont Assemblages in Termite Hindguts

The species of flagellates vary with each species of termites. A particularly thorough documentation of these associations was undertaken by Yamin (1979). This study included several species of termites from the families Kalotermitidae and Rhinotermitidae.

Upon examination, certain patterns emerge. Most clearly evident is the presence of the families Devescovinidae and Calonymphidae in the Kalotermitidae, but not in either of the other termite families. Also noteworthy is the presence of Oxymonad flagellates in the family Pyrsonymphidae being restricted to the Rhinotermitidae. The order Spirotrichonymphida appears to be far more prevalent in the Rhinotermitidae than in the Kalotermitidae. However, the genus *Trichonympha* is present in at least one species from all three of the termite families.

In summary, each family of termites appears to have its own set of flagellate families associated with it. Some flagellate groups are more widespread among the termites. The overall pattern appears to be consistent with the coevolution of termite and flagellate lineages, with the possibility of occasional horizontal transfer.

Termite Carbohydrolase Types and Locations

Before comparing these enzymes between termites and other creatures, it is necessary to define certain aspects of their structure and function. First of all, cellulases may be divided into three varieties, based on their mode of action (Breznak and Brune 1994). Any highly cellulolytic organism requires all three of these enzymes from one source or another.

The first class of cellulases consists of exoglucanases (EC 3.2.1.91). These enzymes are processive, binding to the end of a cellulose chain and moving along its length, breaking it down along the way. Most exoglucanases are cellobiohydrolases, meaning that they reduce cellulose to cellobiose, a disaccharide of beta-glucose. These enzymes are most active against crystalline cellulose, where the cellulose chains are arranged in a tightly bound parallel configuration and held in place by hydrogen bonding.

The second class of cellulases consists of the endoglucanases (EC 3.2.1.4). These enzymes bind anywhere along the cellulose chain and break it up randomly, eventually reducing it to cellodextrins, oligosaccharides of beta-glucose. The endoglucanases are most active against amorphous cellulose, where the chains are not arranged in any particular configuration.

The third class of cellulases is the beta-glucosidases (EC 3.2.1.21). These enzymes break cellodextrins down into glucose. They are the final enzymes in the process of glucose liberation from cellulose.

Aside from cellulose, the major polysaccharides in wood are hemicelluloses. The most well known and thoroughly studied of these hemicelluloses is xylan. It is a polymer of xylose, a

pentose sugar, with side molecules of 4-O-methylglucuronic acid. The xylose molecules may have a varied degree of acetylation. There is also the possibility of arabinose being incorporated into the polymer as an additional variety of side group. Since xylan is far more complex than cellulose, there are several more enzymes required for its complete digestion. However, the major enzymes of concern are often simply known as xylanases (EC 3.2.1.8). They hydrolyze the bonds between xylose units in the polymer, much in the same fashion as endoglucanases hydrolyze cellulose. In addition, there are beta-xylosidases (EC 3.2.1.37) which break down the resulting oligosaccharides.

Functionally, the termite appears to have its enzymes spatially segregated. The amorphous cellulose is attacked first in the foregut and midgut. The hindgut symbionts then appear to digest the crystalline cellulose and the bulk of the hemicelluloses (Hogan et al. 1988, Mishra 1991). The byproducts of this digestion, mainly acetate, then diffuse out into the termite tissues.

The xylanases of lower termites have not been specifically characterized. However, the bulk of xylanase and beta-xylosidase activity has been shown to be located in the hindgut in multiple cases (Azuma et al. 1993, Inoue et al. 1997). Mannanase activity appears to also be mainly located in the hindgut.

Termite Carbohydrolase Structural and Functional Characteristics

A vital structural aspect, in the case of cellulases at least, is cleft vs. tunnel geometry. The catalytic site in exoglucanses is located within a tunnel in the enzyme. This allows the cellulose chain to travel through the enzyme much as a thread may travel through the eye of a needle. Of course, this particular needle has a guillotine incorporated into its structure. The cleft geometry, found in endoglucanases, has the catalytic site located in a groove along the enzyme surface.

This allows the enzyme to effectively clamp onto any available location along the cellulose chain.

The next structural aspect concerns retaining enzymes and inverting enzymes. Using cellulases as an example, an inverting cellulase inserts the hydroxyl group in the alpha position, opposite to the bond configuration in the cellulose chain. A retaining cellulase inserts a hydroxyl group in the beta configuration, the same configuration as the bond in the cellulose chain.

Inverting cellulases work in a single step (Fig 1). The catalytic nucleophile removes a hydrogen from a water molecule. The remaining hydroxide is bonded to the sugar molecule. At the same time, the proton donor donates its hydrogen, allowing the oxygen bond between sugars to be broken, separating off as a hydroxyl group on the other sugar. The remaining hydrogen on the sugar is then brought back to the proton donor.

Retaining cellulases work in two steps, commonly referred to as a double displacement mechanism (Davies et al. 1998). First, the catalytic nucleophile forms a covalent bond to the substrate, while the proton donor allows the separation of the leaving group (Fig 2). In the second step, a water molecule interacts with the bonded substrate, donating a hydroxyl group to the substrate and effectively breaking the bond between it and the nucleophilic residue. The remaining proton is accepted by the proton donor, restoring the enzyme to its original configuration.

Another structural consideration is the nature of the proton donor and the catalytic nucleophile. Glycosyl hydrolases function by placing the polysaccharide between two acidic amino acid residues. If these residues are fairly far apart, there is room for a water molecule to be incorporated into the configuration. This results in a direct trade of bonds, leading to an inverting mechanism. If the residues are closer together, there is no room for a water molecule

in an occupied site, and so the sugar becomes temporarily bonded to the nucleophilic residue. After this step, there is room for a water molecule to enter the site, and it is attached in the beta configuration. This leads to a retaining mechanism.

There are only two amino acids with acidic side chains. These are aspartic acid and glutamic acid. Each of these has a carboxyl group at the end of a short carbon chain. The glutamic acid chain is one carbon longer than that of the aspartic acid, and so leaves less space. As a result, nearly every carbohydrolase with a glutamic acid in each of the two key positions is a retaining enzyme, while nearly every one with aspartic acid in each position is an inverting enzyme. In enzymes with one of each residue, the glutamic acid is nearly always the proton donor.

A final consideration, particularly applicable to the exoglucanases, is the positioning of aromatic residues. Aromatic residues at certain locations on the enzyme surface allow the cellulose chain to slide more easily through the catalytic site. This is often noticeable as a row of tryptophan residues located within the tunnel of exoglucanases (Parsiegla et al. 2000). While there are other aspects of structure, such as disulfide bonds, alpha helices and beta sheets, these are generally more significant to enzyme grouping and relatedness.

So far, all of the endogenous cellulases of lower termites have been in glycosyl hydrolase family 9 (Watanabe et al. 1998, Zhou et al. 2007). The enzymes in this family have aspartic acid as a catalytic nucleophile and glutamic acid as a proton donor. They are inverting cellulases with structures consisting mainly of alpha helices. The catalytic site follows the cleft configuration, allowing the endoglucanases to bind anywhere along the cellulose chain. In addition, there appears to be a high density of aromatic residues on the enzyme surface near the catalytic residues.

The second family found in lower termites, family 7, contains both exoglucanases and endoglucanases. Their structures are dominated by beta sheets and are rich in disulfide linkages. They have glutamic acid at both positions of the catalytic site, and are retaining cellulases. Unlike the other cellulases, the exoglucanases in this family have their catalytic sites located in tunnels. In addition, they have several tryptophan residues located along the length of the tunnel. The exoglucanases generally have four tryptophan residues in a row, while the endoglucanases typically have two or three tryptophan residues along the length of the cleft.

So far, these enzymes have been found mainly in the *Coptotermes* symbionts *Pseudotrichonympha grassii* and *Holomastigotoides mirabile*. The majority of sequences recovered have indicated an endoglucanase activity (Watanabe et al. 2002). However, there have been some promising indications of exoglucanases as well (Nakashima et al. 2002).

Glycosyl hydrolase family 45 consists of endoglucanases, which have aspartic acid in both positions in the catalytic site. As may be expected, these are inverting cellulases. Like the family 7 enzymes, their structures are dominated by beta sheets and are rich in disulfide linkages. Like the termite cellulases, they have an open cleft structure. However, they differ significantly from these enzymes in that they have very few alpha helices, and instead consist mainly of beta sheets. In addition, they have several disulfide linkages, while family 9 cellulases tend to have only one or two. Enzymes in family 45 have been found in the protozoan symbionts of *Reticulitermes speratus* (Ohtoko et al., 2000). Two of the clones were localized to *Trichonympha agilis* and *Teranympha mirabilis*. Both of these are large parabasalian flagellates.

Glycosyl hydrolase family 5 contains of exoglucanases, endoglucanases, xylanases, mannanases, and beta-mannosidases. These enzymes have glutamic acid in both positions in the catalytic site and follow a retaining mechanism. These enzymes consist of a combination of

alpha helices and beta sheets. These enzymes have been characterized from *Coptotermes formosanus* symbionts (Inoue et al. 2005).

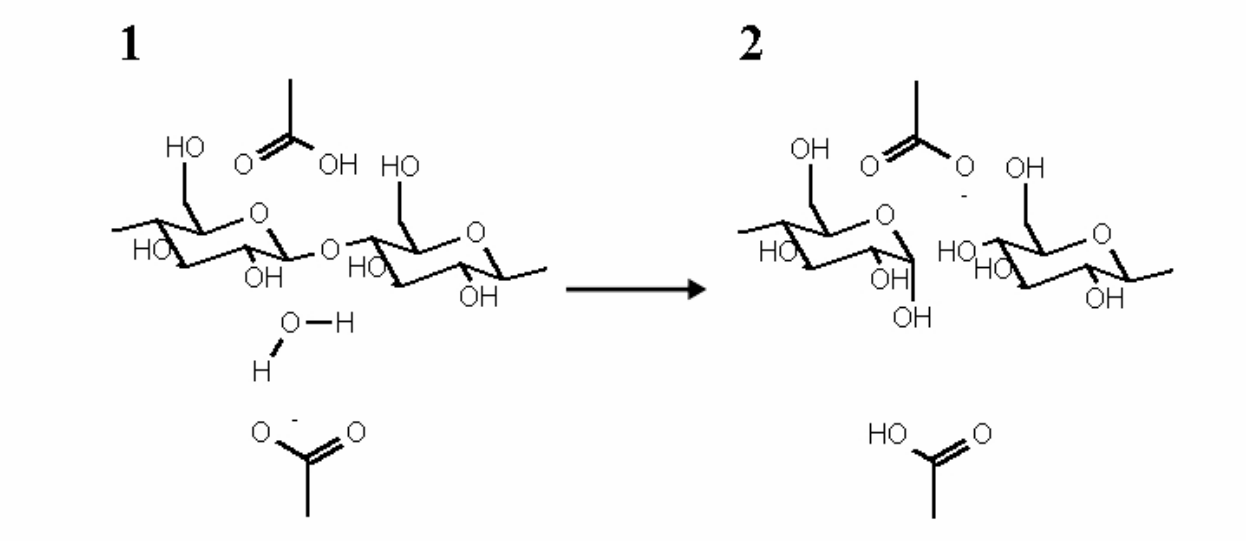

Figure 2-1. Inverting cellulase mechanism.

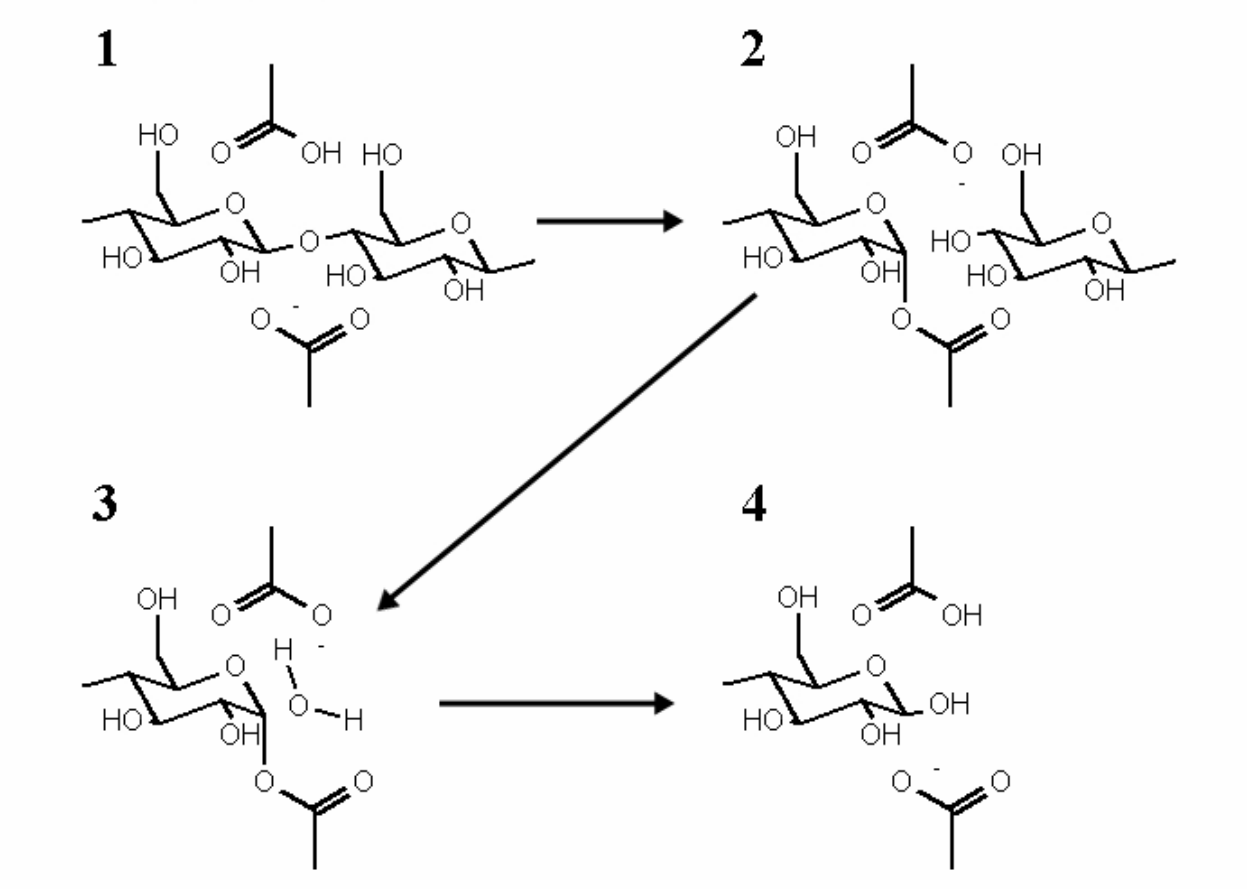

Figure 2-2. Retaining cellulase mechanism.

CHAPTER 3
BUFFER AND PH OPTIMIZATION FOR TERMITE CARBOHYDROLASE ASSAYS

Introduction

The gut of the typical subterranean termite consists of a foregut with a small crop and a proventriculus, a fairly simple midgut and a greatly expanded hindgut containing several species of symbiotic protozoa, fungi, bacteria, and archaea (Yamin 1979, Lewis and Forschler 2004). A pair of salivary glands is also present, emptying into the foregut anterior to the crop. To effectively digest wood, a recalcitrant and nutritionally poor substrate, termites like *R. flavipes* have evolved an array of enzymes. Some carbohydrolases characterized from the Rhinotermitidae have been shown to be endogenous (Watanabe et al. 1998), while others are produced by microbial symbionts (Nakashima et al. 2002a, Watanabe et al. 2002, Ohtoko et al. 2000).

In most prior studies, the gut carbohydrolases of subterranean termites have been assayed using 0.1 M sodium acetate buffer, pH 5.5, as a standard buffer. Substrates have either been based upon the generation of reducing sugars from specific polymers or the hydrolysis of smaller molecules consisting of a monosaccharide or oligosaccharide complexed with p-nitrophenol, a molecule that is yellow when isolated and clear when complexed with a sugar. Prior to using these substrates for carbohydrolase activity evaluations, it was deemed necessary to evaluate a range of possible pH values and buffers to determine whether the standard sodium acetate buffer would produce optimal results.

Our objectives were to determine 1) the optimal buffer types for p-nitrophenol generating substrate-based assays, 2) the optimal buffer types for reducing sugar-dependent assays, 3) the optimal pH values for p-nitrophenol generating substrate-based assays for each major gut region, and 4) the optimal pH values for reducing sugar-dependent assays for each major gut region.

Materials and Methods

Termite Collection, Dissection, and Enzyme Extraction

Reticulitermes flavipes (Kollar) termites were field collected in termite traps consisting of a polyvinyl chloride bucket (20 cm high by 20 cm diam.; Item # 811192-4, Ventura Packaging Inc., Monroeville, Ohio) with 11 holes drilled in the sides and base (3 cm diam.) placed vertically in the ground to a depth of roughly 19 cm and covered with a PVC lid. Three rolls of single-faced corrugated cardboard (10 cm diam. by 20 cm length) were placed into the tube side by side as a food source. Termites were collected from the trap by removal of cardboard rolls, separated from the cardboard, immediately frozen, and kept at -80^{o}C until dissection.

Termites were removed from the freezer and kept on ice until use. Termites were either homogenized intact or dissected before homogenization. In the case of dissections, each termite's gut was removed intact and then separated into three regions: foregut (and salivary glands), midgut and hindgut. The three gut regions were placed into separate 1.5-mL microcentrifuge tubes (Eppendorf, Westbury, NY) containing the appropriate buffer, and kept on ice.

Enzymes were extracted using a method adapted from Inoue et al. (1997). Whole termites or gut contents of each microcentrifuge tube were placed in a 2-mL Tenbroeck glass tissue grinder and manually homogenized on ice. The homogenates were then centrifuged at 14,000 rpm at 4^{o}C for 15 min. The supernatants were collected, frozen, and kept at -80^{o}C until use in the enzyme assays.

Buffer Optimization Assays

Five buffers were prepared at 0.1M in all cases. The buffers and their pH values were as follows: sodium acetate (4.0, 4.5, 5.0, 5.5), sodium phosphate (6.0, 6.5, 7.0, 7.5), MES (methylethyl sulfide; 5.5, 6.0, 6.5), bis-tris (2-[bis(2-hydroxyethyl)imino]-2-(hydroxymethyl)-

1,3-propanediol; 6.0, 6.5, 7.0), and PIPES (piperazine-N,N-bis(2-ethanesulfonic acid); 6.5, 7.0, 7.5). Two substrate solutions were prepared for each of the buffer/pH combinations. The first was 2% carboxymethylcellulose (CMC; Sigma-Aldrich) and the second was 4 mM pNPG. Whole termite extracts were prepared at a concentration of 50 termites/mL.

Endoglucanase assays were conducted using a method adapted from Han et al. (1995). Assays were conducted in clear, flat-bottomed 96-well microplates. In each well, 10 μL of tissue extract was combined with 90 μL of CMC solution. Only the wells more than 2 wells away from the edge of the microplate were used for these assays. Perimeter wells were filled with deionized water (200 μL per well) to add temperature stability and consistency during boiling. Identical plates were prepared as controls.

The enzyme and substrate solutions were allowed to react for 10 min at 23^{o}C. DNSA solution (100 μL), consisting of 1% 3,5-dinitrosalicylic acid (DNSA), 0.4M sodium hydroxide and 30% sodium potassium tartrate, was then added to each enzyme/substrate well of the control plates. After an additional 60 min, the DNSA solution was added in the same manner to the sample plates. In both cases, immediately after addition of DNSA solution, the microplate was placed in boiling water for 10 min to induce an oxidation/reduction reaction with the DNSA, resulting in a color change. All plates were placed on ice immediately after boiling to cool for 15 min. Each microplate was read at 540 nm immediately after cooling using a μQuant Universal Microplate Spectrophotometer (Bio-Tek Instruments, Winooski, VT). Standards were generated using dilutions of glucose in sodium acetate buffer (0.1M, pH 5.5) combined with equal volumes of DNSA solution. Standards were boiled, cooled, and read as described above.

Beta-glucosidase assays were conducted using a method adapted from Han et al. (1995). A solution of 4 mM p-nitrophenyl-β-D-glucopyranoside (pNPG; Sigma-Aldrich) was prepared in

0.1 M sodium phosphate buffer, pH 6.5. Assays were conducted in clear 96-well microplates. In each well, 10 μL of tissue extract was combined with 90 μL of pNPG solution. The reaction was allowed to proceed for 10 min before being placed in a μQuant Universal Microplate Spectrophotometer (Bio-Tek Instruments, Winooski, VT). Absorbance was read at 420 nm every 2 min for 30 min at 23°C. Mean velocities (mOd/s) were recorded. Standards were generated using dilutions of p-nitrophenol.

Gut Region Assays Across PH

Assays were conducted using a method adapted from Han et al. (1995). For endoglucanase assays, both sodium acetate and sodium phosphate CMC solutions were prepared as described above for the buffer optimization assays. Sodium acetate solutions were prepared at pH values of 4, 4.5, 5, and 5.5. Sodium phosphate solutions were prepared at pH values of 6, 6.5, 7, and 7.5. For xylanase assays, solutions consisted of 0.5% beechwood xylan (>90% xylose residues; Sigma-Aldrich) prepared in the same sodium acetate and sodium phosphate buffers. The solutions were boiled approximately 30 min, until xylan particles were no longer visible. The solutions were then centrifuged at 2,500 rpm for 5 min at 23°C and the supernatants were used as the final xylan substrate solutions. Termite tissues were extracted into the eight pH buffers described above. Final tissue extracts consisted of foregut, midgut, and hindgut at a concentration of 50 termite equivalents/mL in all cases.

The assays were conducted as described above for endoglucanase buffer optimization assays. In each well, 10 μL of tissue extract was combined with 90 μl of CMC or xylan solution. CMC solutions were allowed to react for 70 min at 23°C. Xylan solutions were allowed to react for 35 min at 23°C. Control plates were prepared in the same manner, but were allowed to react 10 min for endoglucanase assays or 5 min before xylanase assays before boiling. Standards were

generated using dilutions of glucose for endoglucanase assays and dilutions of xylose for xylanase assays. In both cases the standards were prepared in sodium acetate buffer (0.1M, pH 5.5), combined with equal volumes of DNSA solution, boiled, cooled, and read at 540 nm.

Exoglucanase, beta-glucosidase, and beta-xylosidase assays were conducted using a method adapted from Han et al. (1995). Solutions of 4 mM p-nitrophenyl-β-D-cellobioside (pNPC; Sigma-Aldrich), 4 mM p-nitrophenyl-β-D-glucopyranoside (pNPG; Sigma-Aldrich), and 4 mM p-nitrophenyl-β-D-xylopyranoioside (pNPX; Sigma-Aldrich) were prepared as described above for the buffer optimization assays. Sodium acetate solutions were prepared at pH values of 4, 4.5, 5, and 5.5. Sodium phosphate solutions were prepared at pH values of 6, 6.5, 7, and 7.5. Assays were conducted in clear 96-well microplates. In each well, 10 μL of tissue extract was combined with 90 μL of pNPC solution. The reaction was allowed to proceed for 10 min before being placed in a μQuant Universal Microplate Spectrophotometer (Bio-Tek Instruments, Winooski, VT). Absorbance was read at 420 nm every 2 min for 30 min at 23^{o}C. Mean velocities (mOd/s) were recorded. Standards were generated using dilutions of p-nitrophenol.

Data Analysis

The buffer optimization assays were set up as one-factor designs with a single homogenate for each buffer/pH combination and three technical replicates. The pH assays across gut region were set up as one-factor designs with a single homogenate for each pH and eight technical replicates for each gut region.

For the endoglucanase and xylanase assays, the following formula was used to calculate specific activities;

$$SA = C_S[(A-A_0)/t]/N_T$$

where: SA = specific activity (nmol reducing sugar/termite equivalent/minute), A = absorbance (Od) after 35 min reaction, A_0 = absorbance (Od) for the corresponding control after 5 min reaction, t = time (min), C_S = the coefficient derived from the standard (nmol reducing sugar/mOd), and N_T = the number of termite equivalents per sample.

For the beta-glucosidase and exoglucanase assays, the following formula was used to calculate specific activities;

$$SA = 60C_SV_A/N_T$$

where: SA = specific activity (nmol p-nitrophenol/termite equivalent/min), V_A = mean velocity of absorbance change (mOd/s), C_S = the coefficient derived from the standard (nmol p-nitrophenol/mOd), and N_T = the number of termite equivalents per sample.

For the time/concentration analyses, the mean specific activities were calculated for each combination of time and concentration. The variance in mean specific activity was then calculated among all times for each concentration and among all concentrations for each time. For the remaining assays, means and standard errors were calculated for each enzyme specific activity at each combination of pH and buffer, pH and gut region, or concentration and temperature.

Results

Buffer Optimization Assays

Beta-glucosidase activities increased with pH from 5.5 to 7 (Figure 3-1). Activities were negligible below pH 5.5. MES buffer caused a greater activity level than phosphate or PIPES buffers at pH 6.5. Bis-Tris caused significantly lower activities across its functional pH range. Activities on PIPES buffer were slightly lower than those for phosphate buffer across its functional pH range. With the exception of the bis-tris buffer, the greatest changes in beta-glucosidase activity over pH were seen between pH 5.5 and 7.

Gut Region pH Optimization Assays

Foregut endoglucanase activity was greatest at a pH value of 6.5, with a smaller activity peak at 5.5 (Figure 3-2). Midgut and hindgut endoglucanase activities were greatest at a pH of 5.5. Hindgut activity was significantly lower at pH values above 5.5 and below 5. Changes in foregut endoglucanase activities were high throughout the pH range tested, while the greatest change in hindgut endoglucanase activities was the decline in activity from a pH of 5.5 to 6.

Foregut and midgut beta-glucosidase activities increased with pH up to pH 6.5, then declined at higher pH values (Figure 3-3). Beta-glucosidase activities in the hindgut extract showed a steady increase with pH up to pH 7. All activities were negligible below pH 5.5. Changes in foregut and midgut beta-glucosidase activities were greatest between pH 6 and 6.5. Changes in hindgut beta-glucosidase activities were greatest between pH 5.5 and 7.

Hindgut exoglucanase activities were consistently greater with increasing pH value, following a sigmoid curve (Figure 3-4). The greatest differences in activity were between pH 5.5 and 7. Activity was negligible below a pH of 6.

Hindgut xylanase activity was greatest at pH 4.5 (Figure 3-5). However, there was little difference in hindgut xylanase activities from pH 4 to 5.5. The greatest change in hindgut xylanase activity was seen from a pH of 5.5 to 7.5.

Discussion

Activity Magnitudes

Three qualities of enzymatic activity are of particular interest when selecting buffers for termite carbohydrolase assays. The first is the magnitude of activity yielded by a given buffer. Experiments with a goal of quantifying enzymatic activity may underestimate actual activities if the buffer does not maximize in vitro enzyme activity levels. Lower levels of actual activity may

be missed altogether with some buffers. For comparative studies, lower overall activities may make differences in activity levels more difficult to distinguish.

For pNPG-based beta-glucosidase assays, the highest activities ranged from 6.5 to 7.5 on MES, PIPES, and sodium phosphate buffers. Sodium acetate and bis-tris buffers yielded far lower activities. In the past, investigators have used pH 5.5 sodium acetate buffer for pNPG-based termite beta-glucosidase assays (Inoue et al. 1997). This may have caused beta-glucosidase activity to be underestimated within the termite.

For symbiont exoglucanase from the hindgut, activity was greatest on pH 7.5 sodium phosphate buffer. The activity of symbiont xylanase from the hindgut was greatest on pH 4.5 sodium acetate buffer, and nearly as high on all sodium acetate buffers tested.

Activity Changes with pH

The second quality of interest is the rate of change in activity with changing pH. If the selected buffer is in a region of high change, smaller changes in buffer pH will cause greater changes in activity levels. This can increase variation in experimental results and, in extreme cases, cause difficulties with experimental repeatability. Therefore, the optimal buffer should maximize activity within a region of minimal change in activity with change in pH.

Exoglucanase activity declined sharply below pH 6.5 sodium phosphate buffer and was nearly nonexistent on sodium acetate buffer. However, xylanase activity declined sharply with increasing pH on sodium phosphate buffer.

Differences in Gut Regions

Different activity maxima were seen for different gut regions in beta-glucosidase and endoglucanase assays. In addition, pH ranges with the highest changes in activity differed between gut regions for beta-glucosidase and endoglucanase assays.

In the case of beta-glucosidases, the differences were mainly seen between the endogenous enzymes from the foregut and midgut, and the symbiont enzymes from the hindgut. In contrast, the differences in the endoglucanase assays were mainly seen between the foregut and the hindgut, with midgut activity levels far lower than those seen in the other gut regions. Because of these differences, the optimal buffers for carbohydrolase assays may not be the same for endogenous (termite) and symbiont produced enzymes.

Conclusions

Based upon our experimental data, the optimal pH buffer for predicting overall xylanase or endoglucanase activities is pH 5.5 sodium acetate buffer, which is the standard buffer used in past termite carbohydrolase assays (Hogan et al. 1988, Inoue et al. 1997). Although endoglucanase activity shifts considerably from pH 5.5 to pH 6, the proximity of pH 5.5 sodium acetate buffer to this shift presents no concern. In this case, the observed shift is not only between two pH values, but also occurs in a shift between sodium acetate and sodium phosphate buffers.

Previous experiments have generally used pH 5.5 sodium acetate buffer as a universal buffer for termite carbohydrolase assays. Based on our p-nitrophenol-generating assays, the optimal pH buffer for predicting overall beta-glucosidase activity using pNPG is pH 6.5 sodium phosphate buffer. Both beta-glucosidase and exoglucanase activities on pH 6.5 sodium phosphate buffer are roughly 10-fold higher than on pH 5.5 sodium acetate buffer. The optimal pH buffer for predicting exoglucanase activity or hindgut beta-glucosidase activity is pH 7.5 sodium phosphate buffer. Hindgut exoglucanase activity is roughly 15-fold higher and beta-glucosidase activity roughly 20-fold higher on pH 7.5 buffer than on pH 5.5 buffer. We believe that prior pNPG or pNPC-based assays using pH 5.5 sodium acetate buffer may have underestimated actual beta-glucosidase or exoglucanase activity levels within the termites.

Based upon these findings, we intend to conduct pNPC, pNPG, and pNPX-based assays using 0.1 M sodium phosphate buffer, pH 6.5, in the following chapters. We intend to conduct DNSA-based assays using 0.1 M sodium acetate buffer, pH 5.5.

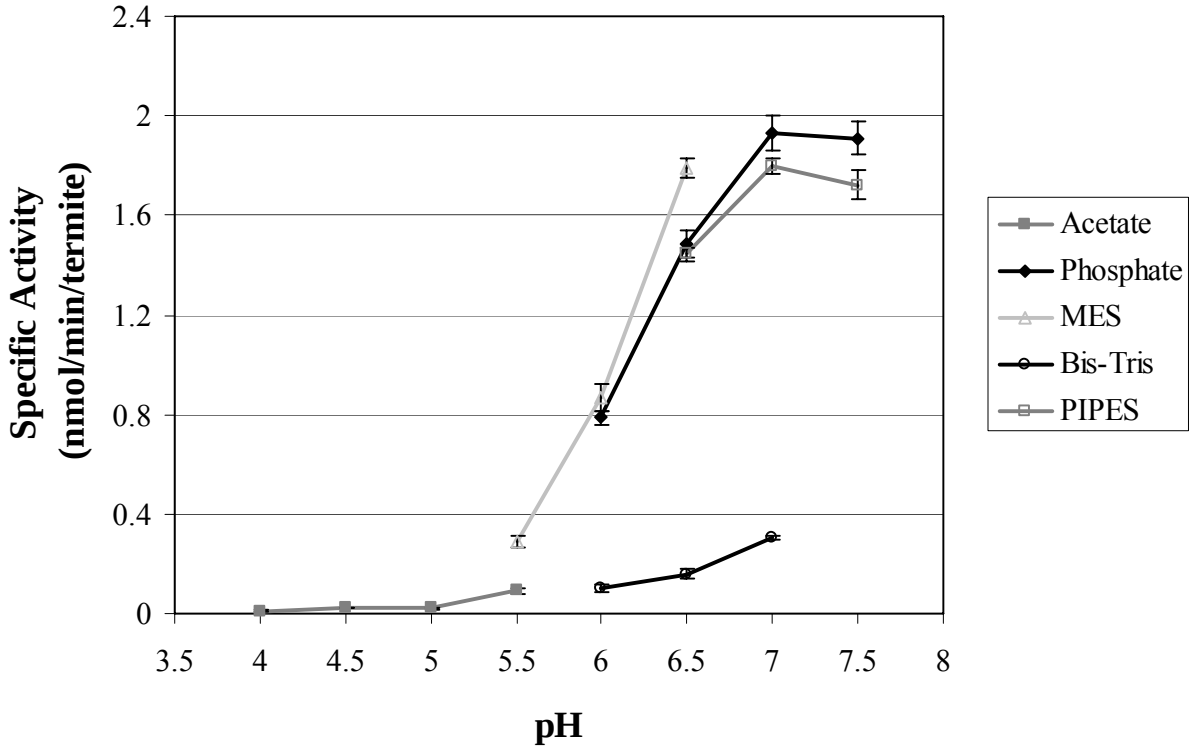

Figure 3-1. Beta-glucosidase activities in *Reticulitermes flavipes* workers across pH and buffer.

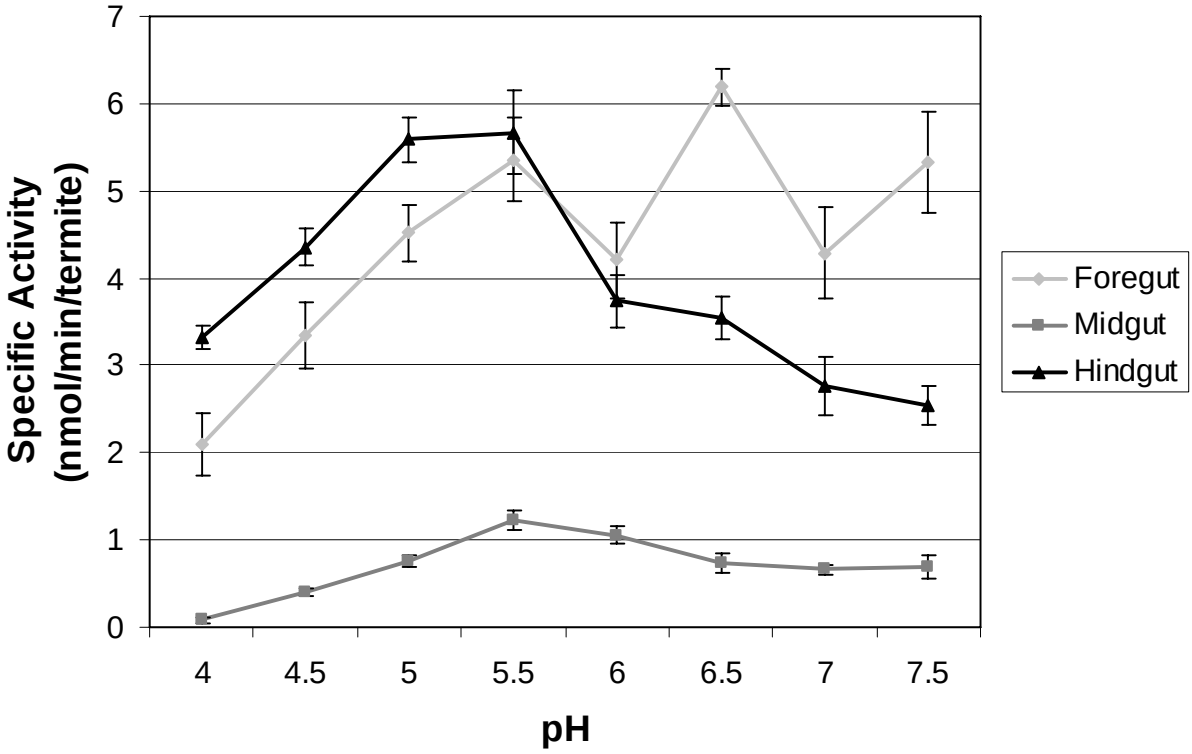

Figure 3-2. Endoglucanase activities in different gut regions of *Reticulitermes flavipes* workers across pH.

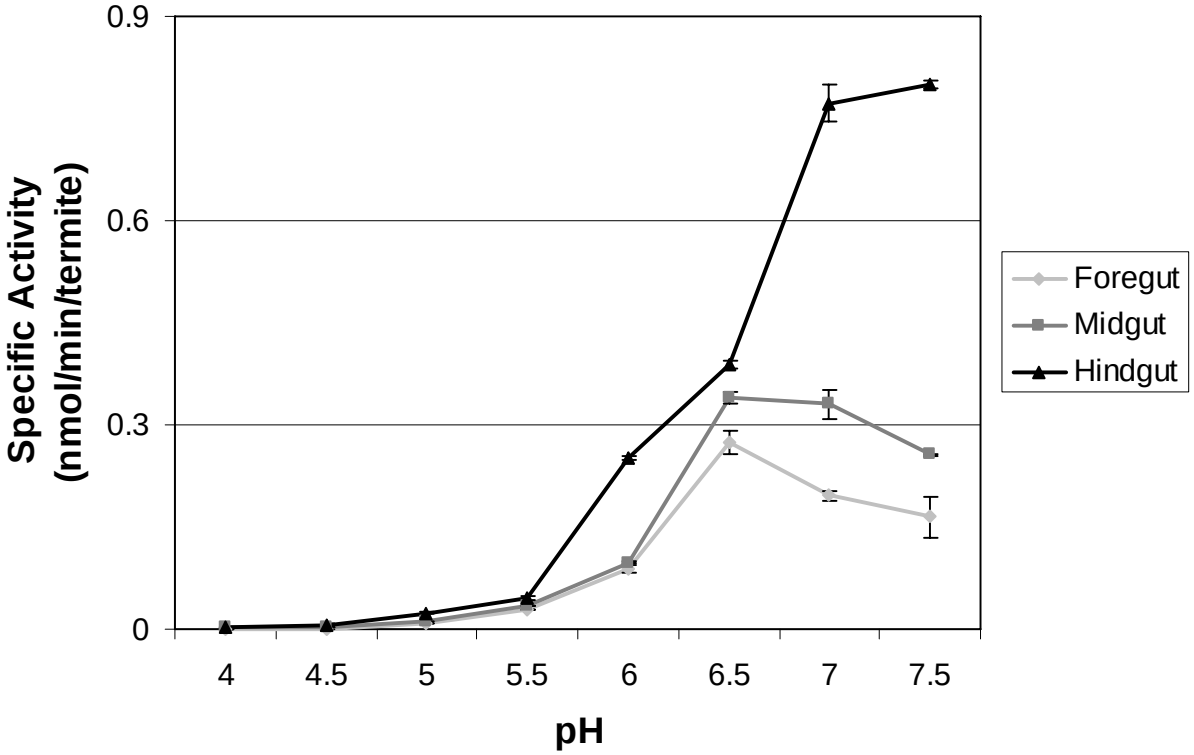

Figure 3-3. Beta-Glucosidase activities in different gut regions of Reticulitermes flavipes workers across pH.

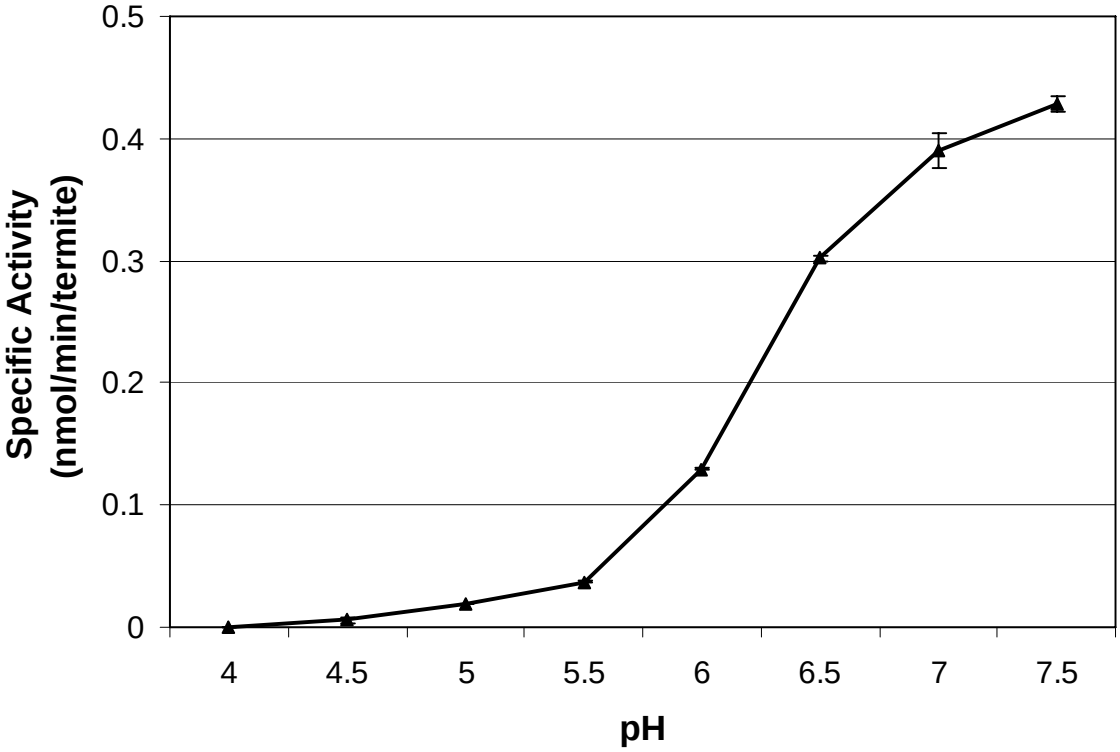

Figure 3-4. Hindgut exoglucanase activities in *Reticulitermes flavipes* workers across pH.

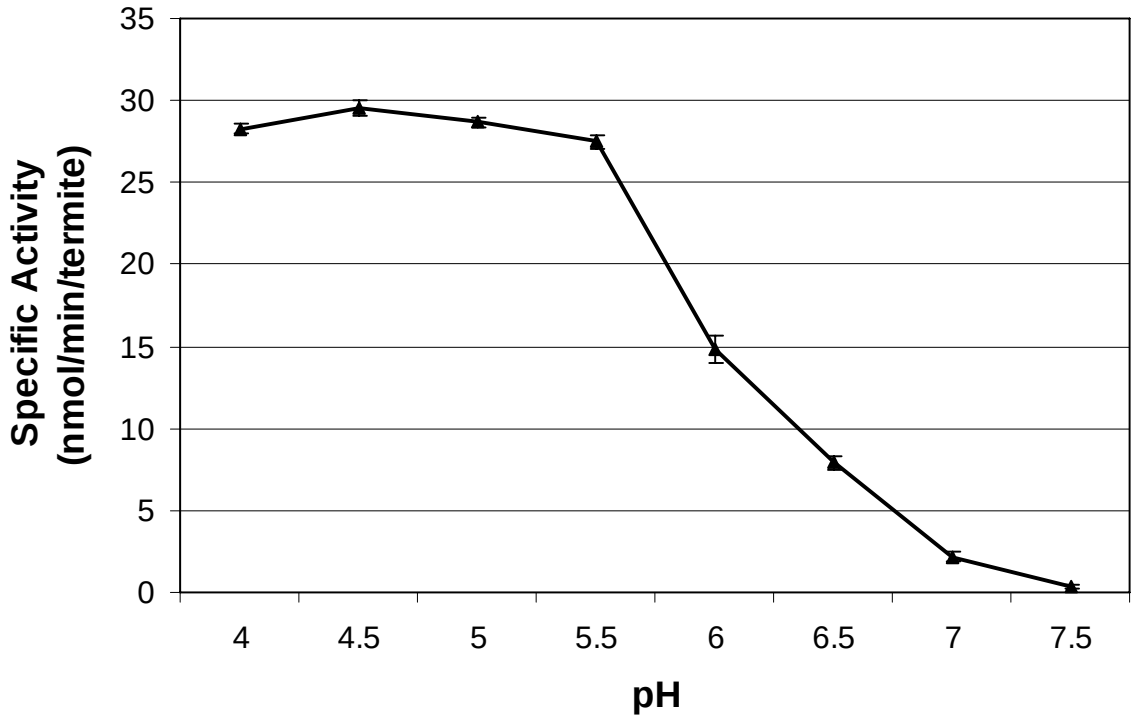

Figure 3-5. Hindgut xylanase activities in *Reticulitermes flavipes* workers across pH.

CHAPTER 4
CARBOHYDROLASE ACTIVITY PATTERNS IN *RETICULITERMES FLAVIPES* AND *COPTOTERMES FORMOSANUS* WORKERS AND SOLDIERS

Introduction

Reticulitermes flavipes (Kollar) and *Coptotermes formosanus* (Shiraki) are two of the most economically significant pest termites in North America. Both are subterranean termites, but *R. flavipes* is a native termite with relatively small colonies and a low soldier ratio while *C. formosanus* is an invasive termite with relatively large colonies and a higher soldier ratio. Such differences in colony structure may be accompanied by differences in gut carbohydrolase profiles.

Termites and their symbionts are known to produce three major types of cellulase which work together to digest cellulose. Exoglucanase (EC 3.2.1.91) cleaves the cellulose chain from the ends, typically producing cellobiose, and is most active against crystalline cellulose. Endoglucanase (EC 3.2.1.4) cleaves the cellulose chain randomly along its length and is most active against amorphous cellulose. Beta-Glucosidase (3.2.1.21) cleaves cellobiose and other small cellulose fragments, hydrolyzing them to glucose. Xylanolytic enzymes include xylanases (EC 3.2.1.8) which internally cleave the xylan chain and beta-xylosidases (EC 3.2.1.37) which cleave xylan oligosaccharides into xylose.

The objectives of this study were 1) determine the patterns of cellulolytic and xylanolytic enzymes in different termite gut regions, 2) determine the relative cellulolytic and xylanolytic activities of *R. flavipes* and *C. formosanus* workers, 3) determine the relative cellulolytic and xylanolytic activities of *R. flavipes* and *C. formosanus* soldiers, and 4) determine the relative cellulolytic and xylanolytic activities of the soldiers of each species compared to the workers.

Materials and Methods

Termite Collection, Dissection, and Enzyme Extraction

Termites were field collected in First Line Smartdisc monitors (FMC Corporation) in Charleston, South Carolina. Termites were collected from the trap by removal of the wood (southern yellow pine) in the bait stations and brought to the University of Florida. Termites were then placed in feeding bioassays. Termites were collected, frozen and kept at -80^{o}C until dissection. Collections were restricted to a single colony for each species to eliminate colony as a source of variation in enzyme activities.

Two buffers were prepared for use in the dissections: 0.1 M sodium acetate buffer, pH 5.5, and 0.1 M sodium phosphate buffer, pH 6.5. Sodium acetate buffer was used for the endoglucanase and xylanase assays, while sodium phosphate buffer was used for the exoglucanase, beta-glucosidase and beta-xylosidase assays.

Termites were removed from the freezer and kept on ice until dissection. Each termite's gut was removed intact and separated into three regions: foregut (and salivary glands), midgut, and hindgut. A single enzyme extract was prepared from dissected termites from each feeding treatment for each assay type using an experimental design similar to the recent previous termite carbohydrolase experiments (Hogan et al. 1988, Inoue et al. 1997, Nakashima et al. 2002b). For the endoglucanase and xylanase assays, 25 termites of each caste and species were dissected in sodium acetate buffer. For the exoglucanase, beta-glucosidase and beta-xylosidase assay, 25 termites of each caste and species were dissected in sodium phosphate buffer. The three gut regions were placed into separate 1.5 mL microcentrifuge tubes (Eppendorf) containing the appropriate buffer, and kept on ice. Final concentrations were 50 termite gut regions per mL in all cases.

Enzymes were extracted using a method adapted from Inoue et al. (1997). 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^{o}C for 15 min. The supernatants were collected, frozen, and kept at -80^{o}C until use in the enzyme assays.

Termite Photography

Guts and frontal glands from both termite species were dissected into 0.1 M sodium phosphate buffer, pH 6.5, using the methods described above. Termites were then photographed on an auto-montage system (Syncroscopy, Frederick, MD).

Soluble Protein Assays

Assays for soluble protein content were conducted using the Bradford reagent. Enzyme extracts (10 μL) were combined with 250 μL of Bradford reagent (Bio-Rad, Hercules, CA) in a 96-well microplate. Absorbances were then read at 595 nm using a μQuant Universal Microplate Spectrophotometer (Bio-Tek Instruments, Winooski, VT). Standards were generated using BSA standards (Bio-Rad, Hercules, CA) combined with the Bradford reagent in the same proportion as the enzyme extracts.

Endoglucanase Assays

The endoglucanase assays were conducted using a method adapted from Han et al. (1995). A 2% solution of carboxymethylcellulose (CMC; Sigma-Aldrich) was prepared in 0.1 M sodium acetate buffer, pH 5.5.

Assays were conducted in clear 96-well microplates. In each well, 10 μL of tissue extract was combined with 90 μL of CMC solution. The solutions were allowed to react for 70 min at 23^{o}C. DNSA solution (100 μL) consisting 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 placed on ice for 15 min. After cooling, each microplate was read at 540 nm using a μQuant Universal Microplate Spectrophotometer (Bio-Tek Instruments, Winooski, VT). Similar 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). A 10 min reaction was used as a control to correct for any differences in initial sample reaction rates due to incomplete mixture of enzyme and substrate solutions. Standards were generated using dilutions of glucose. Only the wells in the middle of the microplate were used for these assays. Perimeter wells were filled with deionized water (200 μL per well) to add temperature stability and consistency during boiling.

Exoglucanase Assays

Exoglucanase assays were conducted using a method adapted from Han et al. (1995). A solution of 4 mM p-nitrophenyl-β-D-cellobioside (pNPC; Sigma-Aldrich) was prepared in 0.1 M sodium phosphate buffer, pH 6.5. Assays were conducted in clear 96-well microplates. In each well, 10 μL of tissue extract was combined with 90 μL of pNPC solution. The reaction was allowed to proceed for 10 min before being placed in a μQuant Universal Microplate Spectrophotometer (Bio-Tek Instruments, Winooski, VT). Absorbance was read at 420 nm every 2 min for 30 min at 23^{o}C. Mean velocities (mOd/s) were recorded. Standards were generated using dilutions of p-nitrophenol.

Beta-Glucosidase Assays

Beta-glucosidase assays were conducted using a method adapted from Han et al. (1995). A solution of 4 mM p-nitrophenyl-β-D-glucopyranoside (pNPG; Sigma-Aldrich) was prepared in 0.1 M sodium phosphate buffer, pH 6.5. Assays were conducted in clear 96-well microplates. In each well, 10 μL of tissue extract was combined with 90 μL of pNPG solution. The reaction was

allowed to proceed for 10 min before being placed in a μQuant Universal Microplate Spectrophotometer (Bio-Tek Instruments, Winooski, VT). Absorbance was read at 420 nm every 2 min for 30 min at 23°C. Mean velocities (mOd/s) were recorded. Standards were generated using dilutions of p-nitrophenol.

Xylanase Assays

The xylanase assays were conducted using a method adapted from Han et al. (1995). A 0.5% solution of beechwood xylan (>90% xylose residues; Sigma-Aldrich) was prepared in 0.1 M sodium acetate buffer, pH 5.5. The solution was boiled approximately 30 min, until xylan particles were no longer visible. The solution was centrifuged at 1250 g for 5 min at 23°C and the supernatant was used as the xylan stock solution.

Assays were conducted in clear 96-well microplates. In each well, 10 μL of tissue extract was combined with 90 μL of xylan solution. The solutions were allowed to react for 35 min at 23°C. DNSA solution (100 μL) consisting 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 placed on ice for 15 min. After cooling, each microplate was read at 540 nm using a μQuant Universal Microplate Spectrophotometer (Bio-Tek Instruments, Winooski, VT). Similar control plates were allowed to react for 5 min to allow for passive mixing of solutions before boiling with DNSA solution (Zhou et al. 2007). A 5 min reaction was used as a control to correct for any differences in initial sample reaction rates due to incomplete mixture of enzyme and substrate solutions. Standards were generated using dilutions of xylose. Only the wells in the middle of the microplate were used for these assays. Perimeter wells were filled with deionized water (200 μL per well) to add temperature stability and consistency during boiling.

Beta-Xylosidase Assays

Beta-xylosidase assays were conducted using a method adapted from Han et al. (1995). A solution of 4 mM p-nitrophenyl-β-D-xylopyranoside (pNPX; Sigma-Aldrich) was prepared in 0.1 M sodium phosphate buffer, pH 6.5. Assays were conducted in clear 96-well microplates. In each well, 10 μL of tissue extract was combined with 90 μL of pNPX solution. The reaction was allowed to proceed for 10 min before being placed in a μQuant Universal Microplate Spectrophotometer (Bio-Tek Instruments, Winooski, VT). Absorbance was read at 420 nm every 2 min for 30 min at 23^{o}C. Mean velocities (mOd/s) were recorded. Standards were generated using dilutions of p-nitrophenol.

The experiment with formulated diets was set up as a two-factor design with four replicates. The two factors were gut region and diet treatments. One microplate well was assayed for each replicate of each gut region/diet combination. Three gut sections and five diet treatments were assayed for each replicate, yielding a total of 15 wells per replicate.

Data Analysis

Experiments were set up as one-factor designs with two homogenates for each caste/species combination; one homogenate for endoglucanase and xylanase assays and another for exoglucanse, beta-glucosidase, and beta-xylosidase assays. The endoglucanase assays had 6 technical replicates for each gut region while the other assays had 4 technical replicates for each gut region. The protein assays had 3 technical replicates for each gut region, species and caste.

For the endoglucanase and xylanase assays, the following formula was used to calculate specific activities;

$$SA = C_S[(A-A_0)/t]/N_T$$

where: SA = specific activity (nmol reducing sugar per termite equivalent per min), A = absorbance (Od) after 35 min reaction, A_0 = absorbance (Od) for the corresponding control after 5 min reaction, t = time (min), C_S = the coefficient derived from the standard (nmol reducing sugar/mOd), and N_T = the number of termite equivalents per sample.

For the exoglucanase, beta-glucosidase, and beta-xylosidase assays, the following formula was used to calculate specific activities;

$$SA = 60C_SV_A/N_T$$

where: SA = specific activity (nmol p-nitrophenol per termite equivalent per min), V_A = mean velocity of absorbance change (mOd/s), C_S = the coefficient derived from the standard (nmol p-nitrophenol/mOd), and N_T = the number of termite equivalents per sample.

The data were analyzed using analysis of variance. Student-Neuman-Keuls test, $\alpha = 0.05$, was used to separate the mean activities within each gut region (SAS Institute 2001).

Results

Termite Gut and Frontal Gland Comparisons

Soldiers in both species had smaller, less developed hindguts than workers (Figure 4-1). Compared to *R. flavipes* workers, *C. formosanus* workers had a smaller crop, a larger rectum, and a small internal sclerotization just behind the malpighian tubules. The frontal gland in *C. formosanus* soldiers was far larger and apparently more developed than that in *R. flavipes* soldiers (Figure 4-2). Formosan soldier termites had a prolonged pharynx, to accommodate the enlarged frontal gland.

Worker Cellulolytic Activities

Endoglucanase activities were mainly confined to the foregut and hindgut (Table 4-1). Activities were significantly greater in the *R. flavipes* worker foregut than in the *C. formosanus* worker foregut, but they were significantly greater in the *C. formosanus* midgut and hindgut.

Exoglucanase activities were almost exclusive to the hindgut, with *C. formosanus* worker activities being significantly greater in all gut regions (Table 4-2). Beta-glucosidase activities were distributed across the gut, being most prominent in the midgut *in C. formosanus* workers and most prominent in the hindgut in *R. flavipes* workers (Table 4-3). Differences in Beta-glucosidase activities were significant in all three gut regions among all species-caste combinations.

Worker Xylanolytic Activities

Xylanase activities were almost exclusive to the hindgut, with no significant differences between worker hindgut xylanase activities (Table 4-4). Beta-xylosidase activities were mainly restricted to the hindgut, and were significantly higher in *C. formosanus* workers in all gut regions (Table 4-5).

Soldier Cellulolytic Activities

Soldier cellulolytic activities were lower than those seen in workers in most cases. Endoglucanase activities were mainly confined to the hindgut, with midgut and hindgut activities significantly greater in *R. flavipes* soldiers (Table 4-1). Exoglucanase activities were mainly confined to the hindgut, and hindgut activities were significantly greater in *R. flavipes* soldiers (Table 4-2). Beta-glucosidase activities were distributed across the gut, being most prominent in the midgut and significantly greater in *R. flavipes* soldier hindguts (Table 4-3).

Soldier Xylanolytic Activities

Soldier xylanolytic activities were lower than those seen in workers in most cases. Xylanase activities were almost exclusive to the hindgut, and were significantly higher in the *R. flavipes* soldier hindgut (Table 4-4). Beta-xylosidase activities were mainly restricted to the hindgut, and were significantly higher in the *R. flavipes* soldier hindgut, while midgut activities were significantly higher in the *C. formosanus* soldier hindgut (Table 4-5).

Gut Soluble Protein Levels

The hindgut extracts had far more soluble protein than the foregut or midgut extracts in all cases except for the *C. formosanus* soldier extract in pH 5.5 buffer (Table 4-6). This difference was smaller, but the hindgut still contained more protein than the foregut or midgut, even for the pH 5.5 *C. formosanus* soldier extract. Soldier protein levels were universally lower than worker protein levels for each gut region. *C. formosanus* worker extracts had higher protein levels than *R. flavipes* worker extracts in most cases. *C. formosanus* soldier extracts had lower protein levels than *R. flavipes* soldier extracts in all cases. The extracts in pH 5.5 buffer had lower protein levels than the extracts in pH 6.5 buffer.

Discussion

The patterns of cellulolytic enzymes within the *R. flavipes* workers appeared to indicate a processive degradation of amorphous cellulose along the gut. Endoglucanase activity was seen to be higher in the foregut than in the midgut, whereas beta-glucosidase activity appeared to increase progressively through the gut.

The majority of exoglucanase and xylanase activities were located in the hindgut of workers soldiers of both species assayed. Similar exoglucanase patterns have been observed in *Coptotermes lacteus* (Hogan et al. 1988). This indicates a large dependence of the termite on its hindgut symbionts for the digestion of crystalline cellulose and xylan. Our findings are consistent with prior characterizations of symbiont exoglucanases in *C. formosanus* and *R. flavipes* (Watanabe et al. 2002, Nakashima et al. 2002a, Zhou et al. 2007). This localization of exoglucanase and xylanase activities to the hindgut is significant, as up to 70% of wood cellulose may be crystalline in nature and xylan may make up as much as 25% of wood dry weight (Biermann 1996). This could explain past observations of reliance on hindgut symbionts (Cleveland 1924) in spite of the characterization of endogenous cellulases in *Reticulitermes* and

Coptotermes termites (Watanabe et al. 1998, Nakashima et al. 2002b, Zhou et al. 2007). It should be noted that, thus far, no endogenous cellulases in characterized lower termites have been exoglucanases.

Formosan subterranean termites are widely regarded as more aggressive foragers than eastern subterranean termites, with far larger colonies. Their soldiers have a larger frontal gland than the soldiers of the eastern subterranean termite, producing a significant amount of chemical exudate in defense of the colony (need to take photos of the frontal glands). This increased metabolic demand, combined with the larger ratio of soldiers to workers, roughly 1:10 as opposed to 1:100 in the case of *R. flavipes*, would seem to necessitate a more active system for the digestion of wood and similar materials.

This is precisely what the results seem to indicate among the workers, especially in the case of exoglucanase and beta-glucosidase. While the overall distributions of carbohydrolase activities are similar in these two species, there are a couple of differences; heightened enzyme activities as mentioned above and the partial redistribution of endoglucanase from the foregut to the midgut and hindgut in *C. formosanus* workers.

A reverse pattern was seen in the soldiers, with the *C. formosanus* soldiers having far less enzyme activity in the case of endoglucanase, exoglucanase and xylanase. However, *C. formosanus* soldier beta-glucosidase and beta-xylosidase were similar to the levels seen in *R. flavipes* soldiers. The former three carbohydrolases are known to break down longer chains, thus working in the earlier parts of cellulose and xylan degradation. The latter two break down oligosaccharides, working in the final parts of cellulose and xylan degradation.

With its greatly enlarged frontal gland, the *C. formosanus* soldier has relatively little room for its digestive system. Since the *C. formosanus* workers are so proficient at wood digestion,

there is little need for the earlier enzymes in the soldier digestive systems. Therefore, it makes sense that these soldiers' limited digestive capacity would be almost entirely focused on the later phases of wood digestion. This is supported by the less extreme case of the *R. flavipes* soldiers, which also have relatively high levels of beta-glucosidase and beta-xylosidase compared with the other three enzymes.

Formosan subterranean termites are not only more aggressive foragers than eastern subterranean termites, but their digestive systems are apparently more capable of digesting wood, particularly crystalline cellulose. This would suggest the possibility of natural cellulase inhibitors or other obstacles to wood digestion in their native range. Some plants are known to produce cellulase inhibitors in the form of proteins or polyphenols (Sineiro et al. 1997, York et al. 2004). However, the increased digestive capacity seen in *C. formosanus* workers could also simply be a result of the need to support a larger proportion of soldiers, as termite soldiers are incapable of feeding themselves and Formosan soldiers have relatively little internal digestive capacity.

Although there were clearly differences in overall activity levels, the patterns of cellulolytic and xylanolytic enzyme activities were similar between the two species assayed, in spite of the fact that these species were in two different genera from two different continents. This suggests that findings regarding cellulose and xylan digestion in one species within the Rhinotermitidae may be cautiously applied to the rest of the family. Our findings also suggest a processive mechanism of amorphous cellulose degradation with a reliance on the termite symbionts for the digestion of xylan and crystalline cellulose. This corroborates the need of these termite species for their hindgut symbionts in spite of their production of endogenous

cellulolytic and xylanolytic enzymes. This pattern of digestion will be revisited in workers of both species in subsequent chapters.

Table 4-1. A comparison of endoglucanase activities in *Reticulitermes flavipes* and *Coptotermes formosanus* workers and soldiers

Caste	Species	Foregut	Midgut	Hindgut
worker	*R. flavipes*	4.70 ± 0.31a	1.39 ± 0.08b	5.21 ± 0.28b
worker	*C. formosanus*	2.84 ± 0.22b	1.91 ± 0.19a	8.17 ± 0.51a
soldier	*R. flavipes*	0.36 ± 0.07c	1.22 ± 0.11b	2.81 ± 0.16c
soldier	*C. formosanus*	0.00 ± 0.01c	0.23 ± 0.03c	0.79 ± 0.07d

Endoglucanase activities are in nmol reducing sugar per termite equivalent per min. Means within a column followed by the same letter are not significantly different, (Student-Neuman-Keuls Means Separation, $\alpha = 0.05$, SAS Institute 2001), n = 6 replicates.

Table 4-2. A comparison of exoglucanase activities in *Reticulitermes flavipes* and *Coptotermes formosanus* workers and soldiers

Caste	Species	Foregut	Midgut	Hindgut
worker	*R. flavipes*	0.0109 ± 0.0001b	0.0121 ± 0.0026c	0.3862 ± 0.0067b
worker	*C. formosanus*	0.0278 ± 0.0002a	0.1182 ± 0.0033a	0.6728 ± 0.0140a
soldier	*R. flavipes*	0.0013 ± 0.0006d	0.0409 ± 0.0008b	0.2184 ± 0.0037c
soldier	*C. formosanus*	0.0037 ± 0.0002c	0.0377 ± 0.0017b	0.1014 ± 0.0019d

Exoglucanase activities are in nmol p-nitrophenol per termite equivalent per min. Means within a column followed by the same letter are not significantly different, (Student-Neuman-Keuls Means Separation, $\alpha = 0.05$, SAS Institute 2001), n = 4 replicates.

Table 4-3. A comparison of beta-glucosidase activities in *Reticulitermes flavipes* and *Coptotermes formosanus* workers and soldiers

Caste	Species	Foregut	Midgut	Hindgut
worker	*R. flavipes*	0.147 ± 0.004b	0.176 ± 0.004a	0.655 ± 0.008b
worker	*C. formosanus*	0.272 ± 0.003a	0.891 ± 0.033c	0.876 ± 0.020a
soldier	*R. flavipes*	0.037 ± 0.001c	0.611 ± 0.008b	0.348 ± 0.001c
soldier	*C. formosanus*	0.056 ± 0.018c	0.560 ± 0.016b	0.168 ± 0.002d

Beta-glucosidase activities are in nmol p-nitrophenol per termite equivalent per min. Means within a column followed by the same letter are not significantly different, (Student-Neuman-Keuls Means Separation, $\alpha = 0.05$, SAS Institute 2001), n = 4 replicates.

Table 4-4. A comparison of xylanase activities in *Reticulitermes flavipes* and *Coptotermes formosanus* workers and soldiers

Caste	Species	Foregut	Midgut	Hindgut
worker	*R. flavipes*	-0.04 ± 0.02a	0.02 ± 0.02b	24.18 ± 0.37a
worker	*C. formosanus*	-0.01 ± 0.04a	0.48 ± 0.11a	25.13 ± 0.43a
soldier	*R. flavipes*	-0.03 ± 0.03a	0.21 ± 0.08b	21.51 ± 0.27b
soldier	*C. formosanus*	-0.03 ± 0.01a	0.01 ± 0.01b	00.99 ± 0.18c

Xylanase activities are in nmol reducing sugar per termite equivalent per min. Means within a column followed by the same letter are not significantly different, (Student-Neuman-Keuls Means Separation, $\alpha = 0.05$, SAS Institute 2001), n = 4 replicates.

Table 4-5. A comparison of beta-xylosidase activities in *Reticulitermes flavipes* and *Coptotermes formosanus* workers and soldiers

Caste	Species	Foregut	Midgut	Hindgut
worker	*R. flavipes*	0.0056 ± 0.0006bc	0.0072 ± 0.0003c	0.0399 ± 0.0006b
worker	*C. formosanus*	0.0075 ± 0.0006a	0.0014 ± 0.0004a	0.0645 ± 0.0011a
soldier	*R. flavipes*	0.0049 ± 0.0004c	0.0011 ± 0.0003b	0.0216 ± 0.0003c
soldier	*C. formosanus*	0.0067 ± 0.0003ab	0.0015 ± 0.0004a	0.0134 ± 0.0002d

Beta-xylosidase activities are in nmol p-nitrophenol per termite equivalent per min. Means within a column followed by the same letter are not significantly different, (Student-Neuman-Keuls Means Separation, $\alpha = 0.05$, SAS Institute 2001), n = 4 replicates.

Table 4-6. A comparison of soluble protein concentrations in *Reticulitermes flavipes* and *Coptotermes formosanus* worker and soldier gut extracts

pH	Caste	Species	Foregut	Midgut	Hindgut
5.5	Worker	*R. flavipes*	1.304 ± 0.018	1.126 ± 0.046	6.954 ± 0.208
5.5	Worker	*C. formosanus*	0.944 ± 0.052	1.714 ± 0.032	11.312 ± 0.060
5.5	Soldier	*R. flavipes*	0.420 ± 0.001	0.676 ± 0.014	3.808 ± 0.156
5.5	Soldier	*C. formosanus*	0.256 ± 0.001	0.354 ± 0.016	0.622 ± 0.078
6.5	Worker	*R. flavipes*	2.250 ± 0.060	4.192 ± 0.128	15.726 ± 0.852
6.5	Worker	*C. formosanus*	0.181 ± 0.014	3.952 ± 0.088	21.522 ± 0.228
6.5	Soldier	*R. flavipes*	1.232 ± 0.030	2.234 ± 0.052	9.832 ± 0.500
6.5	Soldier	*C. formosanus*	0.296 ± 0.032	0.556 ± 0.016	2.312 ± 0.052

Concentrations are in μg per termite gut region, n = 3 replicates.

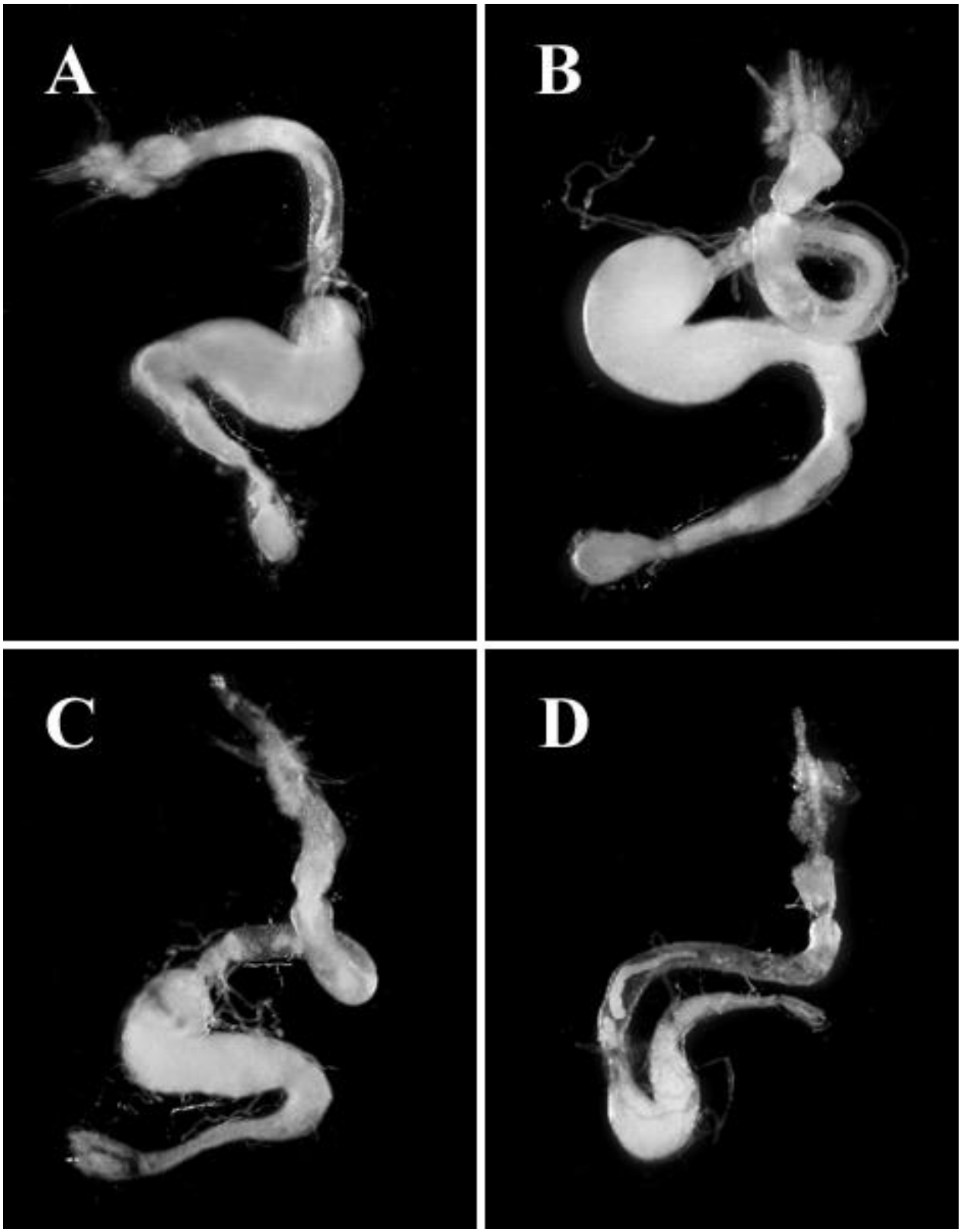

Figure 4-1. Digestive systems of: A) *R. flavipes* worker, B) *C. formosanus* worker, C) *R. flavipes* soldier, and D) *C. formosanus* soldier.

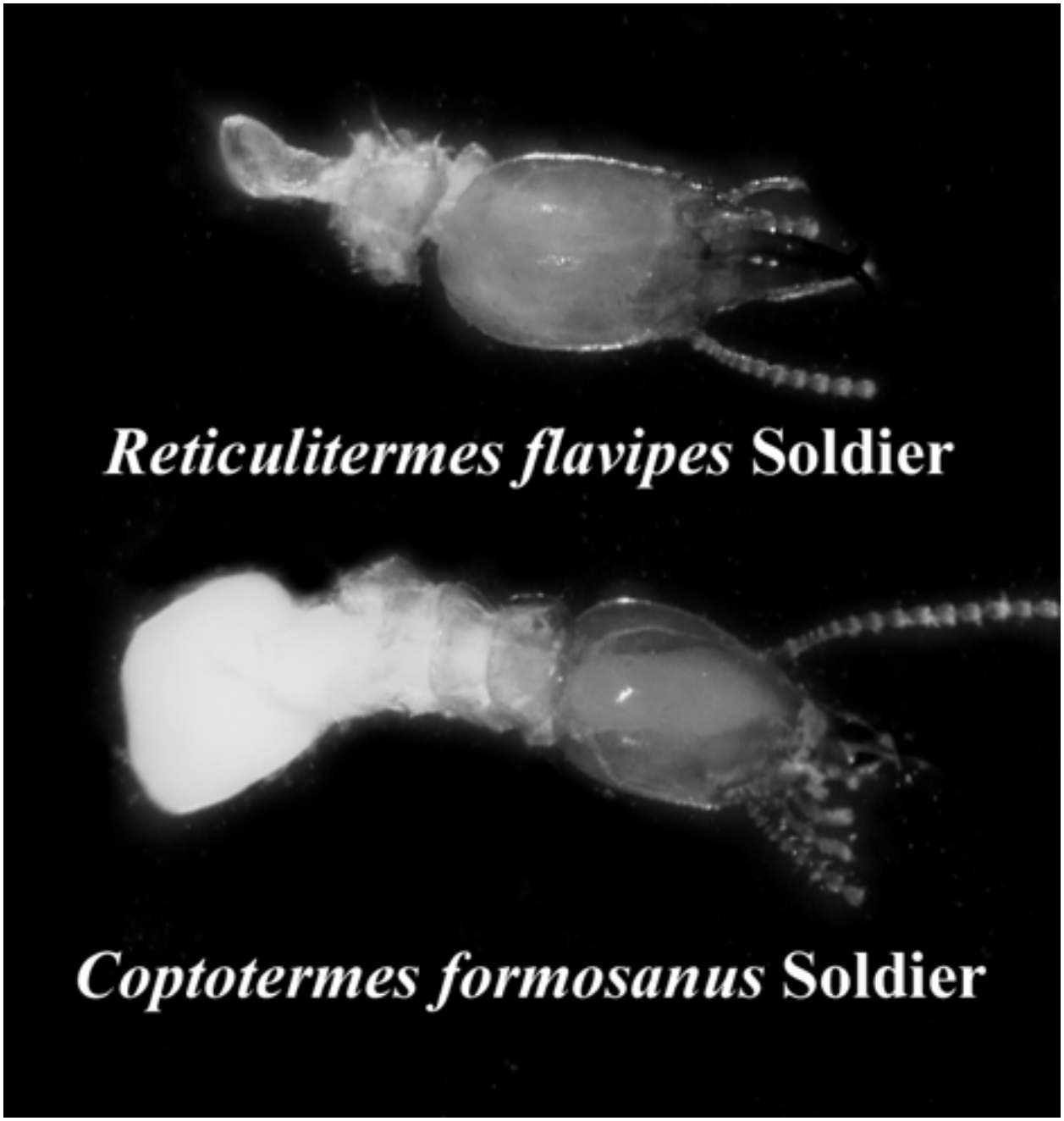

Figure 4-2. Heads and frontal glands of a *R. flavipes* soldier, and a *C. formosanus* soldier.

CHAPTER 5
CHANGES IN *RETICULITERMES FLAVIPES* GUT CELLULOLYTIC ACTIVITIES IN RESPONSE TO DIET

Introduction

Subterranean termites, such as *Reticulitermes flavipes* (Kollar), subsist largely on a diet of wood and similar material (Noirot and Noirot-Timothee 1969). Wood is not a substance that most animals are capable of digesting to any significant degree. Termites are well known for the ability to digest cellulose with the aid of microbial hindgut symbionts.

In wood, cellulose chains are typically arranged in parallel bundles known as microfibers which are embedded in a matrix of lignin and hemicelluloses. The cellulose in the microfibers may be broadly divided into two types: crystalline and amorphous. The crystalline form of cellulose consists of tightly aligned parallel chains, held in a specific configuration by hydrogen bonding. In the amorphous form of cellulose, the chains are more randomly arranged, and not so closely bound together.

There are three major types of cellulase which work together to digest cellulose. Exoglucanase (EC 3.2.1.91) cleaves the cellulose chain from the ends, typically producing cellobiose, and is most active against crystalline cellulose. Endoglucanase (EC 3.2.1.4) cleaves the cellulose chain randomly along its length and is most active against amorphous cellulose. Beta-Glucosidase (3.2.1.21) cleaves cellobiose and other small cellulose fragments, hydrolyzing them to glucose.

The gut of *R. flavipes* and related termites consists of a foregut with a small crop and a proventriculus, a fairly simple midgut, and a greatly expanded hindgut containing several species of symbiotic protozoa, fungi, bacteria, and archaea (Yamin 1979, Lewis and Forschler 2004). A pair of salivary glands is also present, emptying into the foregut anterior to the crop. To effectively digest wood, a chemically demanding and nutritionally poor substrate, termites like

R. flavipes have evolved an array of enzymes. Some of the cellulases characterized from the Rhinotermitidae have been shown to be endogenous, being produced naturally by the termite (Watanabe et al. 1998, Watanabe et al. 1997). Others have been shown to be symbiotic in origin, produced by flagellate symbionts within the hindgut (Nakashima et al. 2002a, Watanabe et al. 2002, Ohtoko et al. 2000). Zhou et al. (2007) demonstrated that exoglucanase activity is largely localized to the hindgut of *R. flavipes*, strongly implicating the resident flagellates as the major 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 on pure cellulose or pure xylan, as opposed to wood. It is probable that, with changes in protozoan populations on differing diets, the levels of different cellulase activities may also change.

Subterranean termites may encounter a number of potential food sources during foraging. These may be broadly grouped into hardwoods, softwoods, and processed cellulosic materials like paper. These three groups vary mainly in their hemicellulose content. In homes, hardwoods may include furniture, flooring, and trim components. Structural timbers are nearly always derived from softwoods.

Our objectives were to: 1) confirm the patterns of R. flavipes worker cellulolytic activity seen in Chapter 4, 2) determine how the three major cellulase activities found in the gut of *R. flavipes* workers change in response to three different simulated field diets.

Materials and Methods

Termite Collection

R. flavipes termites were field collected in termite traps consisting of a PVC bucket (20 cm high by 20 cm diam.; Item # 811192-4, Ventura Packaging Inc., Monroeville, Ohio) with 11 holes drilled in the sides and base (3 cm diam.) placed vertically in the ground to a depth of

roughly 19 cm and covered with a PVC lid. Three rolls of single-faced corrugated cardboard (20 cm long by 10 cm diam.) were placed into the bucket side by side as a food source. Termites were collected from the trap by removal of cardboard rolls, separated from the cardboard, and either placed in feeding bioassays or immediately frozen and kept at -80^{o}C until dissection. Collections were restricted to a single colony to eliminate colony as a source of variation in enzyme activities.

Termite Diets and Feeding

Three diets were prepared for the termites to represent the probable food sources they would encounter in the field. These simulated field diets were as follows: red oak (Quercus spp.), pine (Pinus spp.), and filter paper. Wood diets were generated by drilling into craft wood boards (0.635 x 5.08 x 60.96 cm) with a 2.54 cm spade drill bit. Sawdust was collected and weighed. Filter paper consisted of a weighed number of crumpled cellulose filter paper disks (42.5 mm diameter, Whatman, grade 4).

Each field diet (20 g) was added to a loosely capped 250 mL glass bottle (Pyrex) with 5 mL of deionized water. Field diets were not made from sterile materials. Because of this, the bottles with field diets were autoclaved on a liquid cycle (30 min, 122^{o}C) to sterilize the diets and the bottles were then allowed to cool. Termites were added (~300 workers and 3 soldiers per bottle) after the bottles had cooled and kept in the dark at 21^{o}C for 6 wk, with deionized water (2 mL) added every 2 wk. Termites were collected, frozen and kept at -80^{o}C until dissection.

Termite Dissection and Enzyme Extraction

Two buffers were prepared for use in the dissections: 0.1 M sodium acetate buffer, pH 5.5, and 0.1 M sodium phosphate buffer, pH 6.5. Sodium acetate buffer was used for the endoglucanase assay, while sodium phosphate buffer was used for the exoglucanase and beta-glucosidase assays.

Termites were removed from the freezer and kept on ice until dissection. Each termite's gut was removed intact and separated into three regions: foregut (and salivary glands), midgut, and hindgut. A single enzyme extract was prepared from a minimum of 35 dissected termites from each feeding treatment for each assay type using an experimental design similar to previous termite carbohydrolase experiments (Hogan et al. 1988, Inoue et al. 1997, Nakashima et al. 2002b). For the endoglucanase assay, 50 termites from each feeding treatment were dissected in sodium acetate buffer. For the exoglucanase and beta-glucosidase assays, 35 termites from each feeding treatment were dissected in sodium phosphate buffer. The three gut regions were placed into separate 1.5 mL microcentrifuge tubes (Eppendorf) containing the appropriate buffer, and kept on ice. Final concentrations were 50 termite gut regions per mL in all cases.

Enzymes were extracted using a method adapted from Inoue et al. (1997). 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^{o}C for 15 min. The supernatants were collected, frozen, and kept at -80^{o}C until use in the enzyme assays.

Endoglucanase Assays

The endoglucanase assays were conducted using a method adapted from Han et al. (1995). A 2% solution of carboxymethylcellulose (CMC; Sigma-Aldrich) was prepared in 0.1 M sodium acetate buffer, pH 5.5.

Assays were conducted in clear 96-well microplates. In each well, 10 μL of tissue extract was combined with 90 μL of CMC solution. The solutions were allowed to react for 70 min at 23^{o}C. DNSA solution (100 μL) consisting 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 placed on ice for 15 min. After cooling, each

microplate was read at 540 nm using a μQuant Universal Microplate Spectrophotometer (Bio-Tek Instruments, Winooski, VT). Similar 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). A 10 min reaction was used as a control to correct for any differences in initial sample reaction rates due to incomplete mixture of enzyme and substrate solutions. Standards were generated using dilutions of glucose. Only the wells in the middle of the microplate were used for these assays. Perimeter wells were filled with deionized water (200 μL per well) to add temperature stability and consistency during boiling. For all replicates, the control plates were used to adjust for 540 nm absorbance in gut extracts and were replicated an equal number of times to the assay plates, with one microplate well for each replicate.

Exoglucanase and Beta-glucosidase Assays

The exoglucanase and beta-glucosidase assay was conducted using a method adapted from Han et al. (1995). Solutions of 4 mM p-nitrophenyl-β-D-cellobioside (pNPC) and 4 mM p-nitrophenyl-β-D-glucopyranoside (pNPG) were prepared in 0.1 M sodium phosphate buffer, pH 6.5. Assays were conducted in clear 96-well microplates. In each well, 10 μL of tissue extract was combined with 90 μL of pNPC or pNPG solution. The reaction was allowed to proceed for 10 min before being placed in a μQuant Universal Microplate Spectrophotometer (Bio-Tek Instruments, Winooski, VT). Absorbance was read at 420 nm every 2 min for 30 min at 23^{o}C. Mean velocities (mOd/s) were recorded. Standards were generated using dilutions of p-nitrophenol.

Data Analysis

The endoglucanase assays were set up as a one-factor split-plot design with four technical replicates for each gut region, and one microplate per replicate. Four microplate wells were

assayed for each replicate of each gut region/diet combination. The exoglucanase and beta-glucosidase assays were set up as one-factor designs with four technical replicates for each gut region. Two homogenates were used for each gut/diet combination; one homogenate for endoglucanase assays and another for exoglucanse and beta-glucosidase assays.

For the endoglucanase assays, the following formula was used to calculate specific activities;

$SA = C_S[(A-A_0)/t]/N_T$

where: SA = specific activity (nmol reducing sugar per termite equivalent per min), A = absorbance (Od) after 35 min reaction, A_0 = absorbance (Od) for the corresponding control after 5 min reaction, t = time (min), C_S = the coefficient derived from the standard (nmol reducing sugar/mOd), and N_T = the number of termite equivalents per sample.

For the exoglucanase and beta-glucosidase assays, the following formula was used to calculate specific activities;

$SA = 60C_SV_A/N_T$

where: SA = specific activity (nmol p-nitrophenol per termite equivalent per min), V_A = mean velocity of absorbance change (mOd/s), C_S = the coefficient derived from the standard (nmol p-nitrophenol/mOd), and N_T = the number of termite equivalents per sample.

The field diet data were analyzed using a mixed model analysis of variance. Fixed effects were diet treatment and gut region. The Tukey-Kramer adjustment ($\alpha = 0.05$) was used to separate the mean activities on each diet within each gut region (SAS Institute 2001).

Results

Termite Gut Observations

During dissection, after the termites had been fed on the various diets, the color of the termite guts reflected the color of the different diets. This was especially evident in the enlarged

hindguts (Fig. 1), which were typically filled with a mixture of partially digested food and microbes. Termites fed on red oak had brownish-orange gut contents. Those fed on pine had pale yellow gut contents. Those fed on paper had white gut contents. The termites collected from the field and immediately frozen for dissection had relatively dark gut contents.

Endoglucanase Activities

Most of the endoglucanase activity was located in the foregut and the hindgut, with very little in the midgut (Table 5-1). Foregut endoglucanase activity in the field-collected termites was slightly lower than the foregut activity seen on paper, and greater than activity seen on the other two diets (Table 5-1). Among the three field diets, foregut activity was highest on paper and lowest on oak. Midgut endoglucanase activity in the field-collected termites was higher than the midgut activity seen on all three diets. Among the three field diets, midgut activity was highest on pine and lowest on paper. Hindgut endoglucanase activity in the field-collected termites was higher than the hindgut activity seen on all three diets. Among the three field diets, hindgut activity was highest on oak and lowest on paper.

Exoglucanase Activities

Foregut exoglucanase activity in the field collected termites was significantly higher than the foregut activity seen all three diets (Table 5-2). Among the three diets, foregut activity was highest on paper and lowest on oak. Midgut exoglucanase activity in the field collected termites was intermediate between the midgut activities seen on oak and pine. Among the three diets, midgut activity was highest on paper and lowest on oak. Hindgut exoglucanase activity in the field collected termites was higher than the hindgut activity seen on all three diets. Among the three diets, hindgut activity was highest on oak and lowest on paper. The activity seen on pine was nearly as high as that seen on oak.

Beta-glucosidase Activities

Foregut beta-glucosidase activity in the field collected termites was intermediate between the foregut activities seen on paper and pine (Table 5-3). Among the three diets, foregut activity was highest on paper and lowest on oak. Midgut beta-glucosidase activity in the field collected termites was intermediate between the midgut activities seen on paper and pine. Among the three diets, midgut activity was highest on paper and lowest on oak. Hindgut beta-glucosidase activity in the field collected termites was higher than the hindgut activity seen on all three diets. Among the three diets, hindgut activity was highest on oak and lowest on paper.

Discussion

The overall gut morphology is consistent with that described for *R. flavipes* in previous studies. The appearance of the termite guts upon dissection, particularly the hindguts, indicated that the termites had fed on their respective diets. Based on the relatively dark gut contents of the field termites, as well as the presence of dark oval objects in some cases, it is possible that these termites were feeding on partially decayed materials.

Based on the observed locations of the activities, it is evident that both endoglucanases and beta-glucosidases are produced by both the termite and its symbionts. Endogenous endoglucanases are produced in the foregut, while symbiont endoglucanases are produced in the hindgut. Beta-glucosidase activities have been seen throughout the termite gut, leading to the conclusion that endogenous beta-glucosidases are produced in the foregut and midgut, while symbiont beta-glucosidases are produced in the hindgut.

The overall cellulase activity pattern found in this study was similar to that described by Inoue et al. (1997) for R. speratus, but the levels of activity are roughly 10 to 100-fold lower in this study. These different results are due to a number of factors. First, rather than an exoglucanase assay, Inoue et al. (1997) performed a total cellulase assay, so these data cannot be

directly compared. In addition, Inoue et al. used tetrazolium blue rather than DNSA to determine the reducing sugars generated by endoglucanase. The advantage of tetrazolium blue is that it is highly sensitive to reducing sugars. However, tetrazolium blue reacts continuously at room temperature and the reacted reagent eventually precipitates out of solution. The DNSA reagent requires boiling to react with reducing sugars. Therefore, it is possible to get more precise reaction times, reducing the variance of the results. The enzymatic reactions in their study were conducted at a higher incubation temperature (25^{o}C) than ours (23^{o}C).

In addition, we cannot directly compare the results because the two assays were carried out on different species. The Inoue study used R. speratus collected from logs, while our study used *R. flavipes* collected from cardboard traps. When these differences in methods are taken into account, our findings are consistent with those of Inoue et al. (1997).

The overall endoglucanase activities did not differ significantly among the three diets, but the source of the enzymes did. The activities of both endogenous endoglucanase, produced by the termite in the foregut and midgut, and symbiotic endoglucanase, produced by microbes in the hindgut, varied among diets. Endogenous activities were highest on paper and lowest on oak, but the exact reverse trend was seen with the symbiont endoglucanase activities, which were highest on the oak diet and lowest on paper. Based upon these data, it is probable that either the termite is changing its endogenous enzyme output in response to fluctuations in its symbiont enzymes, or else the symbiont population is fluctuating in response to the levels of endogenous termite enzymes.

If the termite is responding to changes in its symbiont community composition, then some qualities of the diet must be driving the symbiont population changes. It has previously been demonstrated that symbionts are responsible for virtually all of the xylanolytic activity seen in *R.*

flavipes worker termites (Zhou et al. 2007, Smith and Koehler 2007). It is reasonable to suppose that the xylan content of a given diet may affect the symbiont populations, and therefore affect the activity levels of the enzymes produced by these symbionts. All symbiont enzyme activities were significantly higher on diets with a higher xylan content. Hindgut activities in all cases were the highest on the oak diet. Hardwoods typically contain more than twice as much xylan as softwoods (Pettersen 1984). At the same time, hindgut activities were lowest on the paper diet, a diet completely lacking in xylan.

Alternatively, the quality of the cellulose content in the diet may be affecting the termite's ability to digest it without the aid of symbionts. While the termite produces its own endoglucanases and beta-glucosidases, it relies on its symbionts for at least the majority of its exoglucanase production. This means that the termite is most likely capable of degrading amorphous cellulose, but relies heavily upon its symbionts to digest crystalline cellulose. The proportion of crystalline cellulose may be anywhere from 50% to 70% of total cellulose content in different wood species (Biermann 1996). The process of making paper partially denatures the cellulose microfibers in wood, and may increase the ratio of amorphous cellulose to crystalline cellulose. The increase in endogenous cellulolytic activities on the paper diet may therefore be due to greater availability of substrates that the termite can digest without the aid of its symbionts. With more of this digestion and absorption achieved before the food reaches the hindgut, the resident symbiont population may be reduced by the more limited nutrient availability.

Studies on *Reticulitermes speratus* (Azuma et al. 1993, Inoue et al. 1997), *Reticulitermes virginicus* (Cook and Gold 2000) and *Coptotermes formosanus* (Mannesmann 1972, Waller and La Fage 1987) have demonstrated significant changes in the hindgut protozoan communities in

response to different diets, including different wood species, pure cellulose and pure xylan. It is probable that the hindgut enzyme changes in *R. flavipes* are due to changes in the protozoan populations. Alternatively, there is the possibility that individual termite symbionts change their levels of enzyme production in response to differing diets.

It is apparent that *R. flavipes* workers are quite capable of digesting cellulose, following the same pattern seen in Chapter 4. Crystalline cellulose is mainly digested by hindgut symbionts while 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 them to efficiently utilize 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 also makes termite control by means of cellulase inhibition more difficult.

Table 5-1. Endoglucanase activities in the three gut regions of *Reticulitermes flavipes* workers in response to different field diets

Diet	Foregut	Midgut	Hindgut
Field	9.286 ± 0.165a	0.471 ± 0.107a	8.187 ± 0.938a
Paper	9.649 ± 0.246a	0.216 ± 0.102a	5.789 ± 0.610c
Pine	8.311 ± 1.048ab	0.328 ± 0.098a	6.765 ± 0.166bc
Oak	7.438 ± 0.664b	0.291 ± 0.185a	7.663 ± 0.842ab

Endoglucanase activities are in nmol reducing sugar per termite equivalent per min. Means within a column followed by the same letter are not significantly different, (Tukey-Kramer Adjusted Means Separation, $\alpha = 0.05$, SAS Institute 2001), n = 4 replicates.

Table 5-2. Exoglucanase activities in the three gut regions of *Reticulitermes flavipes* workers in response to different field diets

Diet	Foregut	Midgut	Hindgut
Field	0.0121 ± 0.0004a	0.0062 ± 0.0011bc	0.1654 ± 0.0026a
Paper	0.0062 ± 0.0006b	0.0083 ± 0.0002a	0.1011 ± 0.006d
Pine	0.0044 ± 0.0004c	0.0078 ± 0.0002ab	0.1486 ± 0.0032c
Oak	0.0031 ± 0.0008c	0.0054 ± 0.0002c	0.1554 ± 0.0032b

Exoglucanase activities are in nmol p-nitrophenol per termite equivalent per min. Means within a column followed by the same letter are not significantly different, (Tukey-Kramer Adjusted Means Separation, $\alpha = 0.05$, SAS Institute 2001), n = 4 replicates.

Table 5-3. Beta-glucosidaase activities in the three gut regions of *Reticulitermes flavipes* workers in response to different field diets

Diet	Foregut	Midgut	Hindgut
Field	0.1196 ± 0.0017b	0.2128 ± 0.0016a	0.3229 ± 0.0026a
Paper	0.1681 ± 0.0016a	0.2161 ± 0.0036a	0.2289 ± 0.0067d
Pine	0.0991 ± 0.0023c	0.1725 ± 0.0056b	0.2674 ± 0.0077c
Oak	0.0888 ± 0.0015d	0.1445 ± 0.0026c	0.3037 ± 0.0042b

Beta-glucosidase activities are in nmol p-nitrophenol per termite equivalent per min. Means within a column followed by the same letter are not significantly different, (Tukey-Kramer Adjusted Means Separation, $\alpha = 0.05$, SAS Institute 2001), n = 4 replicates.

CHAPTER 6
CHANGES IN *RETICULITERMES FLAVIPES* GUT XYLANOLYTIC ACTIVITIES IN RESPONSE TO DIET

Introduction

Subterranean termites, such as *Reticulitermes flavipes* (Kollar), subsist largely on a diet of wood and similar material (Noirot and Noirot-Timothee 1969). Wood is not a substance that most animals are capable of digesting to any significant degree. Termites are well known for the ability to digest cellulose, but there are other polysaccharides in wood, such as xylan, which the termites may utilize in order to get more energy out of this food source.

In wood, cellulose chains are typically arranged in parallel bundles known as microfibers, which are embedded in a matrix composed mainly of hemicelluloses and lignin. Hemicelluloses are polysaccharides like cellulose, but their chemical structures are far more variable. One of the well-studied varieties of hemicellulose is xylan, which is mainly a polymer of the pentose sugar xylose. Xylan is the predominant hemicellulose in hardwood. Softwood typically is much lower in xylan content, along with significant quantities of other hemicelluloses (Pettersen 1984).

Subterranean termites may encounter a number of potential food sources during foraging. These may be broadly grouped into hardwoods, softwoods, and man-made cellulosic materials like paper. These three groups vary mainly in their xylan content, with hardwoods containing the most xylan while man-made cellulosic materials contain virtually no hemicellulose. In homes, hardwoods may include furniture, flooring, and trim components. Structural timbers are nearly always derived from softwoods.

The gut of *R. flavipes* and related termites consists of a foregut with a small crop and a proventriculus, a fairly simple midgut and a greatly expanded hindgut containing several species of symbiotic protozoa, fungi, bacteria, and archaea (Yamin 1979, Lewis and Forschler 2004). A pair of salivary glands is also present, emptying into the foregut anterior to the crop. To

effectively digest wood, a chemically demanding and nutritionally poor substrate, termites like *R. flavipes* have developed an array of enzymes. Some carbohydrolases characterized from the Rhinotermitidae have been shown to be endogenous (Watanabe et al. 1998), while others are produced by microbial symbionts (Nakashima et al. 2002a, Watanabe et al. 2002, Ohtoko et al. 2000). Xylanolytic enzymes include xylanases (EC 3.2.1.8) which internally cleave the xylan chain and beta-xylosidases (EC 3.2.1.37) which cleave xylan oligosaccharides into xylose. Inoue et al. (1997) demonstrated that xylanase activity is largely localized to the hindgut of *Reticulitermes speratus* (Kolbe), strongly implicating the resident protozoa as the major agents of xylan digestion. In the same study, they showed that the protozoan composition of the R. speratus hindgut significantly changes when the termites are fed on pure cellulose or pure xylan, as opposed to wood. Through similar experiments in differential feeding, Azuma et al. (1993) showed that, within the termite *R. speratus*, protozoan symbionts in the genera *Pyrsonympha* and *Dinenympha* take part in xylan digestion. It is probable that, with the changes in protozoan populations, the capacity for xylan digestion may also change to adapt to the xylan content of the termite diet.

Our objectives were to determine 1) if *R. flavipes* can digest xylan, 2) the relative activities of xylanase and beta-xylosidase in different regions of the gut of *R. flavipes* workers, 3) possible correlations between these activities and dietary xylan content in five formulated cellulose/xylan diets, and 4) how these activities change in response to three different field diets.

Materials and Methods

Termite Collection

R. flavipes termites were field collected in Gainesville, Florida in termite traps consisting of a PVC bucket (20 cm high by 20 cm diam.; Item # 811192-4, Ventura Packaging Inc., Monroeville, Ohio) with 11 holes drilled in the sides and base (3 cm diam.) placed vertically in

the ground to a depth of roughly 19 cm and covered with a PVC lid. Three rolls of single-faced corrugated cardboard (20 cm long by 10 cm diam.) were placed into the bucket side by side as a food source. Termites were collected from the trap by removal of cardboard rolls, separated from the cardboard, and either placed in feeding bioassays or immediately frozen and kept at -80^{o}C until dissection. Termites were collected from one colony for field diet assays and another colony for formulated diet assays. Collections were restricted to a single colony in each case to eliminate colony as a source of variation in enzyme activities.

Termite Diets and Feeding

Five formulated diets consisting of microcrystalline cellulose and beechwood xylan (>90% xylose residues) (Sigma-Aldrich, Atlanta, GA) were prepared for the termites: 0%, 5%, 10%, 20%, and 40% xylan. The remainder of the diet was composed of cellulose in all cases. The cellulose and xylan were mixed together and water (1.5 mL/g) was added to make a paste. This paste was thoroughly kneaded to achieve homogeneity.

For each formulated diet, a glass jar (125 mL, straight-sided, Fisherbrand, Fisher Scientific, Pittsborough, PA) was prepared with 80 g of moist builders sand (10% moisture). The bottom was cut out of a plastic cup (5.92 mL Soufflé cup, Solo Cup Company, Urbana, IL), leaving approximately 2 mm of cup wall intact. Formulated diet (1 g) was placed in the cup bottom and this was placed on top of the sand in the jar. Termites were added (~200 workers and 2 soldiers per jar) and kept in the dark at 21^{o}C for 2 wk. Termites were collected, frozen and kept at -80^{o}C until dissection.

Three diets were prepared for the termites to represent the probable food sources they would encounter in the field. These field diets were as follows: red oak (Quercus spp.), pine (Pinus spp.), and filter paper. Wood diets were generated by drilling into craft wood boards (0.635 x 5.08 x 60.96 cm, TOMS) with a 2.54 cm spade drill bit. Sawdust was collected and

weighed. Filter paper consisted of a weighed number of crumpled cellulose filter paper disks (42.5 mm diameter, Whatman, grade 4).

Each field diet (20 g) was added to a loosely capped 250 mL glass bottle (Pyrex) with 5 mL of deionized water. Unlike the formulated diets, the field diets were not made from sterile materials. Because of this, the bottles with field diets were autoclaved on a liquid cycle (30 min, 122^{o}C) to sterilize the diets and the bottles were then allowed to cool. Termites were added (~300 workers and 3 soldiers per bottle) after the bottles had cooled and kept in the dark at 21^{o}C for 6 wk, with deionized water (2 mL) added every 2 wk. Termites were collected, frozen and kept at -80^{o}C until dissection.

Termite Dissection and Enzyme Extraction

Two buffers were prepared for use in the dissections: 0.1 M sodium acetate buffer, pH 5.5, and 0.1 M sodium phosphate buffer, pH 6.5. Sodium acetate buffer was used for the xylanase assay, while sodium phosphate buffer was used for the beta-xylosidase assays.

Termites were removed from the freezer and kept on ice until dissection. Each termite's gut was removed intact and separated into three regions: foregut (and salivary glands), midgut, and hindgut. A single enzyme extract was prepared from dissected termites from each feeding treatment for each assay type using an experimental design similar to previous termite carbohydrolase experiments (Hogan et al. 1988, Inoue et al. 1997, Nakashima et al. 2002b). For the xylanase assay, 50 termites from each feeding treatment were dissected in sodium acetate buffer. For the beta-xylosidase assays, 35 termites from each feeding treatment were dissected in sodium phosphate buffer. The three gut regions were placed into separate 1.5 mL microcentrifuge tubes (Eppendorf) containing the appropriate buffer, and kept on ice. Final concentrations were 50 termite gut regions per mL in all cases.

Enzymes were extracted using a method adapted from Inoue et al. (1997). 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^{o}C for 15 min. The supernatants were collected, frozen, and kept at -80^{o}C until use in the enzyme assays.

Xylanase Assays

The xylanase assays were conducted using a method adapted from Han et al. (1995). A 0.5% solution of beechwood xylan (>90% xylose residues; Sigma-Aldrich) was prepared in 0.1 M sodium acetate buffer, pH 5.5. The solution was boiled approximately 30 min, until xylan particles were no longer visible. The solution was centrifuged at 1250 g for 5 min at 23^{o}C and the supernatant was used as the xylan stock solution.

Assays were conducted in clear 96-well microplates. In each well, 10 μL of tissue extract was combined with 90 μL of xylan solution. The solutions were allowed to react for 35 min at 23^{o}C. DNSA solution (100 μL) consisting 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 placed on ice for 15 min. After cooling, each microplate was read at 540 nm using a μQuant Universal Microplate Spectrophotometer (Bio-Tek Instruments, Winooski, VT). Similar control plates were allowed to react for 5 min to allow for passive mixing of solutions before boiling with DNSA solution (Zhou et al. 2007). A 5 min reaction was used as a control to correct for any differences in initial sample reaction rates due to slow mixture of enzyme and substrate solutions. Standards were generated using dilutions of xylose. Only the wells in the middle of the microplate were used for these assays. Perimeter wells were filled with deionized water (200 μL per well) to add temperature stability and consistency during boiling. For all replicates, the control plates were used to adjust for 540 nm

absorbance in gut extracts and were replicated an equal number of times to the assay plates, with one microplate well for each replicate.

Beta-Xylosidase Assays

Beta-xylosidase assays were conducted using a method adapted from Han et al. (1995). A solution of 4 mM p-nitrophenyl-β-D-xylopyranoside (pNPX; Sigma-Aldrich) was prepared in 0.1 M sodium phosphate buffer, pH 6.5. Assays were conducted in clear 96-well microplates. In each well, 10 μL of tissue extract was combined with 90 μL of pNPX solution. The reaction was allowed to proceed for 10 min before being placed in a μQuant Universal Microplate Spectrophotometer (Bio-Tek Instruments, Winooski, VT). Absorbance was read at 420 nm every 2 min for 30 min at 23^{o}C. Mean velocities (mOd/s) were recorded. Standards were generated using dilutions of p-nitrophenol.

Data Analysis

The xylanase assays were set up as one-factor split-plot designs with seven technical replicates per gut region for each formulated diet, four technical replicates per gut region for each simulated field diet, and one microplate per replicate. Four microplate wells were assayed for each replicate of each gut region/diet combination. The beta-xylosidase assays were set up as one-factor designs with four technical replicates per gut region for each formulated diet and simulated field diet. Two homogenates were used for each gut/diet combination; one homogenate for xylanase assays and another for beta-xylosidase assays.

For the xylanase assays, the following formula was used to calculate specific activities;

$SA = C_S[(A-A_0)/t]/N_T$

where: SA = specific activity (nmol reducing sugar per termite equivalent per min), A = absorbance (Od) after 35 min reaction, A_0 = absorbance (Od) for the corresponding control after

5 min reaction, t = time (min), C_S = the coefficient derived from the standard (nmol reducing sugar/mOd), and N_T = the number of termite equivalents per sample.

For the beta-xylosidase assays, the following formula was used to calculate specific activities;

$$SA = 60C_SV_A/N_T$$

where: SA = specific activity (nmol p-nitrophenol per termite equivalent per min), V_A = mean velocity of absorbance change (mOd/s), C_S = the coefficient derived from the standard (nmol p-nitrophenol/mOd), and N_T = the number of termite equivalents per sample.

The field diet data were analyzed using a mixed model analysis of variance. Fixed effects were diet treatment and gut region. The Tukey-Kramer adjustment ($\alpha = 0.05$) was used to separate the mean activities on each diet within each gut region (SAS Institute 2001).

Results

Termite Gut Observations

During dissection, after the termites had been fed on the various diets, the color of the termite guts reflected the color of the different diets. This was especially evident in the enlarged hindguts which were typically filled with a mixture of partially digested food and resident microbes. Termites fed on formulated diets showed an increasing brown shade in their gut contents on diets containing more xylan. Termites fed on red oak had brownish-orange gut contents. Those fed on pine had pale yellow gut contents. Those fed on paper had white gut contents. The termites collected from the field and immediately frozen for dissection had relatively dark gut contents.

Xylanase Activities

Both foregut and midgut xylanase activities were insignificant in comparison with hindgut xylanase activity on all of the formulated diets (Figure 6-1). Hindgut xylanase activity was

significantly greater in termites kept on 20% and 40% xylan diets than in termites kept on 0%, 5% and 10% xylan diets (Figure 6-1). Although there was higher activity in termites fed on 5% xylan than 10% xylan, the difference was not significant.

Foregut xylanase activity was greater in the field-collected termites than activities in termites fed on any of the three field diets (Table 6-1). Foregut activities on pine and paper diets differed significantly, with activity on pine being the highest and activity on paper being the lowest. Midgut xylanase activity in field-collected termites was between the activities on pine and oak diets. Among the field diets, midgut activity was significantly highest on pine and lowest on paper. Hindgut xylanase activity was >92% of total xylanase activity among the different diets. The hindgut activity of field-collected termites was slightly higher than that on paper, but not significantly so. The hindgut activity differed significantly among the three field diets, being highest on oak and lowest on paper.

Beta-Xylosidase Activities

Overall beta-xylosidase activity showed a general increase from termites fed 0% xylan to termites fed 40% xylan (Figure 6-2). There were no significant differences among the foregut beta-xylosidase activities on the formulated diets. Midgut beta-xylosidase activities were greatest in termites fed 10% xylan. Activities were intermediate and nearly equal on 5% and 20% xylan, and they were lowest and nearly equal on 0% and 40% xylan. Hindgut beta-xylosidase activities showed a steady increase on the formulated diets from 5% xylan to 40% xylan (Figure 6-2). Activities in termites fed 20% and 40% xylan were significantly greater than those in termites fed 0% and 5% xylan. In addition, beta-xylosidase activities in termites fed 40% xylan were significantly greater than activities in termites fed 10% xylan.

There were no significant differences among the foregut beta-xylosidase activities in termites fed field diets (Table 6-2). Midgut beta-xylosidase activity was lowest on the field-

collected termites, which did not differ significantly from the activity on pine. Among the diets, midgut activity was highest on paper and lowest on pine. Similar to xylanase activity, beta-xylosidase activity was predominantly located in the hindgut. However, this was not as pronounced as with xylanase, as hindgut beta-xylosidase activity ranged from ~50 to 75% of total beta-xylosidase activity. Hindgut activity of the field-collected termites was intermediate between the activities on oak and pine diets. Among the field diets, activity was highest on oak and lowest on paper. Hindgut beta-xylosidase activities on all four of these treatments were significantly different from one another.

Discussion

The overall gut morphology of *R. flavipes* workers was similar to that described for *Zootermopsis* (Child 1946) and is consistent with that found in other lower termites (Noirot and Noirot-Timothee 1969). The appearance of the termite guts upon dissection, particularly the hindguts, indicated that the termites had fed on their respective diets. Based on the relatively dark gut contents of the field-collected termites, it was apparent that these termites were feeding on relatively dark materials, such as cardboard or pine bark mulch, associated with the termite traps.

Among the termites in the colony fed on formulated diets, both xylanase and beta-xylosidase total activities were significantly higher on diets with higher xylan content. The hindgut activities in particular, which formed the majority of total activities, also followed this pattern. A similar pattern was seen in termites from the colony fed on field diets. All xylanolytic activities were highest on the oak diet. Hardwoods typically consist of roughly 20% xylan, twice as much as the typical softwood xylan content of roughly 10% (Biermann 1996). At the same time, hindgut activities were lowest on the paper diet, a diet completely lacking in xylan. Just as termites fed on 20% xylan showed far greater xylanase activity than termites fed

on 10% xylan, so there was a similar jump in activity from pine fed termites to oak fed termites. There was a much smaller gap between the hindgut xylanase activities of paper fed and pine fed termites, as there was a much smaller gap between the activities of termites fed 0% and 10% xylan. The hindgut beta-xylosidase patterns were also consistent between termites fed on formulated diets and termites fed on field diets.

Our data showed that the majority of xylanase activity was in the *R. flavipes* hindgut. This is consistent with previous findings where xylanase activities were almost exclusively located in the hindgut of *R. speratus* and *Coptotermes heimi* (Wasmann), and associated with the symbionts (Inoue et al. 1997, Mishra 1991). Therefore, it was evident that *R. flavipes* workers follow a typical xylan digestion pattern for subterranean termites, where this hemicellulose is mainly digested by hindgut symbionts.

The overall digestion pattern of xylanase and beta-xylosidase activity found in our study using the field termites was similar to that found by Inoue et al. (1997), but the levels of activity are roughly 10 to 100-fold lower in our study. These differences may be due to differences in assay reagents or differences in termite species. Inoue et al. (1997) used tetrazolium blue rather than DNSA to determine the reducing sugars generated by xylanase. We used the DNSA reagent because it requires boiling to react with reducing sugars, in contrast with tetrazolium blue, which reacts continuously at room temperature. This boiling requirement allowed more precise control of reaction times, limiting the overestimation of activity. Our findings are consistent with those of Inoue et al. (1997) when the differences in methods are taken into account. Inoue et al. (1997) observed no beta-xylosidase activity in the foregut of *R. speratus*, in contrast with our findings of minor beta-xylosidase activity in the foregut of *R. flavipes*. We cannot directly compare these results because the two assays were carried out on different species of termite; the Inoue et al.

(1997) study used *R. speratus* collected from logs, while our study used *R. flavipes* collected from cardboard traps.

The low hindgut xylanolytic activities on the paper diet and the 0% xylan formulated diet were consistent with the absence of xylan in the paper diet. However, significant xylanase and beta-xylosidase activities were still present even in the absence of dietary xylan. This suggests the presence of symbionts that constitutively produce xylanases and beta-xylosidases, but are capable of subsisting solely on a cellulose diet. The relatively low hindgut xylanolytic activity in the field-collected termites, similar to the activity on the paper diet, was most likely due either to feeding on the cardboard in the termite traps, or feeding on some form of softwood such as pine mulch.

Symbiont xylanase and beta-xylosidase activities change significantly in response to diet, consistent with xylan content. It is apparent that the community of hindgut symbionts is able to adapt to a wide range of dietary xylan content. Studies on *R. speratus* (Azuma et al. 1993, Inoue et al. 1997), *Reticulitermes virginicus* (Banks) (Cook and Gold 2000) and *Coptotermes formosanus* (Shiraki) (Mannesmann 1972, Waller and La Fage 1987) have demonstrated significant changes in the hindgut protozoan communities in response to different diets. Many of these studies focused on feeding termites different wood species. It is therefore probable that the xylanolytic enzyme changes in *R. flavipes* are due to changes in hindgut symbiont populations. Alternatively, it is possible that these enzyme changes are due to changes in xylanolytic enzyme production within the hindgut symbionts.

R. flavipes workers are capable of digesting xylan, following the same pattern seen in Chapter 4, where xylan is mainly digested in the hindgut. The xylanolytic activities of the two termite colonies we investigated changed to accommodate dietary xylan content, most likely by

changes in the hindgut symbiont communities. These termites' capacity for xylan digestion allows them to gain more energy from a wood diet, and their flexibility allows them to efficiently utilize diets of varying xylan content.

Compared to cellulose, xylan is often overlooked as a starting compound for the production of alternative fuels. However, xylan may comprise up to a quarter of wood, depending on the species, and a significant proportion of other plant materials used for alternative fuel production. These termites and their symbionts may provide candidate enzymes for the degradation of xylan in this process.

Table 6-1. Xylanase activities in the three gut regions of *Reticulitermes flavipes* workers in response to different field diets

Diet	Foregut	Midgut	Hindgut
Field	0.833 ± 0.048a	0.258 ± 0.064a	12.672 ± 0.249c
Paper	0.062 ± 0.044b	0.030 ± 0.078a	12.436 ± 0.260c
Pine	0.216 ± 0.051ab	0.324 ± 0.088a	13.672 ± 0.335b
Oak	0.017 ± 0.045ab	0.196 ± 0.052a	22.953 ± 0.315a

Xylanase activities are in nmol reducing sugar per termite equivalent per min. Means within a column followed by the same letter are not significantly different, (Tukey-Kramer Adjusted Means Separation, $\alpha = 0.05$, SAS Institute 2001), n = 4 replicates.

Table 6-2. Beta-xylosidase activities in the three gut regions of *Reticulitermes flavipes* workers in response to different field diets

Diet	Foregut	Midgut	Hindgut
Field	2.50 ± 0.22a	4.08 ± 0.21b	21.08 ± 0.20b
Paper	2.63 ± 0.05a	9.25 ± 0.09a	11.91 ± 0.32d
Pine	1.82 ± 0.23a	5.70 ± 0.09b	16.91 ± 0.32c
Oak	2.49 ± 0.05a	7.04 ± 0.32b	28.26 ± 1.00a

Beta-xylosidase activities are in pmol p-nitrophenol per termite equivalent per min. Means within a column followed by the same letter are not significantly different, (Tukey-Kramer Adjusted Means Separation, $\alpha = 0.05$, SAS Institute 2001), n = 4 replicates.

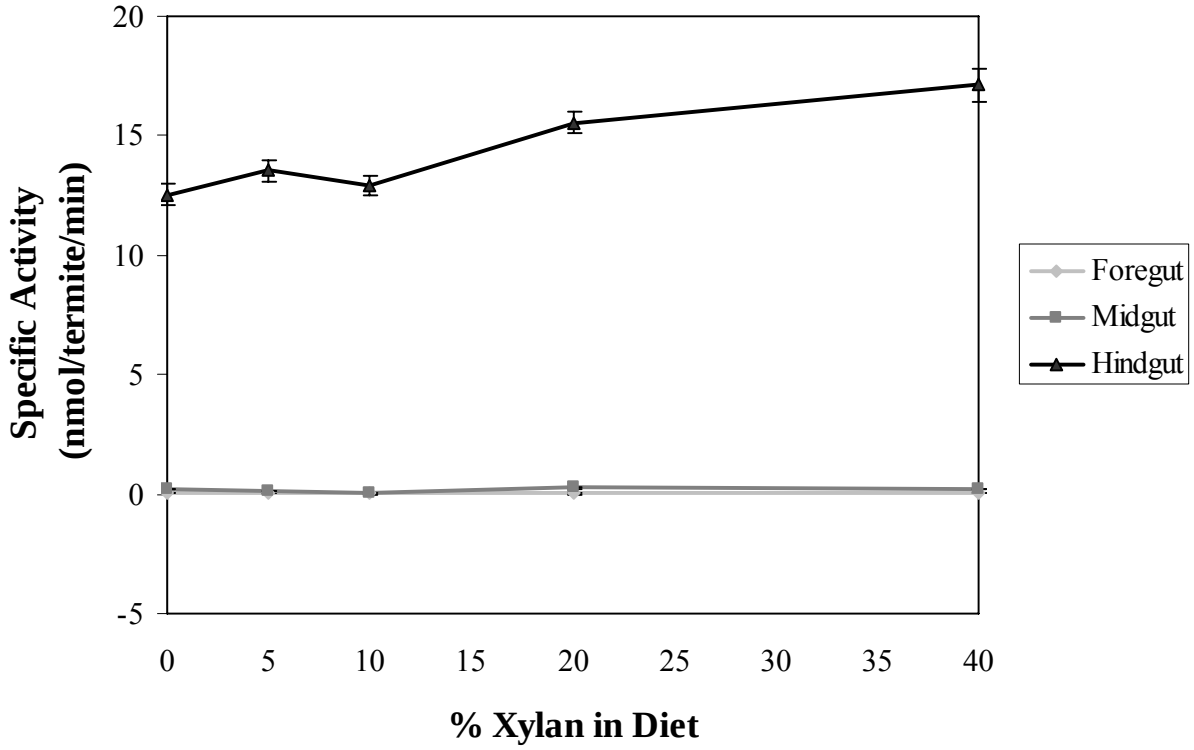

Figure 6-1. Xylanase activities in different gut regions of *Reticulitermes flavipes* in response to dietary xylan content.

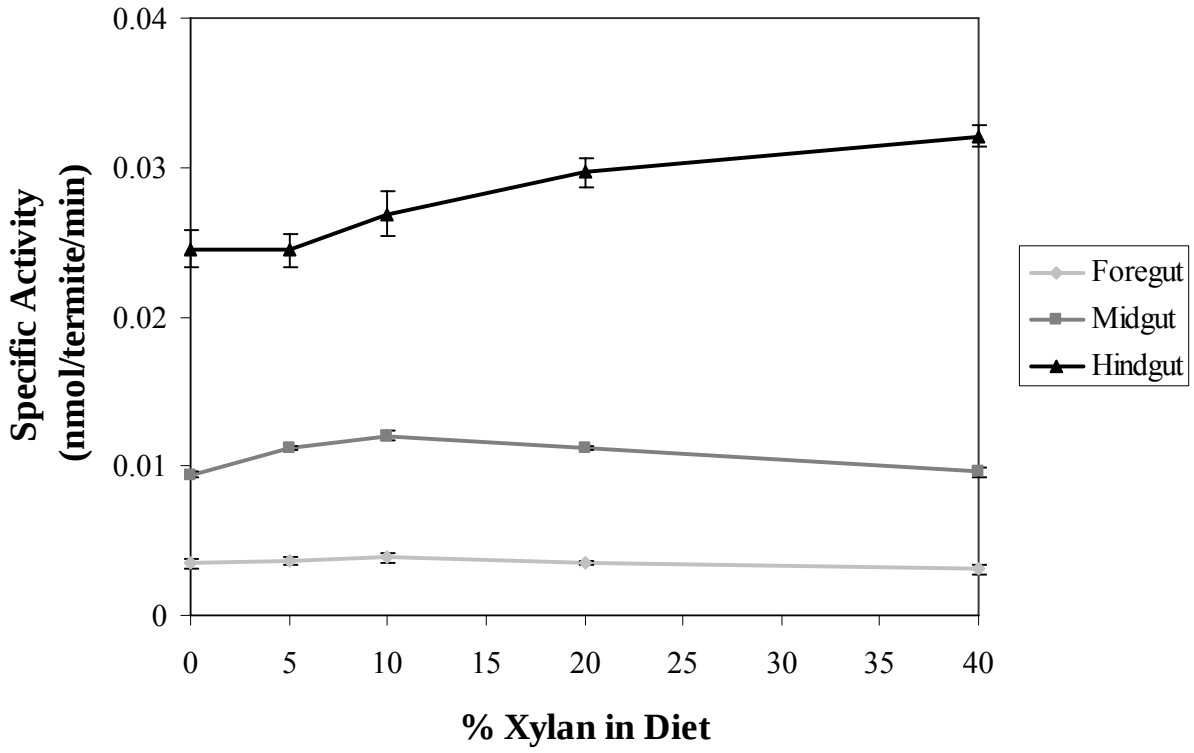

Figure 6-2. Beta-Xylosidase activities in different gut regions of *Reticulitermes flavipes* in response to dietary xylan content.

CHAPTER 7
CHANGES IN *COPTOTERMES FORMOSANUS* GUT CELLULOLYTIC ACTIVITIES IN RESPONSE TO DIET

Introduction

Subterranean termites, such as *Coptotermes formosanus* (Shiraki), subsist largely on a diet of wood and similar material. Wood is not a substance that most animals are capable of digesting to any significant degree. Termites are well known for the ability to digest cellulose with the aid of microbial hindgut symbionts.

In wood, cellulose chains are typically arranged in parallel bundles known as microfibers which are embedded in a matrix of lignin and hemicelluloses. The cellulose in the microfibers may be broadly divided into two types: crystalline and amorphous. The crystalline form of cellulose consists of tightly aligned parallel chains, held in a specific configuration by hydrogen bonding. In the amorphous form of cellulose, the chains are more randomly arranged, and not so closely bound together.

There are three major types of cellulase which work together to digest cellulose. Exoglucanase (EC 3.2.1.91) cleaves the cellulose chain from the ends, typically producing cellobiose, and is most active against crystalline cellulose. Endoglucanase (EC 3.2.1.4) cleaves the cellulose chain randomly along its length and is most active against amorphous cellulose. Beta-Glucosidase (3.2.1.21) cleaves cellobiose and other small cellulose fragments, hydrolyzing them to glucose.

The gut of *C. formosanus* and related termites consists of a foregut with a small crop and a proventriculus, a fairly simple midgut, and a greatly expanded hindgut containing four major species symbiotic protozoa, as well as several species of bacteria and archaea (Yamin 1979). A pair of salivary glands is also present, emptying into the foregut anterior to the crop. To effectively digest wood, a recalcitrant and nutritionally poor substrate, termites like *C.*

formosanus have developed an array of enzymes. Some of the cellulases characterized from the Rhinotermitidae have been shown to be endogenous (Watanabe et al. 1998, Zhou et al. 2007), while others are produced by microbial symbionts (Nakashima et al. 2002a, Watanabe et al. 2002, Ohtoko et al. 2000). Zhou et al. (2007) demonstrated that exoglucanase activity is largely localized to the hindgut of *C. formosanus*, strongly implicating the resident flagellates as the major 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 on pure cellulose or pure xylan, as opposed to wood. It is probable that, with changes in protozoan populations on differing diets, the levels of different cellulase activities may also change.

Subterranean termites may encounter a number of potential food sources during foraging. These may be broadly grouped into hardwoods, softwoods, and processed cellulosic materials like paper. These three groups vary mainly in their hemicellulose content. In homes, hardwoods may include furniture, flooring, and trim components. Structural timbers are nearly always derived from softwoods.

Our objectives were to determine: 1) the distribution of the three major cellulase activities within the gut of *C. formosanus* workers and 2) how these activities change in response to three different simulated field diets.

Materials and Methods

Termite Collection

Termites were field collected in First Line Smartdisc monitors (FMC Corporation) in Charleston, South Carolina. Termites were collected from the trap by removal of the wood (southern yellow pine) in the bait stations and brought to the University of Florida. Termites

were then placed in feeding bioassays. Collections were restricted to a single colony to eliminate colony as a source of variation in enzyme activities.

Termite Diets and Feeding

Three diets were prepared for the termites to represent the probable food sources they would encounter in the field. These simulated field diets were as follows: red oak (Quercus spp.), pine (Pinus spp.), and filter paper. Wood diets were generated by drilling into craft wood boards (0.635 x 5.08 x 60.96 cm) with a 2.54 cm spade drill bit. Sawdust was collected and weighed. Filter paper consisted of a weighed number of crumpled cellulose filter paper disks (42.5 mm diameter, Whatman, grade 4).

For each simulated field diet, a mason jar (# mL, brand) was prepared with 40 g of moist builders sand (10% moisture). Diet (10 g) was then added with 40 mL of deionized water and the jar was loosely capped. The jars with simulated field diets were autoclaved on a liquid cycle (30 min, 122^{o}C) to sterilize the diets and the jars were then allowed to cool. Termites were added (~270 workers and 30 soldiers per jar) after the jars had cooled and kept in the dark at 21^{o}C for 6 wk. Termites were collected, frozen and kept at -80^{o}C until dissection.

Termite Dissection and Enzyme Extraction

Two buffers were prepared for use in the dissections: 0.1 M sodium acetate buffer, pH 5.5, and 0.1 M sodium phosphate buffer, pH 6.5. Sodium acetate buffer was used for the endoglucanase assay, while sodium phosphate buffer was used for the exoglucanase and beta-glucosidase assays.

Termites were removed from the freezer and kept on ice until dissection. Each termite's gut was removed intact and separated into three regions: foregut (and salivary glands), midgut, and hindgut. A single enzyme extract was prepared from dissected termites from each feeding treatment for each assay type using an experimental design similar to the recent classic termite

carbohydrolase experiments (Hogan et al. 1988, Inoue et al. 1997, Nakashima et al. 2002b). For the endoglucanase assay, 25 termites from each feeding treatment were dissected in sodium acetate buffer. For the exoglucanase and beta-glucosidase assays, 25 termites from each feeding treatment were dissected in sodium phosphate buffer. The three gut regions were placed into separate 1.5 mL microcentrifuge tubes (Eppendorf) containing the appropriate buffer, and kept on ice. Final concentrations were 50 termite gut regions per mL in all cases.

Enzymes were extracted using a method adapted from Inoue et al. (1997). 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^{o}C for 15 min. The supernatants were collected, frozen, and kept at -80^{o}C until use in the enzyme assays.

Endoglucanase Assays

The endoglucanase assays were conducted using a method adapted from Han et al. (1995). A 2% solution of carboxymethylcellulose (CMC; Sigma-Aldrich) was prepared in 0.1 M sodium acetate buffer, pH 5.5.

Assays were conducted in clear 96-well microplates. In each well, 10 μL of tissue extract was combined with 90 μL of xylan solution. The solutions were allowed to react for 70 min at 23^{o}C. DNSA solution (100 μL) consisting 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 placed on ice for 15 min. After cooling, each microplate was read at 540 nm using a μQuant Universal Microplate Spectrophotometer (Bio-Tek Instruments, Winooski, VT). Similar 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). A 10 min reaction was used as a control to correct for any differences in initial sample reaction

rates due to incomplete mixture of enzyme and substrate solutions. Standards were generated using dilutions of glucose. Only the wells in the middle of the microplate were used for these assays. Perimeter wells were filled with deionized water (200 μL per well) to add temperature stability and consistency during boiling. For all replicates, the control plates were used to adjust for 540 nm absorbance in gut extracts and were replicated an equal number of times to the assay plates, with one microplate well for each replicate.

Exoglucanase and Beta-glucosidase Assays

The exoglucanase and beta-glucosidase assay was conducted using a method adapted from Han et al. (1995). Solutions of 4 mM p-nitrophenyl-β-D-cellobioside (pNPC) and 4 mM p-nitrophenyl-β-D-glucopyranoside (pNPG) were prepared in 0.1 M sodium phosphate buffer, pH 6.5. Assays were conducted in clear 96-well microplates. In each well, 10 μL of tissue extract was combined with 90 μL of pNPC or pNPG solution. The reaction was allowed to proceed for 10 min before being placed in a μQuant Universal Microplate Spectrophotometer (Bio-Tek Instruments, Winooski, VT). Absorbance was read at 420 nm every 2 min for 30 min at 23^{o}C. Mean velocities (mOd/s) were recorded. Standards were generated using dilutions of p-nitrophenol.

Data Analysis

The endoglucanase assays were set up as a one-factor design with four technical replicates for each gut region. Four microplate wells were assayed for each replicate of each gut region/diet combination. The exoglucanase and beta-glucosidase assays were set up as one-factor designs with four technical replicates for each gut region. Two homogenates were used for each gut/diet combination; one homogenate for endoglucanase assays and another for exoglucanse and beta-glucosidase assays.

For the endoglucanase assays, the following formula was used to calculate specific activities;

$SA = C_S[(A-A_0)/t]/N_T$

where: SA = specific activity (nmol reducing sugar per termite equivalent per min), A = absorbance (Od) after 35 min reaction, A_0 = absorbance (Od) for the corresponding control after 5 min reaction, t = time (min), C_S = the coefficient derived from the standard (nmol reducing sugar/mOd), and N_T = the number of termite equivalents per sample.

For the exoglucanase and beta-glucosidase assays, the following formula was used to calculate specific activities;

$SA = 60C_SV_A/N_T$

where: SA = specific activity (nmol p-nitrophenol per termite equivalent per min), V_A = mean velocity of absorbance change (mOd/s), C_S = the coefficient derived from the standard (nmol p-nitrophenol/mOd), and N_T = the number of termite equivalents per sample.

The field diet data were analyzed using a mixed model analysis of variance. Fixed effects were diet treatment and gut region. The Tukey-Kramer adjustment ($\alpha = 0.05$) was used to separate the mean activities on each diet within each gut region (SAS Institute 2001).

Results

Termite Gut Observations

During dissection, after the termites had been fed on the various diets, the color of the termite guts reflected the color of the different diets. This was especially evident in the enlarged hindguts, which were typically filled with a mixture of partially digested food and resident microbes. Termites fed on red oak had brownish-orange gut contents. Those fed on pine had pale yellow gut contents. Those fed on paper had white gut contents.

Endoglucanase Activities

Most of the endoglucanase activity was located in the foregut and the hindgut, with relatively little in the midgut (Table 7-1). Among the three diets, there were no significant differences in foregut activities. Midgut activities were significantly lower on the paper diet than on the other two diets. Hindgut activities were significantly higher on the oak diet than on the other two diets.

Exoglucanase Activities

Most of the exoglucanase activity was located in the hindgut, with very little in the foregut or midgut (Table 6-2). Hindgut exoglucanase activity was significantly different among all three diets, being highest on oak and lowest on pine.

Beta-glucosidase Activities

Most of the beta-glucosidase activity was located in the midgut and hindgut, with relatively little in the foregut (Table 6-3). Foregut beta-glucosidase activity was significantly different among all three diets, being highest on pine and lowest on paper. Midgut beta-glucosidase activity was significantly different among all three diets, being highest on pine and lowest on oak. Hindgut beta-glucosidase activity was significantly different among all three diets, being highest on oak and lowest on paper.

Discussion

The overall gut morphology observed is consistent with that described for *C. formosanus* in previous studies. The appearance of the termite guts upon dissection, particularly the hindguts, indicated that the termites had fed on their respective diets.

Based on the observed locations of the activities, it is evident that both endoglucanases and beta-glucosidases are produced by both the termite and its symbionts. Endogenous endoglucanases are produced in the foregut, and such endoglucanases have been characterized in

a number of termite species from the genera *Reticulitermes* and *Coptotermes* (Watanabe et al. 1998, Nakashima et al. 2002b). The endoglucanase activities observed in the hindgut are most likely derived from the hindgut flagellates, as a number of endoglucanases have been characterized from these *C. formosanus* symbionts (Nakashima et al., 2002a; Watanabe et al., 2002b). Beta-glucosidase activities have been seen throughout the termite gut, leading to the conclusion that endogenous beta-glucosidases are produced in the foregut and midgut, while symbiont beta-glucosidases are produced in the hindgut.

In contrast to the other cellulolytic activities, exoglucanase activity appears to be almost entirely due to the symbionts. This is evident based on the almost exclusive distribution of exoglucanase activity within the hindgut. Similar patterns have been observed in *Coptotermes lacteus* (Hogan et al. 1988).

It is possible that endogenous cellulase expression is inducible by, or at least responsive to, the quality of dietary cellulose. While the termite produces its own endoglucanases and beta-glucosidases, it primarily relies on its symbionts for exoglucanase production. This means that the termite is most likely capable of degrading amorphous cellulose, but relies upon its symbionts to digest crystalline cellulose. The increase in endogenous cellulolytic activities on the paper diet may therefore be due to greater availability of substrates, such as amorphous cellulose, that the termite can digest without the aid of its symbionts.

Symbiont cellulase activities change significantly in response to diet, increasing significantly in wood as opposed to paper, and in oak as opposed to pine. It is apparent that the community of hindgut symbionts is able to adapt to a range of diets, with differences in cellulose quality and hemicellulose content.

Studies on *Reticulitermes speratus* (Azuma et al. 1993, Inoue et al. 1997), *Reticulitermes virginicus* (Cook and Gold 2000) and *Coptotermes formosanus* (Mannesmann 1972, Waller and La Fage 1987) have demonstrated significant changes in the hindgut protozoan communities in response to different diets, including different wood species, pure cellulose and pure xylan. It is probable that the hindgut enzyme changes in *C. formosanus* are due to these changes in its protozoan communities.

It is apparent that *C. formosanus* workers are quite capable of digesting cellulose, following the same pattern seen in Chapter 4. Crystalline cellulose is mainly digested by hindgut symbionts in while amorphous cellulose and cellodextrins are digested by both the termites and its symbionts. Moreover, the balance of endogenous versus symbiont cellulolytic activities changes in response to their diet, most likely by changes in the hindgut protozoan communities as well as the termite enzyme expression. This flexibility allows these termites to efficiently utilize a variety of wood species and wood-derived materials which have different qualities of cellulose. This capacity for adaptation also makes termite control by means of cellulase inhibition more difficult.

Table 7-1. Endoglucanase activities in the three gut regions of *Coptotermes formosanus* workers in response to different field diets

Diet	Foregut	Midgut	Hindgut
Paper	4.38 ± 0.48a	2.91 ± 0.21b	4.85 ± 0.77b
Pine	4.52 ± 0.52a	2.82 ± 0.25b	7.94 ± 0.60a
Oak	6.28 ± 0.75a	4.06 ± 0.36a	8.70 ± 0.87a

Endoglucanase activities are in nmol reducing sugar per termite equivalent per min. Means within a column followed by the same letter are not significantly different, (Tukey-Kramer Adjusted Means Separation, $\alpha = 0.05$, SAS Institute 2001), n = 4 replicates.

Table 7-2. Exoglucanase activities in the three gut regions of *Coptotermes formosanus* workers in response to different field diets

Diet	Foregut	Midgut	Hindgut
Paper	0.022 ± 0.003a	0.099 ± 0.002a	0.448 ± 0.027b
Pine	0.019 ± 0.001a	0.099 ± 0.002a	0.371 ± 0.036c
Oak	0.018 ± 0.002a	0.074 ± 0.002b	0.589 ± 0.047a

Exoglucanase activities are in nmol p-nitrophenol per termite equivalent per min. Means within a column followed by the same letter are not significantly different, (Tukey-Kramer Adjusted Means Separation, $\alpha = 0.05$, SAS Institute 2001), n = 4 replicates.

Table 7-3. Beta-glucosidaase activities in the three gut regions of *Coptotermes formosanus* workers in response to different field diets

Diet	Foregut	Midgut	Hindgut
Paper	0.155 ± 0.003c	0.852 ± 0.005b	0.489 ± 0.005c
Pine	0.221 ± 0.003a	0.907 ± 0.016a	0.623 ± 0.012b
Oak	0.168 ± 0.001b	0.770 ± 0.010c	0.728 ± 0.009a

Beta-glucosidase activities are in nmol p-nitrophenol per termite equivalent per min. Means within a column followed by the same letter are not significantly different, (Tukey-Kramer Adjusted Means Separation, $\alpha = 0.05$, SAS Institute 2001), n = 4 replicates.

CHAPTER 8
CHANGES IN *COPTOTERMES FORMOSANUS* GUT XYLANOLYTIC ACTIVITIES IN RESPONSE TO DIET

Introduction

Subterranean termites, such as *Coptotermes formosanus* (Shiraki), subsist largely on a diet of wood and similar material. Wood is not a substance that most animals are capable of digesting to any significant degree. Termites are well known for the ability to digest cellulose, but there are other polysaccharides in wood, such as xylan, which the termites may utilize in order to get more energy out of this food source.

In wood, cellulose chains are typically arranged in parallel bundles known as microfibers, which are embedded in a matrix composed mainly of hemicelluloses and lignin. Hemicelluloses are polysaccharides like cellulose, but their chemical structures are far more variable. One of the well-studied varieties of hemicellulose is xylan, which is mainly a polymer of the pentose sugar xylose. Xylan is the predominant hemicellulose in hardwood. Softwood typically is much lower in xylan content, along with significant quantities of other hemicelluloses (Pettersen 1984).

Subterranean termites may encounter a number of potential food sources during foraging. These may be broadly grouped into hardwoods, softwoods, and man-made cellulosic materials like paper. These three groups vary mainly in their xylan content, with hardwoods containing the most xylan while man-made cellulosic materials contain virtually no hemicellulose. In homes, hardwoods may include furniture, flooring, and trim components. Structural timbers are nearly always derived from softwoods.

The gut of *C. formosanus* and related termites consists of a foregut with a small crop and a proventriculus, a fairly simple midgut and a greatly expanded hindgut containing four main species of symbiotic protozoa, as well as several species of bacteria and archaea (Yamin 1979). A pair of salivary glands is also present, emptying into the foregut anterior to the crop. To

effectively digest wood, a recalcitrant and nutritionally poor substrate, termites like *C. formosanus* have developed an array of enzymes. Some carbohydrolases characterized from the Rhinotermitidae have been shown to be endogenous (Watanabe et al. 1998), while others are produced by microbial symbionts (Nakashima et al. 2002a, Watanabe et al. 2002, Ohtoko et al. 2000). Xylanolytic enzymes include xylanases (EC 3.2.1.8) which internally cleave the xylan chain and beta-xylosidases (EC 3.2.1.37) which cleave xylan oligosaccharides into xylose. Inoue et al. (1997) demonstrated that xylanase activity is largely localized to the hindgut of *Reticulitermes speratus* (Kolbe), strongly implicating the resident protozoa as the major agents of xylan digestion. In the same study, they showed that the protozoan composition of the R. speratus hindgut significantly changes when the termites are fed on pure cellulose or pure xylan, as opposed to wood. Through similar experiments in differential feeding, Azuma et al. (1993) showed that, within the termite *R. speratus*, protozoan symbionts in the genera *Pyrsonympha* and *Dinenympha* take part in xylan digestion. It is probable that, with the changes in protozoan populations, the capacity for xylan digestion may also change to adapt to the xylan content of the termite diet.

Our objectives were to determine 1) if *C. formosanus* can digest xylan, 2) the relative activities of xylanase and beta-xylosidase in different regions of the gut of *C. formosanus* workers, 3) possible correlations between these activities and dietary xylan content in five formulated cellulose/xylan diets, and 4) how these activities change in response to three different simulated field diets.

Materials and Methods

Termite Collection

Termites were field collected in First Line Smartdisc monitors (FMC Corporation) in Charleston, South Carolina. Termites were collected from the trap by removal of the wood

(southern yellow pine) in the bait stations and brought to the University of Florida. Termites were then placed in feeding bioassays. Collections were restricted to a single colony for assays with formulated diets and a single colony for assays with simulated field diets to eliminate colony as a source of variation in enzyme activities.

Termite Diets and Feeding

Five formulated diets consisting of microcrystalline cellulose and beechwood xylan (>90% xylose residues) (Sigma-Aldrich, Atlanta, GA) were prepared for the termites: 10%, 20%, and 40% xylan. The remainder of the diet was composed of cellulose in all cases. The cellulose and xylan were mixed together and water (1.5 mL/g) was added to make a paste. This paste was thoroughly kneaded to achieve homogeneity.

For each formulated diet, a glass jar (125 mL, straight-sided, Fisherbrand, Fisher Scientific, Pittsborough, PA) was prepared with 80 g of moist builders sand (10% moisture). The bottom was cut out of a plastic cup (5.92 mL Soufflé cup, Solo Cup Company, Urbana, IL), leaving approximately 2 mm of cup wall intact. Formulated diet (1 g) was placed in the cup bottom and this was placed on top of the sand in the jar. Termites were added (~180 workers and 20 soldiers per jar) and kept in the dark at 21^{o}C for 2 wk. Termites were collected, frozen and kept at -80^{o}C until dissection.

Three diets were prepared for the termites to represent the probable food sources they would encounter in the field. These field diets were as follows: red oak (Quercus spp.), pine (Pinus spp.), and filter paper. Wood diets were generated by drilling into craft wood boards (0.635 x 5.08 x 60.96 cm) with a 2.54 cm spade drill bit. Sawdust was collected and weighed. Filter paper consisted of a weighed number of crumpled cellulose filter paper disks (42.5 mm diameter, Whatman, grade 4).

For each field diet, a mason jar (# mL, brand) was prepared with 40 g of moist builders sand (10% moisture). Field diet (10 g) was then added with 40 mL of deionized water and the jar was loosely capped. Unlike the formulated diets, the field diets were not made from sterile materials. Because of this, the jars with field diets were autoclaved on a liquid cycle (30 min, 122^{o}C) to sterilize the diets and the jars were then allowed to cool. Termites were added (~270 workers and 30 soldiers per jar) after the jars had cooled and kept in the dark at 21^{o}C for 6 wk. Termites were collected, frozen and kept at -80^{o}C until dissection.

Termite Dissection and Enzyme Extraction

Two buffers were prepared for use in the dissections: 0.1 M sodium acetate buffer, pH 5.5, and 0.1 M sodium phosphate buffer, pH 6.5. Sodium acetate buffer was used for the xylanase assays, while sodium phosphate buffer was used for the beta-xylosidase assays.

Termites were removed from the freezer and kept on ice until dissection. Each termite's gut was removed intact and separated into three regions: foregut (and salivary glands), midgut, and hindgut. A single enzyme extract was prepared from dissected termites from each feeding treatment for each assay type using an experimental design similar to the recent classic termite carbohydrolase experiments (Hogan et al. 1988, Inoue et al. 1997, Nakashima et al. 2002b). For the xylanase assays, 25 termites from each feeding treatment were dissected in sodium acetate buffer. For the beta-xylosidase assays, 25 termites from each feeding treatment were dissected in sodium phosphate buffer. The three gut regions were placed into separate 1.5 mL microcentrifuge tubes (Eppendorf) containing the appropriate buffer, and kept on ice. Final concentrations were 50 termite gut regions per mL in all cases.

Enzymes were extracted using a method adapted from Inoue et al. (1997). 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.

Xylanase Assays

The xylanase assays were conducted using a method adapted from Han et al. (1995). A 0.5% solution of beechwood xylan (>90% xylose residues; Sigma-Aldrich) was prepared in 0.1 M sodium acetate buffer, pH 5.5. The solution was boiled approximately 30 min, until xylan particles were no longer visible. The solution was centrifuged at 1250 g for 5 min at 23°C and the supernatant was used as the xylan stock solution.

Assays were conducted in clear 96-well microplates. In each well, 10 μL of tissue extract was combined with 90 μL of xylan solution. The solutions were allowed to react for 35 min at 23°C. DNSA solution (100 μL) consisting 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 placed on ice for 15 min. After cooling, each microplate was read at 540 nm using a μQuant Universal Microplate Spectrophotometer (Bio-Tek Instruments, Winooski, VT). Similar control plates were allowed to react for 5 min to allow for passive mixing of solutions before boiling with DNSA solution (Zhou et al. 2007). A 5 min reaction was used as a control to correct for any differences in initial sample reaction rates due to slow mixture of enzyme and substrate solutions. Standards were generated using dilutions of xylose. Only the wells in the middle of the microplate were used for these assays. Perimeter wells were filled with deionized water (200 μL per well) to add temperature stability and consistency during boiling. For all replicates, the control plates were used to adjust for 540 nm absorbance in gut extracts and were replicated an equal number of times to the assay plates, with one microplate well for each replicate.

Beta-Xylosidase Assays

Beta-xylosidase assays were conducted using a method adapted from Han et al. (1995). A solution of 4 mM p-nitrophenyl-β-D-xylopyranoside (pNPX; Sigma-Aldrich) was prepared in 0.1 M sodium phosphate buffer, pH 6.5. Assays were conducted in clear 96-well microplates. In each well, 10 μL of tissue extract was combined with 90 μL of pNPX solution. The reaction was allowed to proceed for 10 min before being placed in a μQuant Universal Microplate Spectrophotometer (Bio-Tek Instruments, Winooski, VT). Absorbance was read at 420 nm every 2 min for 30 min at 23°C. Mean velocities (mOd/s) were recorded. Standards were generated using dilutions of p-nitrophenol.

Data Analysis

The xylanase assays were set up as one-factor designs with four technical replicates per gut region for each formulated diet and each simulated field diet. Four microplate wells were assayed for each replicate of each gut region/diet combination. The beta-xylosidase assays were set up as one-factor designs with four technical replicates per gut region for each formulated diet and simulated field diet. Two homogenates were used for each gut/diet combination; one homogenate for xylanase assays and another for beta-xylosidase assays.

For the xylanase assays, the following formula was used to calculate specific activities;

$SA = C_S[(A-A_0)/t]/N_T$

where: SA = specific activity (nmol reducing sugar per termite equivalent per min), A = absorbance (Od) after 35 min reaction, A_0 = absorbance (Od) for the corresponding control after 5 min reaction, t = time (min), C_S = the coefficient derived from the standard (nmol reducing sugar/mOd), and N_T = the number of termite equivalents per sample.

For the beta-xylosidase assays, the following formula was used to calculate specific activities;

$SA = 60C_S V_A / N_T$

where: SA = specific activity (nmol p-nitrophenol per termite equivalent per min), V_A = mean velocity of absorbance change (mOd/s), C_S = the coefficient derived from the standard (nmol p-nitrophenol/mOd), and N_T = the number of termite equivalents per sample.

The field diet data were analyzed using a mixed model analysis of variance. Fixed effects were diet treatment and gut region. The Tukey-Kramer adjustment ($\alpha = 0.05$) was used to separate the mean activities on each diet within each gut region (SAS Institute 2001).

Results

Termite Gut Observations

During dissection, after the termites had been fed on the various diets, the color of the termite guts reflected the color of the different diets. This was especially evident in the enlarged hindguts which were typically filled with a mixture of partially digested food and resident microbes. Termites fed on formulated diets showed an increasing brown shade in their gut contents on diets containing more xylan. Termites fed on red oak had brownish-orange gut contents. Those fed on pine had pale yellow gut contents. Those fed on paper had white gut contents. The termites collected from the field and immediately frozen for dissection had relatively dark gut contents.

Xylanase Activities

Both foregut and midgut xylanase activities were insignificant in comparison with hindgut xylanase activity on all of the formulated diets (Fig. 8-1). Hindgut xylanase activity was significantly greater in termites kept on 40% xylan diets than in termites kept on 10% and 20% xylan diets.

Both foregut and midgut xylanase activities were insignificant in comparison with hindgut xylanase activity on all of the simulated field diets (Table 8-1). Hindgut xylanase activity differed significantly among all of the three simulated field diets, being highest on oak and lowest on paper.

Beta-Xylosidase Activities

Hindgut xylanase activity increased from termites fed 10% xylan to termites fed 40% xylan, differing significantly on all three diets (Fig. 8-2). Midgut beta-xylosidase activities were highest in termites fed 10% xylan, and were significantly lower in termites fed 40% xylan. Activities were nearly equal on 10% and 20% xylan.

Hindgut beta-xylosidase activity differed significantly among all of the three simulated field diets, being highest on oak and lowest on paper (Table 8-2). Midgut beta-xylosidase activity was significantly greater on pine than on the other two simulated field diets.

Discussion

The overall gut morphology observed here for *C. formosanus* workers was similar to that described for *Zootermopsis* (Child 1946) and is consistent with that found in other lower termites (Noirot and Noirot-Timothee 1969). The appearance of the termite guts upon dissection, particularly the hindguts, indicated that the termites had fed on their respective diets.

Among the termites in the colony fed on formulated diets, both xylanase and beta-xylosidase hindgut activities were significantly higher on diets with higher xylan content. A similar pattern was seen in termites fed on field diets. All hindgut xylanolytic activities were highest on the oak diet. Hardwoods typically consist of roughly 20% xylan, twice as much as the typical softwood xylan content of roughly 10% (Biermann 1996). At the same time, hindgut activities were lowest on the paper diet, a diet completely lacking in xylan. Just as termites fed on 40% xylan showed far greater xylanase activity than termites fed on 10% xylan, so there was

a similar jump in activity from pine fed termites to oak fed termites. The hindgut beta-xylosidase patterns were also consistent between termites fed on formulated diets and termites fed on field diets.

Our data showed that the majority of xylanase activity was in the *C. formosanus* hindguts. This is consistent with previous findings where xylanase activities were almost exclusively located in the hindgut of *R. speratus* and *Coptotermes heimi* (Wasmann), and associated with the symbionts (Inoue et al. 1997, Mishra 1991). Therefore, it was evident that *C. formosanus* workers follow an expected xylan digestion pattern for subterranean termites, in which xylan is thought to be mainly digested by hindgut symbionts.

The low hindgut xylanolytic activities on the paper diet were consistent with the absence of xylan. However, significant xylanase and beta-xylosidase activities were still present even in the absence of dietary xylan. This suggests the presence of symbionts that constitutively produce xylanases and beta-xylosidases, but are capable of subsisting solely on a cellulose diet.

Symbiont xylanase and beta-xylosidase activities were observed to change significantly in response to diet, in a manner consistent with xylan content. These findings suggest that the community of hindgut symbionts is able to adapt to a wide range of dietary xylan content. Studies on *R. speratus* (Azuma et al. 1993, Inoue et al. 1997), *Reticulitermes virginicus* (Banks) (Cook and Gold 2000) and *Coptotermes formosanus* (Shiraki) (Mannesmann 1972, Waller and La Fage 1987) have demonstrated significant changes in the hindgut protozoan communities in response to different diets. Many of these studies focused on feeding termites different wood species. It is therefore probable that the xylanolytic enzyme changes in *C. formosanus* are due to changes in hindgut symbiont populations. Alternatively, it is possible that these enzyme changes are due to changes in xylanolytic enzyme production within the hindgut symbionts.

C. formosanus workers are capable of digesting xylan, following the same pattern seen in Chapter 4, where xylan is mainly digested by in the hindgut. The xylanolytic activities of the two termite colonies we investigated changed to accommodate dietary xylan content, most likely by changes in the hindgut symbiont communities. This flexibility allows a termite colony to efficiently utilize a variety of wood species and wood-derived materials.

Compared to cellulose, xylan is often overlooked as a starting compound for the production of alternative fuels. However, xylan may comprise up to a quarter of wood, depending on the species, and a significant proportion of other plant materials used for alternative fuel production. These termites and their symbionts may provide candidate enzymes for the degradation of xylan in this process.

Table 8-1. Xylanase activities in the three gut regions of *Coptotermes formosanus* workers in response to different field diets

Diet	Foregut	Midgut	Hindgut
Paper	0.14 ± 0.06a	0.21 ± 0.01a	12.75 ± 0.15c
Pine	0.00 ± 0.09a	0.20 ± 0.03a	17.98 ± 0.23b
Oak	0.07 ± 0.04a	0.13 ± 0.03a	26.95 ± 0.45a

Xylanase activities are in nmol reducing sugar per termite equivalent per min. Means within a column followed by the same letter are not significantly different, (Tukey-Kramer Adjusted Means Separation, $\alpha = 0.05$, SAS Institute 2001), n = 4 replicates.

Table 8-2. Beta-xylosidase activities in the three gut regions of *Coptotermes formosanus* workers in response to different field diets

Diet	Foregut	Midgut	Hindgut
Paper	0.0020 ± 0.0012a	0.0067 ± 0.0004b	0.0252 ± 0.0011c
Pine	0.0027 ± 0.0009a	0.0127 ± 0.0003a	0.0408 ± 0.0007b
Oak	0.0020 ± 0.0008a	0.0073 ± 0.0003b	0.0623 ± 0.0008a

Beta-xylosidase activities are in nmol p-nitrophenol per termite equivalent per min. Means within a column followed by the same letter are not significantly different, (Tukey-Kramer Adjusted Means Separation, $\alpha = 0.05$, SAS Institute 2001), n = 4 replicates.

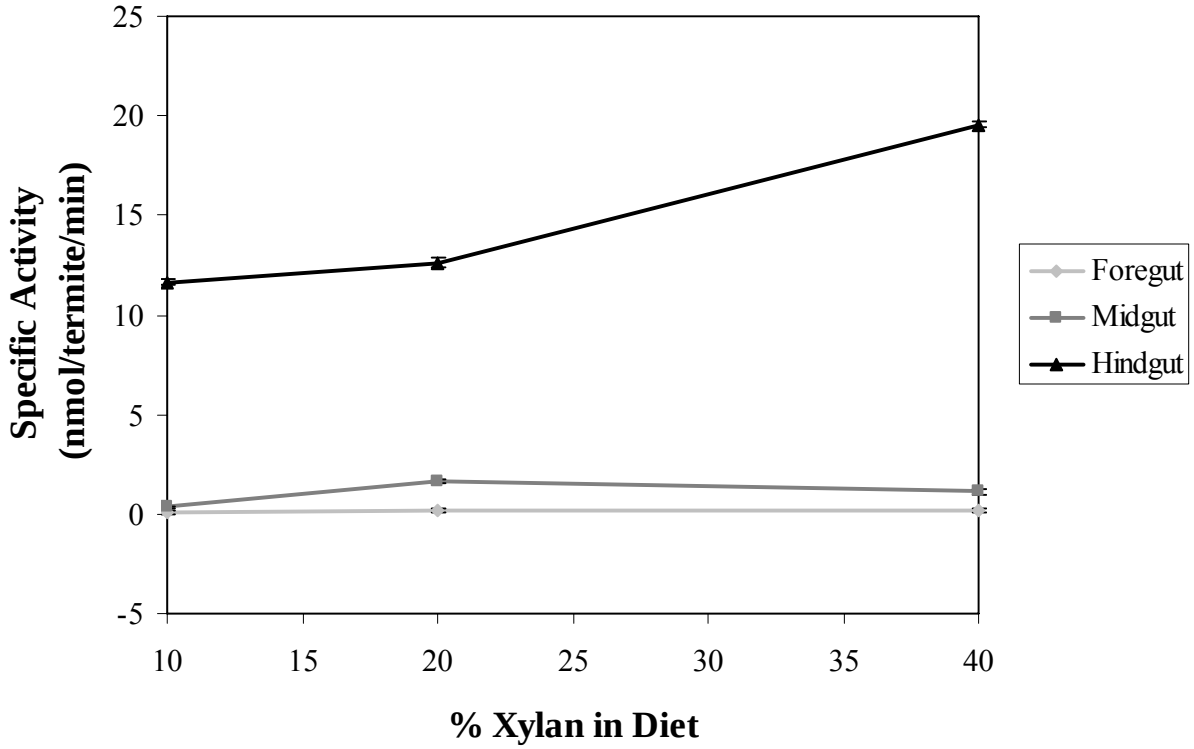

Figure 8-1. Xylanase activities in different gut regions of *Coptotermes formosanus* in response to dietary xylan content.

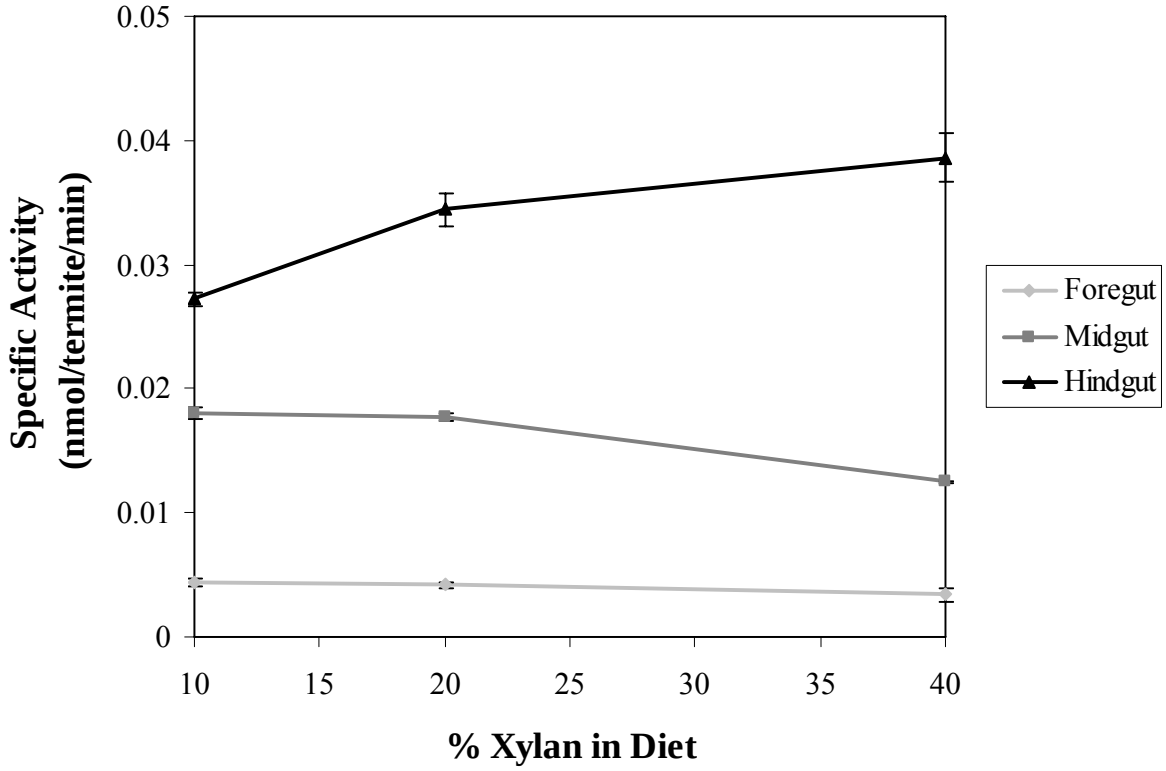

Figure 8-2. Beta-xylosidase activities in different gut regions of *Coptotermes formosanus* in response to dietary xylan content.

CHAPTER 9
CONCLUSION

Termite gut carbohydrolase assays were optimized for buffer and pH, leading to the selection of a 0.1 M pH 5.5 sodium acetate buffer for endoglucanase and xylanase assays and a 0.1M pH 6.5 sodium phosphate buffer for exoglucanase, beta-glucosidase, and beta-xylosidase assays. This was in contrast with the almost universal use of 0.1M pH 5.5 sodium acetate buffer in prior literature.

C. formosanus was found to have a more active array of gut carbohydrolases, particularly exoglucanase and beta-glucosidase, than *R. flavipes*. This was consistent with increased metabolic demands from more aggressive foraging and a larger soldier ratio within *C. formosanus* colonies. Soldier carbohydrolase activities were generally lower than worker carbohydrolase activities, consistent with a caste incapable of feeding itself.

Cellulolytic enzyme activity levels were found to significantly change on differing diets for both *C. formosanus* and *R. flavipes* workers. Xylanolytic enzyme activities were found to change in a manner consistent with dietary xylan content, being increased on diets containing more xylan. This has demonstrated dietary adaptability in both of these species.

The patterns of cellulolytic and xylanolytic enzyme activities and activity changes on differing diets were similar between the two species assayed. General findings regarding cellulose and xylan digestion in one species of Rhinotermitidae may be cautiously applied to the rest of the family. Our findings suggest a processive endogenous mechanism of amorphous cellulose degradation with a reliance on the termite symbionts for the digestion of xylan and crystalline cellulose.

Crystalline cellulose and xylan may make up between 30% and 60% of wood dry weight, while amorphous cellulose may make up between 15% and 25% of wood dry weight. Therefore,

the termite symbionts are vital to effective wood digestion. In addition, while the endogenous termite enzymes may be of use in industrial digestion of amorphous cellulose, the symbiont enzymes should prove far more valuable in the digestion of cellulose and xylan in general.

There is some redundancy with regards to amorphous cellulose digestion in the termites we have investigated, as both the termites and their symbionts produce endoglucanases and beta-glucosidases. This could complicate cellulase inhibition efforts. The enzymes responsible for digestion of crystalline cellulose and xylan are largely produced by the hindgut symbionts, with little contribution from the termite itself. Although this removes one level of redundancy, the presence of multiple symbiont species producing differing enzymes may still complicate cellulase and xylanase inhibition efforts. However, these very redundancies could make termites a robust system to adapt for the industrial degradation of cellulosic waste.

Compared to cellulose, xylan is often overlooked as a starting compound for the production of alternative fuels. However, xylan may comprise up to 25% of wood dry weight, depending on the species, as well as a significant portion of other plant materials used for alternative fuel production. The termites and termite symbionts investigated in this dissertation may provide enzymes for the degradation of xylan in this process.

The balance of endogenous versus symbiont cellulolytic activities appears to change in response to diet in both species, most likely by changes in the hindgut protozoan communities as well as the termite enzyme expression. This capacity for adaptation and partial balancing between the termite and its symbionts could make termite control by means of cellulase inhibition more difficult, but may eventually yield a mechanism to increase efficiency in industrial cellulose degradation.

The xylanolytic activities of the two termite species investigated changed to accommodate dietary xylan content, most likely by changes in the hindgut symbiont communities. These termites' capacity for xylan digestion allows them to gain more energy from a wood diet, and their adaptability allows them to efficiently utilize diets of varying xylan content. This adaptability could make termite xylanase inhibition difficult, but it may provide a means for effective xylan degradation on an industrial scale using living termites or termite symbionts.

The enzymatic mechanisms of wood digestion in these subterranean termite pests are both complex and effective. A greater understanding of these mechanisms may open new avenues in safer termite control, and may certainly improve processes for recycling cellulosic waste and development of alternative fuels.

LIST OF REFERENCES

Astley R. J., Harrington J. J. and Stol, K. A. (1997) Mechanical modeling of wood microstructure, an engineering approach. *IPENZ Trans*. **24**, 21-29

Azuma J. I., Kanai K., Murashima K., Okamura K., Nishimoto K. and Takahashi M. (1993) Studies on digestive system of termites: III. Digestibility of xylan by termite *Reticulitermes speratus* (Kolbe). *Wood Res.* **79**, 41-51.

Biermann, C.J. (1996) *Handbook of Pulping and Papermaking*, *2nd ed*. Academic Press, San Diego, CA.

Breznak J. A. and Brune A. (1994) Role of microorganisms in the digestion of lignocellulose by termites. *Annu. Rev. Entomol.* **39**, 453-487.

Brune A., Miambi E. and Breznak J. A. (1995) Roles of oxygen and the intestinal microflora in the metabolism of lignin-derived phenylpropanoids and other monoaromatic compounds by termites. *Appl. Environ. Microbiol.* **61**, 2688-2695.

Cavalier-Smith T. (1993) Kingdom protozoa and its 18 phyla. *Microbiol. Rev.* **57**, 953-994.

Cleveland L. R. (1924) The physiological and symbiotic relationships between the intestinal protozoa of termites and their host, with special reference to *Reticulitermes flavipes* (Kollar). *Biol. Bull.* **46**, 178-224.

Cleveland L. R. (1925) The method by which *Trichonympha campanula*, a protozoan in the intestine of termites, ingests solid particles of wood for food. *Biol. Bull.* **48**, 282-289.

Child H. J. (1946) The internal anatomy of termites and histology of the digestive tract. In *Termites and Termite Control, 2nd ed.,* eds. C. A. Kofoid, S. F. Light, A. C. Horner, M. Randall, W. B. Herms, and E. E. Bowe, pp. 59-88. University of California Press, Berkeley.

Cook, T. J. and R. E. Gold. (2000). Effects of different cellulose sources on the structure of the hindgut flagellate community in Reticulitermes virginicus (Isoptera: Rhinotermitidae). *Sociobiology* **35**, 119-130.

Davies G. J., Mackenzie L., Varrot A., Dauter M., Brzozowski A. M., Schulein M. and Withers S. G. (1998) Snapshots along an enzymatic reaction coordinate: Analysis of a retaining b-glycoside hydrolase. *Biochem.* **37**, 11707-11713.

Delgado-Viscogliosi P., Viscogliosi E., Gerbod D., Kulda J., Sogin. M. L. and Edgcomb V.P. (2000) Molecular phylogeny of parabasalids based on small subunit rRNA sequences, with emphasis on the Trichomonadinae subfamily. *J. Eukaryot. Microbiol.,* **47**, 70-75.

Eggleton P. (2001) Termites and trees: a review of recent advances in termite phylogenetics. *Insectes Soc*. **48**, 187-193.

Han S. J., Yoo Y. J. and Kang H. S. (1995) Characterization of a bifunctional cellulase and its structural gene. *J. Biol. Chem.* **270**, 26012-26019.

Hogan M.E., Schulz M.W., Slaytor M., Czolu R.T. and O'Brien R.W. (1988) Components of termite and protozoal cellulases from the lower termite, *Coptotermes lacteus* Froggatt. *Insect Biochem.* **18**, 45-51.

Inoue T., Murashima K., Azuma J.-L., Sugimoto A. and Slaytor M. (1997) Cellulose and xylan utilization in the lower termite *Reticulitermes speratus*. *J. Insect Physiol.* **43**, 235-242.

Inoue T., Moriya S., Okhuma M. and Kudo T. (2005) Molecular cloning and characterization of a cellulase gene from a symbiotic protist of the lower termite, *Coptotermes formosanus*. *Gene.* **349**, 67-75.

Lewis J. L. and Forschler B. T. (2004) Protist communities from four castes and three species of Reticulitermes (Isoptera: Rhinotermitidae). *Ann. Entomol. Soc. Am.* **97**, 1242-1251.

Mannesmann R. (1972) Relationship between different wood species as a termite food source and the reproduction rate of termite symbionts. *Z. Angew. Entomol.* **72**, 116-128.

Mishra S. C. (1991) Carbohydrases in the gut of the termite *Coptotermes heimi* (Wasm.) (Rhinotermitidae), their origin, distribution and evolutionary significance. *Ann.Entomol*. **9**, 41-46.

Mishra S. C. and Sen-Sarma P. K. (1979) Studies on deterioration of wood by insects. III. Chemical composition of faecal matter, nest material and fungus comb of some Indian termites. *Mater. Organismen*. **14**, 1-14.

Moriya S., Dacks J. B., Takagi A., Noda S., Ohkuma M., Doolittle W. F. and Kudo T. (2003) Molecular phylogeny of three oxymonad genera: *Pyrsonympha*, *Dinenympha* and *Oxymonas*. *J. Eukaryot. Microbiol.,* **50**, 190-197.

Nakashima K., Watanabe H. and Azuma J.-I. (2002a) Cellulase genes from the parabasalian symbiont *Pseudotrichonympha grassii* in the hindgut of the wood-feeding termite *Coptotermes formosanus*. *Cell. Mol. Life Sci.* **59**, 1554-1560.

Nakashima K., Watanabe H., Saitoh H., Tokuda G. and Azuma J.-I. (2002b) Dual cellulose-digesting system of the wood-feeding termite, *Coptotermes formosanus* Shiraki. *Insect Biochem. Molec.* **32**, 777-784.

Noirot C., and Darlington J. (2002) Termite nests: architecture, regulation and defence. In *Termites: evolution, sociality, symbioses, ecology,* eds. T. Abe, D. E. Bignell, and M. Higashi, pp. 121-139. Kluwer Academic Publishers, Boston.

Noirot C., and Noirot-Timothee C. (1969) The digestive system. In *Biology of Termites,* eds. K. Krishna and F. M. Weesner, pp. 49-88. Academic Press, New York.

Ohkuma M., Ohtoko K., Iida T., Tokura M., Moriya S., Inoue, T., Usami R., Horikoshi K. and Kudo T. (2000) Phylogenetic identification of hypermastigotes, *Pseudotrichonympha*, *Spirotrichonympha*, *Holomastigotoides*, and parabasalian symbionts in the hindgut of termites. *J. Eukaryot. Microbiol.* **47**, 249-259.

Ohtoko K., Ohkuma M., Moriya S., Inoue T., Usami R., and Kudo T. (2000) Diverse genes of cellulase homologues of glycosyl hydrolase family 45 from the symbiotic protists in the hindgut of the termite *Reticulitermes speratus. Extremophiles* **4**, 343-349.

Parsiegla G., Reverbel-Leroy C., Tardif C., Belaich J. P., Driguez H. and Haser R. (2000) Crystal structures of the cellulase Cel48F in complex with inhibitors and substrates give insights into its processive action. *Biochem.* **39**, 11238-11246.

Pettersen R. C. (1984) The chemical composition of wood. *Adv. Chem. Ser*. **207**, 57-126.

Potrikus C. J. and Breznak J. A. (1980) Anaerobic degradation of uric acid by gut bacteria of termites. *Appl. Environ. Microb.* **40**, 125-132.

Potter M. F. (2004) Termites. In *Handbook of pest control, 9th ed.,* eds. A. Mallis, S. A. Hedges and D. Moreland, pp. 217-361. GIE Media, Inc., Richfield.

SAS Institute. (2001) SAS Institute, Cary, N.C.

Smith J. A. and Koehler P. G. (2007) Changes in Reticulitermes flavipes (Isoptera: Rhinotermitidae) Gut Xylanolytic Activities in Response to Dietary Xylan Content. *Ann. Entomol. Soc. Am.* **100**, 568-573.

Sineiro J., Dominguez H., Nunez M. J. and Lema J. M. (1997) Inhibition of cellulase activity by sunflower polyphenols. *Biotechnol. Lett.* **19**, 521-524.

Timell T. E. (1964) Wood Hemicelluloses. Part I. *Adv. Carbohyd. Chem. Bi.* **19**, 247-302.

Waller D. A. and La Fage J. P. (1987) Food quality and foraging response by the subterranean termite *Coptotermes formosanus* Shiraki (Isoptera: Rhinotermitidae). *B. Entomol. Res.* **77**, 417-424.

Watanabe H., Noda H., Tokuda G. and Lo N. (1998) A cellulase gene of termite origin. *Nature* **394**, 330-331.

Watanabe H., Nakashima K., Saito H. and Slaytor M. (2002) New endo-beta-1,4-glucanases from the parabasalian symbionts, *Pseudotrichonympha grassii* and *Holomastigotoides mirabile* of *Coptotermes* termites. *Cell Molec. Life Sci.* **59**, 1983-1992.

Wenzel M., Schonig I., Berchtold M., Kampfer P. and Konig H. (2002) Aerobic and facultatively anaerobic cellylolytic bacteria from the gut of the termite *Zootermopsis angusticollis*. *J. Appl. Micribiol.* **92**, 32-40.

Whistler R.L. and Chen C.C. (1991) Hemicelluloses. In *Wood structure and composition*, eds. M. Lewin and I. S. Goldstein pp. 287-320. International Fiber Science and Technology Series, Vol. II. Marcel Decker, Inc., New York.

Yamin M. A. (1979) Flagellates of the orders Trichmonadida Kirby, Oxymonadida Grasse, and Hypermastigida Grassi and Foa reported from lower termites (Isoptera families Mastotermitidae, Kalotermitidae, Hodotermitidae, Termopsidae, Rhinotermitidae, and Serritermitidae) and from the wood-feeding roach *Cryptocercus* (Dictyoptera: Cryptocercidae). *Sociobiology* **4**, 5-119.

York W. S., Qin Q. and Rose J. K. C. (2004) Proteinaceous inhibitors of endo-β-glucanases. *Biochim. Biophys. Acta* **1696**, 223-233.

Zhou X., Smith J. A., Oi F. M., Koehler P. G., Bennett G. W. and Scharf M. E. (2007) Correlation of cellulase gene expression and cellulolytic activity throughout the gut of the termite *Reticulitermes flavipes*. *Gene* **395**, 29-39.

BIOGRAPHICAL SKETCH

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