Metabolism of Cellulose in Wood in the Lower Termite *Coptotermes formosanus* Shiraki (Isoptera: Rhinotermitidae)

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CONTENTS

INTRODUCTION

One of the most important groups of organisms to destroy wood used in buildings or other constructions are the termites. The mechanism of wood their digestion is not fully understood yet. It is important to clarify the digestion mechanism to develop new and more specific termite control methods instead of the currently used termiticidal chemicals of broad-spectrum.

Three classes of enzymes, endo-1,4-β-glucanase, exo-1,4-β-glucanase (exo-cellobiohydrolase (CBH) or exo-1,4-β-glucosidase), and β-glucosidase, are found both in fungal (Wood, 1991) and bacterial (Rapp and Beermann, 1991) cellulolytic systems. Among these cellulolytic enzymes the presence of CBH or endo-1,4-β-glucanase tightly absorbed on cellulose should be necessary in reactions of solubilization of crystalline cellulose (Klesov, 1991).

A cellulolytic system in the lower termites, *Coptotermes formosanus* Shiraki and *Reticulitermes speratus* Kolbe, has been proposed (Yamaoka and Nagatani, 1975; Yoshimura *et al.*, 1992). They reported the followings a) endo-1,4-ß-glucanase is secreted from the salivary glands of the termites and is also produced in protozoa present in the hindgut, and exists in the gut fluid of the termites; b) most of the exo-1,4-ß-glucanase is found in the bodies of the protozoa, and very little in the gut fluid of the termites. Then the following theory on the cellulose digestion in the lower termites based on the above reports is still widely believed although support for this theory has grown more tentative since the topic was reviewed by Martin (1991) and Slaytor (1992): ingested crystalline cellulose is initially and partially degraded by enzymes produced by the termites themselves in the region anterior to the hindgut; the partially degraded cellulose is endocytosed by the symbiotic protozoa in the hindgut and hydrolyzed to glucose in the bodies of the protozoa by synergistic actions of enzymes produced by both the termites and symbiotic protozoa (Breznak and Brune, 1994). The theory means the following: crystalline cellulose can be

only partially hydrolyzed and not to glucose in the region anterior to the hindgut of the lower termites and can be completely hydrolyzed to glucose only in the bodies of the symbiotic protozoa in the hindgut, in other words, glucose originating from the cellulose is produced only in the bodies of the protozoa.

The higher termites (some 75 % of all termite species) can hydrolyze and metabolize the cellulose in wood. These termites lack protozoa but harbor a dense and diverse array of bacteria in their alimentary tract. Therefore the higher termites are thought to have a cellulose-degrading system that is independent of protozoa. The system acts on the cellulases produced by both the bacteria and the termites themselves. In the higher termite *Nasutitermes walkeri* Hill, most of the cellulose hydrolysis occurs in the foregut and midgut, and most of the glucose produced is absorbed by the midgut (Hogan *et al.*, 1988).

It is a quite waste of energy that the glucose is produced by the hydrolysis of crystalline cellulose in the bodies of the protozoa in the hindgut of the lower termites and oxidized to organic acids including acetic acid in the bodies, and the organic acids, especially acetic acid, are excreted out of the protozoa, transported across the gut wall and used as a carbon source for the biosynthesis of glucose essential for termites. Being based on the viewpoint, I have speculated that the lower termites as well as the higher termites endogeneously produced a complete cellulase system capable of hydrolyzing crystalline cellulose to glucose in the region anterior to the hindgut and sufficient glucose for the termites' needs is produced by the cellulase system with injected crystalline cellulose in the midgut, the site of metabolite absorption.

It has been reported that termites are unable to use glucose directly as a source of carbon and energy, since they lack the pyruvate dehydrogenase complex for converting pyruvate to acetyl-coenzyme A (acetyl-CoA). Rather, since termites have acetyl-CoA synthetase to convert acetate to acetyl-CoA and all of the enzymes for glycolysis and the tricarboxylic acid (TCA) cycle, they have been reported to utilize acetate that is produced by microorganisms in the hindgut and transferred into the body of the termite through the

hindgut epithelium (Breznak and Brune, 1994; Hogan *et al.*, 1985; O'Brien and Breznak, 1984). Recently, this mechanism has been revised for higher termites: since termites have malate dehydrogenase (decarboxylating) as the anaplerotic enzyme of the TCA cycle, some of the pyruvate produced during the glycolysis of glucose is converted to malate and metabolized via the TCA cycle; thus, termites can use a portion of the glucose in their bodies as an energy source (Slaytor *et al.*, 1997). According to this theory, termites still depend on acetate produced by microbiota in the hindgut as the source of acetyl-CoA for the TCA cycle. In aerobic organisms with abundant glucose supplies, the production of acetyl-CoA from acetate produced by hindgut microorganisms, rather than from pyruvate, is energetically inefficient. If termites possess the pyruvate dehydrogenase complex, they could oxidize glucose completely, in a more energetically efficient process, without assistance from hindgut microorganisms.

The objective of this dissertation is to clarify whether crystalline cellulose is hydrolyzed to glucose in the foregut and midgut as well as in the hindgut of lower termite *C. formosanus* and to determine whether pyruvate dehydrogenase complex is active in tissue of the termite. Digestibility of major wood components in termite with little or no ligninolytic enzyme and the mechanism of degradation of wood-cell wall in the termite's digestive system are discussed in Chapter 1. Distribution of exo-1,4-ß-glucanase, which is used in this dissertation to represent a cellulase component able to produce reducing sugar from Avicel, endo-1,4-ß-glucanase, and ß-glucosidase activities in the termite is described together with distribution of glucose, trehalose, and acetate in Chapter 2. Rates of hydrolysis of natural cellulose in the termite tissue and the hindgut are also discussed in connection with cellulase activities in each tissue. Characteristics of cellulase components isolated from the termite tissue are compared with those from the hindgut in Chapter 3. Finally, in Chapter 4, pyruvate dehydrogenase complex in the termite tissue and the hindgut is assayed by using [1-^{14}C]- and [2-^{14}C]-labeled pyruvate. Acetyl-CoA

synthetase from these tissues is also assayed to compare the rates of acetyl-CoA production by acetyl-CoA synthetase and by the pyruvate dehydrogenase complex.

Chapter 1 DEGRADATION OF WOOD COMPONENTS BY *COPTOTERMES FORMOSANUS* SHIRAKI

1.1 Introduction

Lower termites harbor diverse populations of cellulolytic protozoa and bacteria in their alimentary tracts (Breznak, 1982; Breznak, 1984; Breznak and Brune, 1994; Malburg *et al.*, 1992; Prins and Kreulen, 1991; Slaytor, 1992). The symbiotic protozoa play an important role in digesting cellulosic materials (Yoshimura *et al.*, 1993a, 1993b, 1993c) because most of species of the protozoa have endo-1,4-ß-glucanase (EC 3.2.1.4), exo-1,4-ß-glucanase, and ß-glucosidase (EC 3.2.1.21) (Odelson and Breznak, 1985; Yamin and Trager, 1979), while another protozoon is closely related to wood-feeding activities of termites (Yoshimura *et al.*, 1994). But relevance of cellulases produced by termites and gut bacteria to cellulose digestion should not be neglected because cellulase activities have been found in salivary glands (Slaytor, 1992; Yamaoka and Nagatani, 1975), some cellulolytic bacteria were isolated from the alimentary tracts of termites and cultured (French, 1975; Mannesmann, 1972; Paul *et al.*, 1993; Thayer, 1976), and the majority (about 75%) of termites are higher termites which contain bacteria but no protozoa in their alimentary tract.

The relationships between the lower termites and gut symbionts in the digestion of cellulosic material have been thought as follows: a large portion of exo-1,4-ß-glucanases is present in the gut of a termite, and they are produced within the body of the protozoa in the gut, but little or no exo-1,4-ß-glucanases exist in the gut fluid of the termite; cellulose or wood pieces are endocytosed by symbiotic protozoa in the hindgut of the termite and degraded to glucose *in situ;* acetate and other organic acids excreted into the hindgut of the termite by the protozoa are important carbon and energy sources for the termite (Breznak, 1982; Eaton and Hale, 1993).

Lower termites were found to have very little or no lignin-degrading abilities (Cookson, 1987). This suggests that these termites have little or no ligninolytic enzyme.

One of the objects of this Chapter was to clarify which wood component is mostly utilized by the lower termites on the basis of the digestibility coefficients for the monosaccharides and lignin in wood. Another object was to account for how wood is degraded and digested in the gut of the lower termites without ligninolytic enzymes (Itakura *et al.*, 1995).

1.2 Materials and Methods

1.2.1 Termites

Worker caste termites of *C. formosanus*, collected from a laboratory colony maintained in the Faculty of Agriculture of Kinki University for about ten years, were used. Termites were fed at 28°C on Japanese red pine wood placed on moist dental plaster for about a week before use.

1.2.2 Recovery of fecal material

Five round holes of 5 mm diameters were made in the bottom of each cylindrical plastic container which was 80 mm in diameter and 75 mm in height. Dental plaster (25 g) with 26% moisture content was uniformly spread on the bottom of each container and air-dried.

Blocks of Japanese red pine sapwood (25 x 25 x 10 mm) were oven-dried at 60°C and weighed. A block was placed on the dental plaster in each of the containers. The containers were placed on dampened cotton, and 200 worker caste termites were kept in

each one at 28°C. The fecal material was dislodged from the surfaces of the blocks, the dental plaster, and from the walls of the containers with a spatula. The fecal material recovered and the blocks remaining were oven-dried and weighed. The recovery percentage of the fecal material containing plaster was calculated according to the following equation:

$R_P = W_P/W_S \times 100$,

R_P: Recovery percentage of fecal material contaminated by plaster (%),

W_P: Fecal material contaminated by plaster (mg),

W_S: Weight loss of block (mg).

1.2.3 Determination of the plaster containing the fecal material to calculate the amount of net fecal material

The calcium content in the hydrolysate obtained by the procedure for the determination of Klason lignin in fecal material contaminated by plaster was determined on a Z-6100 polarized Zeeman atomic absorption spectrophotometer (Hitachi, Ltd.).

The plaster content was calculated as $CaSO_4$ from the calcium content and was used to calculate the recovery of the net fecal material.

1.2.4 Determination of lignin in the fecal material

Klason lignin included in the fecal material contaminated by plaster was determined according to JIS (Japan Industrial Standard) P8008-1976, except that the hydrolysis with dilute sulfuric acid at the reflux temperature for 4 h was replaced by that at 120°C for 40 min in an autoclave.

The acid-soluble lignin content was determined by means of UV (ultraviolet) spectroscopy. Thirty liter $\cdot g^{-1} \cdot cm^{-1}$ at 240 nm was used as the absorptivity (Sudo *et al.*, 1976).

1.2.5 Determination of the carbohydrate composition of fecal material

Monosaccharides included in the hydrolysate obtained during the assay of Klason lignin were determined according to the alditol acetate method (Sawardeker *et al.*, 1965) modified by Misaki (1974). Alditol acetates derived from the monosaccharides were determined by gas-liquid chromatography.

1.2.6 Coefficient of digestibility by termites for the different components in wood

As termites fed mainly on the earlywood in wood, the digestibility coefficient for each wood component in the wood ingested by the termites was calculated based on the compositions of the components of the earlywood in the control wood. The termite-digestibility coefficient (TDC) for each wood component (X) was calculated by the following formula:

$$\text{TDC (X)} = \frac{\text{Amount of X in wood ingested} - \text{amount of X in fecal material}}{\text{Amount of X in wood ingested by termites}} \times 100.$$

1.2.7 Observation of the shape of wood existing in the guts of termites

Blocks of Japanese red pine sapwood (25 x 25 x 3 mm) were dyed with remazole brilliant blue R salt by the method of Thomas and Zeikus (1980). Twenty worker caste termites were kept with three pieces of dyed blocks under the conditions described in **1.2.2** for two days. The guts of the termites were pulled out from their posterior ends by a pair of tweezers. The contents of the guts were squeezed out from the guts, dispersed into distilled water, and observed under a light microscope.

1.2.8 Hydrolysis of wood with cellulase and the determination of the digestibility coefficients for the different components of the wood

The chips of the earlywood of Japanese red pine were separated by cutting them off from the wood, in about 5 mm thicknesses, with a knife, and then they were ground for 4 h to fine particles of less than 50 μm in size in a vibrating sample mill, TI-100 (Heiko Seisakusho, Ltd.). A mixture of 1000 mg of the wood meal and 100 or 300 mg of a commercial cellulase "Cellulase Onozuka R-10" (Yakult Pharmaceutical Ind. Co., Ltd.) in 30 ml of 0.1 M sodium acetate buffer (SAB), pH 5.0, had been shaken at 37°C for 96 h. The wood meal residue was collected by filtration, washed with distilled water (3 x 10 ml), oven-dried, and weighed. The relative sugar composition and the Klason lignin of the remaining wood meal were determined according to the method described in **1.2.5** and **1.2.4**, respectively. The digestibility coefficient (DC) for each component (Y) in the wood was calculated according to the following formula:

$$DC(Y) = \frac{\text{Amount of Y in sound wood} - \text{amount of Y in the remaining wood}}{\text{Amount of Y in sound wood}} \times 100.$$

1.3 Results

1.3.1 Recovery of fecal material from the ingested wood and digestibility of the major wood components in termite

Table 1-1 shows the recovery of the collected fecal material and the net fecal material for each test period. The weight losses of wood blocks increased with increases of

test periods from one week to three weeks. There were no marked variations for the recoveries of the fecal materials during the test period of one to four weeks.

Table 1-1. Amounts of wood ingested by termites, fecal material contaminated with plaster, net fecal material, and recovery percent of the net fecal material.

	1	2	3	4
			(week)	
Wood weight loss (mg)[1)]	121	253	350	352
Fecal material contaminated with plaster (mg)[1)]	52.7	127	162	168
Recovery of fecal material contaminated with plaster (%)[1)]	43.6	50.2	46.3	47.7
Plaster content contained in fecal material (%)[2)]	5.85	5.14	5.58	5.61
Net fecal material (mg)[1)]	49.6	120	153	159
Recovery of net fecal material (%)[1)]	41.0	47.6	43.7	45.1

1) Values represent means of 9 replicates for 1 week, 4 replicates for 2 weeks, 3 replicates for 3 and 4 weeks.

2) Plaster content calculated as $CaSO_4$ from the calcium content in the hydrolysate obtained by the procedure for determination of Klason lignin in fecal material.

Table 1-2 shows the compositions of the lignin and monosaccharides in the hydrolysate of the earlywood of Japanese red pine sapwood and in the collected fecal materials. The lignin contents of the fecal materials were about 1.7 to 1.9 times that in the sound wood, whereas the glucose contents of the fecal materials were about 0.6 times that in the sound wood. The contents of the other monosaccharides in the hydrolysates of fecal

Table 1-2. Composition of monosaccharides and lignin in sound wood and fecal material.

		Sound[1] wood	Fecal material 1	2	3	4
					(week)	
Sample amounts used	(mg)	500	400	400	400	400
Wood or net fecal material	(mg)	500	376.6	379.4	377.7	377.6
Plaster contained in sample	(mg)		23.4	20.6	22.3	22.4
Arabinose	(mg)	14.5	11.2	12.6	12.0	7.6
		(2.90)[3]	(2.97)	(3.32)	(3.18)	(2.01)
Galactose	(mg)	23.9	23.3	31.5	26.4	16.0
		(4.78)	(6.19)	(8.30)	(6.99)	(4.24)
Glucose	(mg)	213.9	87.8	92.5	99.2	99.3
		(42.8)	(23.3)	(24.4)	(26.3)	(26.3)
Mannose	(mg)	64.3	41.0	37.7	36.0	39.7
		(12.9)	(10.9)	(9.94)	(9.53)	(10.5)
Xylose	(mg)	20.8	11.7	12.3	14.1	13.4
		(4.16)	(3.11)	(3.24)	(3.73)	(3.55)
Total amount of monosaccharide	(mg)	337.4	175.0	186.6	187.7	176.0
		(67.5)	(46.5)	(49.2)	(49.7)	(46.6)
Klason lignin	(mg)	125.7	176.5	166.5	164.2	174.7
		(25.1)	(46.9)	(43.9)	(43.5)	(46.3)
A.S.L.[2]	(mg)	2.7	3.6	3.4	2.6	4.3
		(0.54)	(0.96)	(0.90)	(0.69)	(1.1)
Monosaccharide/lignin		2.63	0.97	1.10	1.13	0.98

1) Values represent means of two replicates.

2) Acid-soluble lignin.

3) Values in parentheses present percentage of each component.

materials were almost the same or a little less than those of the sound wood. The ratio of the total monosaccharide content to the lignin content in sound earlywood was 2.6:1, whereas in the fecal materials, it was approximately 1:1. There was little variation in the ratio of each monosaccharide content to the lignin content in the fecal materials over the feed-period of one to four weeks.

Table 1-3 shows the digestibility coefficients of the termite *C. formosanus* for the major components and lignin in the earlywood. The digestibility coefficient for the glucose constituting the cellulose in the wood was the most and in a range of 72 to 78% over the feed period, whereas that of lignin was the smallest and in a range of 17 to 24%. These results indicate that the cellulose in wood is most severely digested, and that the lignin is degraded appreciably during the passage of the fine wood tips ingested by the termite through the digestive tract.

Table 1-3. Digestibility coefficients of termites for the monosaccharides and lignin in wood.

	Digestibility coefficient(%)				
	1	2	3	4	mean
	(week)				
Klason lignin	23.4	17.0	24.2	16.7	20.3
A.S.L.[1)]	27.1	20.9	44.1	8.0	25.0
Arabinose	58.0	45.7	52.1	68.7	56.1
Galactose	46.9	17.6	36.1	59.9	40.1
Glucose	77.7	73.0	73.1	72.2	74.0
Mannose	65.4	63.5	67.7	63.2	65.0
Xylose	69.4	63.1	60.8	61.5	63.7

[1)] Acid-soluble lignin.

There was no tendency for constant increasing or decreasing with incubation time in the coefficient of digestibility for each monosaccharide or for the lignin. Furthermore, there was little variation in the coefficients, of digestibility by the termites for each compound.

1.3.2 Hydrolysis of ground wood meals with cellulase

The photomicrograph of wood meal taken from the hindgut of *C. formosanus* is shown in Figure 1-1. All of the wood particles are smaller than 50 μm in size.

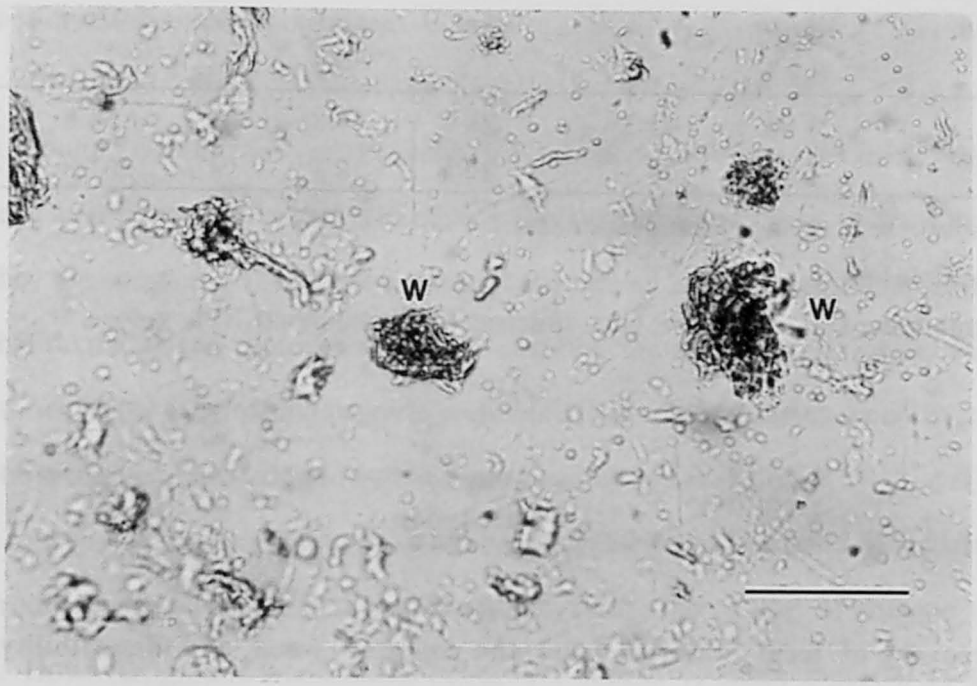

Fig. 1-1. Wood particles taken from the hindgut of *C. formosanus*; (w) wood particle; scale bar is 50 μm.

Table 1-4 shows the coefficients of digestibility of commercial cellulase for the monosaccharides and the lignin in the earlywood of Japanese red pine sapwood. The wood meals of the earlywood, which were smaller than 50 μm in size, were hydrolyzed with the cellulase to such an extent that the weight losses of the wood meals were 36% (360 mg) and 41% (410 mg). The coefficients of digestibility of the cellulase for the glucose, mannose, galactose, xylose, and arabinose in the earlywood were 58%, 62%, 28%, 50%, and 37% respectively at a weight loss of 41%. The digestibility coefficients for the lignin were about

2% at weight losses of 36% and 41%. These values are very small compared to those in the gut of the termite which are in a range of 17% to 24%. Thus, among the major components in wood, the cellulose was most selectively hydrolyzed in mass by the cellulase.

Table 1-4. Digestibility coefficients of cellulase for the neutral monosaccharides and lignin in wood.[1)]

Weight loss of wood (%)	Digestibility coefficient (%)						
	Klason lignin	A.S.L.[2)]	Ara.[3)]	Gal.	Glu.	Man.	Xyl.
36.4	1.84	45.2	28.9	9.86	54.3	56.8	27.2
41.1	1.58	49.3	37.4	28.1	58.3	61.8	49.9

1) Values represent means of three replicates.

2) Acid-soluble lignin.

3) Ara., arabinose; Gal., galactose; Glu., glucose; Man., mannose; Xyl., xylose.

1.4 Discussion

1.4.1 Recovery of fecal material from the ingested wood and digestibility of the termites for the major components in wood

Digestibility coefficient of the termite for glucose (74%) was largest among those for the monosaccharides and lignin in wood. Various enzyme activities in *C. formosanus* and mortality of the termites reared with various carbohydrates have been reported by Kanai and others (1982). They found that the termites cannot utilize hemicelluloses efficiently although activities of glucosidase, galactosidase, mannosidase, xylosidase, and arabinosidase were detected in the termites. These facts are consistent with our results. Thus, cellulose is digested and utilized most efficiently by the termites and hemicelluloses, which are not available for the termites, are decomposed to a significant extent by the above hemicellulolytic enzyme in the termites as shown in Table 1-3.

The cellulose in the wood ingested was digested most preferentially in the guts of the termites, and the lignin was digested appreciably. Very similar results have been reported by Mishra and Sen-sarma (1979). They found that the digestibility coefficients for the cellulose in wood varied from 59% to 86%, depending upon the species of wood, and those for the lignin in wood were in a range of 2% to 13% in *Neotermes bosei*.

1.4.2 Wood hydrolysis with cellulase

Although *Trichoderma* sp. extracellularly produces complete cellulase systems and is capable of well-degrading natural crystalline cellulose substances such as filter paper and Avicel, they are substantially incapable of degrading sound wood because they produce no system that degrades and removes the lignin covering the cellulose; however, the wood exposed to white-rot fungi which degrade preferentially the lignin during wood degradation is susceptible to severe degradation by the deuteromycetes (Tanaka *et al.*, 1988). Thus the cellulose in wood-cell walls is quite resistant to enzymatic attack because of the existence of the lignin covering the cellulose in the wood and preventing the contact of enzyme with the cellulose. However, ground wood-meals being very similar to those ingested by termites and less than 50 μm in size were digested efficiently with the commercial cellulase without any ligninolytic system. Similar results have been reported by some other authors (Matsumura *et al.*, 1977).

Enzymatic saccharification of ground wood meals with various commercially available cellulases and with a cellulase extracted from *C. formosanus* has been reported by Azuma and Koshijima (1984). They found that wood meal (175-246 μm in size, Japanese red pine) was degraded with the termite cellulase although the weight loss of the wood meal treated with the termite cellulase was about a third of that treated with commercial cellulase "Onozuka R-10". Trituration by the termites would increase the cellulose-exposed area to

which cellulase is accessible because the wood ingested by the termites is ground to particles smaller than 50 μm.

The coefficient of digestibility (by the cellulase) of the lignin in the wood meals was about 2% and much less than those in the guts of the termites being in a range of 17% to 24%, suggesting that some ligninolytic systems might be involved in wood digestion in termite guts. However, because the Klason lignin method gives approximate estimates of the lignin content when used in biodegradation studies (Cookson, 1987), the lignin degrading abilities of termites are still equivocal. If a ligninolytic system existed in termites, it would originate from the bacteria existing in the guts of the termites because the only organisms known to degrade lignin directly are some fungi and bacteria.

The recoveries (59% and 64%) of ground wood-meals treated with commercial cellulase were significantly greater than those of fecal material (41 - 48%). This may be because of the greater particle sizes of the ground wood-meals because the extent of the enzymatic hydrolysis of wood meal increases with the reduction of particle size (Matsumura *et al.*, 1977) or to the synergistic effect of the combined action of the cellulolytic and ligninolytic systems in the termites.

1.5 Summary

To clarify the mechanism of wood digestion by the lower termites, the amount of Japanese red pine eaten by *C. formosanus* was measured periodically; fecal material was recovered at the same time and variations in the compositions of the major wood components in the fecal material were determined.

There were no tendencies of constantly increasing or decreasing in both the recovery of the fecal material and the coefficients of digestibility for the major wood components. The digestibility coefficient for glucose constituting the cellulose in wood was largest and in

a range of 72 - 78%, indicating that the cellulose in the wood ingested by the termites was digested most preferentially in the course of its passage through the intestines of the termites.

Wood was ground by the termites into particles less than 50 μm in size. Cellulose in the wood is digested efficiently by means of the combined actions of trituration by the termites and by cellulolytic enzymes existing in the termites.

Chapter 2 DISTRIBUTION OF CELLULASES, GLUCOSE, AND RELATED SUBSTANCES IN THE BODY OF *COPTOTERMES FORMOSANUS* SHIRAKI

2.1 Introduction

In Chapter 1, it was apparent that digestibility coefficient for glucose constituting cellulose in wood was largest and that the cellulose in wood was digested most preferentially by *C. formosanus*. As described in INTRODUCTION, it has been accepted that crystalline cellulose ingested by the lower termites is hydrolyzed to glucose only in the bodies of the symbiotic protozoa and that the termites use acetate excreted from the protozoa into their hindgut as energy and carbon sources. If lower termites endogenously produce a complete cellulase system capable of hydrolyzing crystalline cellulose to glucose in the region anterior to the hindgut, sufficient glucose for the termites' needs is produced by the cellulase system with injected crystalline cellulose in the region.

The objective of this Chapter was to determine if the crystalline cellulose substance ingested by *C. formosanus* is hydrolyzed to glucose in the region ranging from the foregut to the midgut and to verify that the three cellulases essential for hydrolyzation of cellulose are present in the region covering the salivary glands, foregut, and midgut of the termite (Itakura *et al.*, 1997b).

2.2 Materials and Methods

2.2.1 Termites

Worker caste termites of *C. formosanus* were collected from the laboratory colony as described in Chapter 1. Termites were fed at 28°C on Japanese red pine, placed on moist dental plaster, for about a week before use.

2.2.2 Preparation for enzyme assay

2.2.2.1 Enzyme retrieval from the digestive system

Seven worker-caste termites were dissected and salivary glands and whole gut were removed. The whole gut was dissected into foregut, midgut, and hindgut. When the salivary glands were separated from the foregut with tweezers, the crop-contents effused into the thorax. Consequently, for all analyses, the foregut and its contents were combined with the dissected-body. The tissues from seven termites were homogenized in 0.7 ml of the physiological saline (42.6 mM NaCl, 22.1 mM KCl, 3.72 mM $CaCl_2$, 13.6 mM $MgSO_4$, 8.58 mM KH_2PO_4, 15.2 mM K_2HPO_4) in a Potter-Elvehjem homogenizer. The homogenates were centrifuged at 11500 x *g* for 3 min and the supernatants were obtained as crude extracts of the salivary glands, midgut, hindgut, and foregut/dissected-body. All operations were carried out at 0 - 4°C.

2.2.2.2 Enzyme retrieval from the whole body

The gut of the seven worker-caste termites was pulled out through the anus and divided by knife into midgut and hindgut. The gutted-body was divided into head, thorax, and abdomen. Each thorax had whole salivary glands and crop and residual fragments of the midgut where it tore close to the foregut during removal. The midgut, hindgut, head, thorax, and abdomen were immersed in physiological saline (0.7 ml) and homogenized. The homogenates were centrifuged at 11500 x *g* for 3 min and the supernatants were obtained as

crude extracts of the midgut, hindgut, head, thorax, and abdomen. All operations were carried out at 0 - 4°C.

2.2.3 Preparation for hydrolysis of cellulose

The gut of the four hundred worker-caste termites was pulled out and divided into midgut and hindgut. The midgut was combined with the gutted-body. This preparation is hereafter referred as to hindgutted-body preparation. The hindgutted-body and hindgut were immersed in distilled water (5 ml) and homogenized. The homogenates were centrifuged at 19500 x *g* for 20 min. The supernatants were adjusted to 60% saturation with ammonium sulfate and centrifuged again at 19500 x *g* for 30 min. The pellets were dissolved in distilled water (5 ml). The enzyme solutions were desalted on a column of Sephadex G-15, lyophilized and redissolved in 5 ml of distilled water. The resultant solutions were obtained as ammonium sulfate fractions of the hindgutted-body and hindgut. All operations were carried out at 0 - 4°C.

2.2.4 Enzyme assays

One unit of cellulase activity is defined as the amount of enzyme that produces 1 μmol of reducing sugar (expressed as glucose) per minute. An F-test was applied to examine the differences in cellulase activity among salivary glands, midgut, hindgut, and dissected-body, and among head, thorax, abdomen, midgut, and hindgut. Standard error of the mean (SEM) of the each cellulase activity was estimated by t-test.

2.2.4.1 Exo-1,4-ß-glucanase assay

Each crude extract (0.4 ml) or ammonium sulfate fraction (0.2 ml) was incubated with 1 ml of 2% (w/v) Avicel (Funacel SF for thin-layer chromatography, Funakoshi Co., Ltd.) in 0.1 M SAB, pH 5.0, at 30°C for 6 h. The mixtures were centrifuged. The reducing sugar in each of the supernatants was determined by the method of Somogyi (1951). Two controls were used: in one, each extract was inactivated by heating and was incubated in 0.1 M SAB with the substrate; in the other, each crude extract, allowed to remain active, was incubated in 0.1 M SAB without the substrate.

2.2.4.2 Endo-1,4-ß-glucanase assay

Each crude extract (0.4 ml) or ammonium sulfate fraction (0.2 ml) was incubated with 1 ml of 2% (w/v) carboxymethylcellulose (CMC) (CMC sodium salt, Nacalai Tesque, Inc.) in 0.1 M SAB at 30°C for 0.5 h. Determination of reducing sugar formed in the incubation mixtures and the controls was carried out in the same manner as described for **2.2.4.1 exo-1,4-ß-glucanase** except that CMC replaced Avicel as a substrate.

2.2.4.3 ß-glucosidase assay

Each crude extract (0.4 ml) was incubated with 1 ml of 1% (w/v) salicin (Kishida Chemical Co., Ltd.), while each ammonium sulfate fraction (0.2 ml) was incubated with 1 ml of 1% (w/v) *p*-nitrophenyl ß-glucopyranoside (*p*NPG) (Sigma Chemical Co., Missouri), in 0.1 M SAB at 30°C for 1 h. Determination of the reducing sugar formed in the incubation mixtures and the controls was carried out in the same manner as described for **2.2.4.1 exo-1,4-ß-glucanase** except that salicin or *p*NPG replaced Avicel as a substrate.

2.2.5 Rate of hydrolysis of cellulose in hindgut and hindgutted-body

Each ammonium sulfate fraction (1.5 ml) was incubated with 1.5 ml of 2% (w/v) Avicel in 0.1 M SAB at 30°C for 6 h. Aliquots (1 ml) of the mixtures were centrifuged and each supernatant was recovered.

2.2.5.1 Glucose and cellobiose assay

Glucose and cellobiose in each supernatant were converted to their trimethylsilyl (TMS) derivatives, and the TMS derivatives were measured by gas-liquid chromatography (GLC) with a GC-14A gas chromatograph (Shimadzu) on a column (2.1 m x 3 mm id) packed with Silicone SE-30 on chromosorb WAW DMCS 80/100. The oven temperature was programmed from 130°C to 230°C at 5°C/min.

2.2.6 Determination of glucose, trehalose, and acetate in midgut, hindgut, whole gut and gutted-body

The presence of glucose, trehalose, and acetate was determined by two measures. For one set experiments, worker caste termites were dissected and the whole gut was removed. The gut was dipped into water to rinse off the hemolymph adhering to its outer surface and separated into foregut, midgut, and hindgut. For other experiments the gut was pulled from the termites to obtain the gutted body (with the foregut in place) and the pulled gut which comprised most of midgut and the whole hindgut. The whole midgut and whole hindgut from 100 termites and the pulled gut and gutted body from 100 termites were homogenized in separate batches and centrifuged at 11500 x *g* for 3 min, and each supernatant was recovered. All operations were carried out at 0 - 4°C.

2.2.6.1 Glucose and trehalose assay

Glucose and trehalose in each supernatant were converted to their TMS derivatives, and the TMS derivatives were identified and measured by gas chromatography-mass spectrometry with a QP-1000 mass spectrometer (Shimadzu) on the same column and condition as described for **2.2.5.1 Glucose and cellobiose assay**.

2.2.6.2 Acetate assay

Acetate in each supernatant was converted to acetic acid, and the acetic acid was measured by GLC on a column (2.1 m x 3 mm id) packed with Unisol F-200 (GL Sciences). The oven temperature was programmed from 140°C to 180°C at 10°C/min.

2.3 Results

2.3.1 Enzyme activities

Exo-1,4-ß-glucanase, endo-1,4-ß-glucanase, and ß-glucosidase activities in extracts of digestive system segments of *C. formosanus* are compared in Table 2-1. Exo-1,4-ß-glucanase activity was detected in extracts of the salivary glands (19.6%) and midgut (18.1%), as well as of the hindgut(36.4%). Endo-1,4-ß-glucanase activity was highest in the salivary gland extracts; it was significantly higher ($p < 0.05$) than that in the other extracts. ß-Glucosidase activity was highest in the midgut extract; it was significantly higher ($p < 0.05$) than in the other extracts. About 75% of the total activity in the digestive system was detected in the midgut extract.

Enzyme activities for whole-body segment extract are presented in Table 2-2. Exo-1,4-ß-glucanase activities in the thorax, abdomen, and hindgut were significantly higher ($p < 0.05$) than those in the head and midgut, whereas no significant difference ($p > 0.05$) was

Table 2-1. Distribution of cellulase components in the digestive system[1] of the worker caste termite.

	Salivary glands	Midgut (whole)	Hindgut	Foregut/ dissected-body
	Activity (mU / termite) ± SEM[2]			
Exo-1,4-ß-glucanase	0.45 ± 0.11a (19.6)[3]	0.42 ± 0.06a (18.1)	0.85 ± 0.11b (36.4)	0.60 ± 0.11a,b (26.0)
Endo-1,4-ß-glucanase	28.1 ± 1.4c (34.5)	17.2 ± 1.3d (21.1)	14.8 ± 2.1d (18.2)	21.4 ± 2.6d (26.3)
ß-Glucosidase	2.65 ± 0.55e (12.7)	15.7 ± 2.0f (75.5)	0.87 ± 0.12e (4.2)	1.58 ± 0.04e (7.6)

[1] Salivary glands and whole gut of seven worker-caste termites were removed and the gut divided into foregut, midgut, and hindgut. The foregut and dissected-body segments were combined for all analyses.

[2] Values represent means of five replications ± SEM. Values followed by same letter are not significantly different ($p > 0.05$).

[3] Values in parentheses represent relative value (%) of each enzyme activity.

detected among the activities in the thorax, abdomen, and hindgut, or between activities in the head and midgut. The activities in the head and midgut were low but significant. Endo-1,4-ß-glucanase activity was highest in the thorax, significantly higher ($p < 0.05$) than activity in the head, midgut, and hindgut. The activity in the midgut was also significantly higher ($p < 0.05$) than that in the head and hindgut. ß-Glucosidase activity was highest in the midgut. That activity was significantly higher ($p < 0.05$) than activity in all other sections. ß-Glucosidase activity in the thorax was significantly higher ($p < 0.05$) than that in the head, abdomen, and hindgut.

Table 2-2. Distribution of cellulase components in the whole body[1] of the worker caste termite.

	Head	Thorax	Abdomen	Midgut	Hindgut
	Activity (mU / termite) ± SEM[2]				
Exo-1,4-ß-glucanase	0.08 ± 0.01a (7.9)[3]	0.25 ± 0.01b (23.8)	0.30 ± 0.02b (28.3)	0.11 ± 0.01a (11.0)	0.30 ± 0.02b (29.0)
Endo-1,4-ß-glucanase	2.08 ± 0.37c (5.2)	12.5 ± 0.8d (30.9)	11.3 ± 0.5d,e (28.0)	9.87 ± 0.71e (24.4)	4.65 ± 0.77c (11.5)
ß-Glucosidase	0.05 ± 0.04f (0.9)	1.49 ± 0.80g (26.9)	0.35 ± 0.20f,h (6.3)	3.41 ± 0.43g (61.5)	0.25 ± 0.02h (4.5)

[1] Gut was pulled and divided into hindgut and midgut, which had been torn from the foregut. The gutted-body was divided into head, abdomen, and thorax, which comprised the salivary glands, foregut, and residual fragments of the midgut.

[2] Values represent means of five replications ± SEM. Values followed by same letter are not significantly different ($p > 0.05$).

[3] Values in parentheses represent relative value (%) of each enzyme activity.

2.3.2 Rate of hydrolysis of cellulose

Amounts of glucose and cellobiose formed from Avicel during incubation of it with the hindgut and hindgutted-body preparations are presented together with distribution of enzyme activities in Table 2-3. More than 50% of the total exo-1,4-ß-glucanase and endo-1,4-ß-glucanase activities in the termite were detected in the hindgutted-body preparation. Both the hindgut and hindgutted-body preparations hydrolyzed Avicel to glucose and cellobiose to a significant extent. The amount of glucose formed from the mixture of Avicel with the hindgutted-body preparation was about 50% larger than that with the hindgut preparation, when the mixtures had been incubated for 6 h. To form about 42 nmol/termite of cellobiose, it took 6 h in the mixture of Avicel with the hindgut preparation, whereas it took only 3 h with the hindgutted-body preparation.

Table 2-3. Distribution of cellulase components and rate of hydrolysis of crystalline cellulose in the hindgut and hindgutted-body.[1)]

		Hindgut preparation	Hindgutted-body preparation
		Activity (mU / termite)	
Exo-1,4-ß-glucanase		0.137 (47.4)[3)]	0.150 (52.3)
Endo-1,4-ß-glucanase		5.140 (45.4)	6.194 (54.6)
ß-Glucosidase		0.019 (4.1)	0.449 (95.9)
	Incubation period (h)	Amount formed from crystalline cellulose (nmol / termite)	
Glucose	0.1	0.58[2)] (44.6)[3)]	0.72 (55.4)
	3	6.12 (42.3)	8.34 (57.7)
	6	10.7 (40.1)	16.0 (59.9)
Cellobiose	0.1	0.45 (13.0)	3.0 (87.0)
	3	28.5 (40.3)	42.3 (59.7)
	6	42.4 (47.2)	47.4 (52.8)
Total	0.1	1.03 (21.7)	3.72 (78.3)
	3	34.6 (40.6)	50.6 (59.4)
	6	53.1 (45.6)	63.4 (54.4)

1) Gut was pulled out and divided into midgut and hindgut. The midgut was combined with the gutted-body.

2) Values represent means of two replications.

3) Values in parentheses represent relative value (%) of each enzyme activity or amount of saccharide at the same incubation period.

2.3.3 Glucose, trehalose, and acetate distribution

The distributions of glucose, trehalose and acetate in *C. formosanus* are shown in Table 2-4 and 2-5. In pure gut homogenates, about 66% of the glucose and 26% of the trehalose in the gut were detected in the midgut (Table 2-4). Thus most of the glucose in the gut was located in the midgut, including its walls and contents. About 160 nmol of

Table 2-4. Distribution of glucose and trehalose in the digestive system homogenates.

	Glucose	Trehalose
	(nmol / termite)	
Midgut (whole) homogenate	6.00	0.75
Hindgut homogenate	3.15	2.18

Table 2-5. Distribution of glucose, trehalose, and acetate in whole-body homogenates.

	Glucose	Trehalose	Acetate
		(n mol / termite)	
Gut homogenate[1)]	11.4	3.6	8.6
Gutted-body homogenate[2)]	147.5	24.3	2.8

1) Gut homogenate was prepared from the pulled gut, which comprised most of the midgut and the whole hindgut.

2) Gutted-body from which most of midgut and whole hindgut had been removed.

glucose and 28 nmol of trehalose were detected in the whole body homogenate. About 11 - 12 nmol (~ 7%) of glucose and 3 - 4 nmol (~ 13%) of trehalose were detected in the pulled gut (Table 2 - 5). Approximately 11.5 nmol of acetate was detected in the whole body, about 75% of which was found in the gut homogenate, including its walls and contents. Thus most of the glucose (~ 93%) and the trehalose (~ 87%) were present in the gutted-body, but most of the acetate (~ 75%) was present in the gut homogenate.

2.4 Discussion

Exo-1,4-ß-glucanase and endo-1,4-ß-glucanase were detected to significant extents in the salivary glands and midgut. This result is consistent with the results with other lower termites (Veivers *et al.*, 1982), but conflicts with the result by Yoshimura *et al.* (1992),

reporting that most of the exo-1,4-ß-glucanase (87%) present in *C. formosanus* is located in the hindgut. Then they concluded that the lower termites are dependent on the protozoa in the hindgut for the complete hydrolysis of crystalline cellulose to glucose, in the other words the crystalline cellulose ingested is only partially degraded and hydrolyzed to glucose to a negligible extent in the region anterior to the hindgut, and most of glucose originating from the cellulose substance is produced only in the bodies of the protozoa in the hindgut. To examine whether cellulose is completely hydrolyzed only in the hindgut, I determined the amount of glucose and cellobiose formed from Avicel, which had been incubated with the hindgut and hindgutted-body preparations. Both the rates of formation of glucose and cellobiose in the mixture of Avicel with the hindgutted-body preparation were much higher than those with the hindgut preparation. These results were consistent with the fact that more than 50% of each of the hydrolytic enzyme activities detected in the termite was detected in the segments other than the hindgut. Most of ß-glucosidase activity (75.5%) detected in the whole bodies of the termites was detected in the midgut. Consequently crystalline cellulose would be able to be hydrolyzed more efficiently to glucose and cellobiose in the region anterior to the hindgut.

About 66% of the total glucose amount detected in the whole gut were detected in the midgut. This fact also suggests that crystalline cellulose is more efficiently hydrolyzed to glucose in the region anterior to the hindgut than in the hindgut. Similarly, in the higher termite *Nasutitermes walkeri* Hill, most of the cellulose hydrolysis occurs in the foregut and midgut, and most of the glucose produced is absorbed at the midgut (Hogan *et al.*, 1988). It has been proposed that glucose will be absorbed in the midgut in both the lower termites and the higher termites (Slaytor, 1992).

Most of the cellulase activities detected in the thorax would be ascribed to the cellulases existing in the salivary glands and a part of the midgut, for the thorax contains the tissues that show the cellulase activities to significant extents. Most of the exo- and endo-1,4-ß-glucanase activities found in the head would be due to the corresponding cellulases

secreted in the salivary glands, because the salivary glands open into pharynx in the head section (Gokan, 1992).

Part of the exo-1,4-ß-glucanase, endo-1,4-ß-glucanase, and ß-glucosidase activities found in the hindgut would be ascribed to the cellulases in the symbiotic protozoa in the hindgut of the lower termite (Yoshimura *et al.*, 1992). The lower termite devoid of protozoa cannot survive on cellulose (Mauldin *et al.*, 1972). These results indicate that the protozoa play a significant role other than cellulose hydrolysis when the lower termite feeds on natural cellulose substrates.

Cellulase activities detected in the abdomen or the dissected body including the foregut could never be ascribed to the cellulases in the hindgut and the midgut because the abdomen and dissected-body extracts were prepared after the midgut and hindgut had been completely removed. These results suggest that both the cellulases exist in the foregut to significant extents or are produced by the termite itself, or both of the termite and symbiotic micro-organisms.

Large amounts of glucose and trehalose were detected in the gutted-body and to a minor but significant extent in the gut homogenate. Most of the glucose present in the gut exists in the midgut including the gut-wall and contents. This corresponds with the presence of enzymes in the digestive tract above the midgut.

These results suggest that the lower termite *C. formosanus* hydrolyzes cellulose to glucose in the foregut and midgut as well as in the hindgut and the glucose, which is mainly produced in the midgut, is absorbed through the midgut-wall to be used as carbon and energy sources, and that trehalose and glycogen synthesized from the glucose are converted to glucose when glucose is required.

2.5 Summary

Extracts of the digestive system and of the whole body of *C. formosanus* were assayed to determine tissular localization of various cellulases to verify its full cellulolytic system.

About 20%, 18% and 36% of the total exo-1,4-ß-glucanase activity of *C. formosanus* were detected in the salivary glands, midgut, and hindgut, respectively. About a third of the total endo-1,4-ß-glucanase activity in the termite was detected in the salivary glands (34.5%) whereas the relative activities in the midgut and hindgut were 21.1% and 18.2%, respectively. About 75% of the total ß-glucosidase activity in the termite was detected in the midgut. Thus all the necessary cellulases for hydrolysis of natural cellulose to glucose were present in the region ranging from the salivary glands to the midgut in significant amounts. Most of the glucose and trehalose detected in the termite existed in the gutted-body. Most of the glucose detected in the gut existed in the midgut. These results suggest that natural cellulose ingested by the termite is hydrolyzed to oligosaccharides in the region of the foregut and midgut as well as in the hindgut, that oligosaccharides are hydrolyzed to glucose predominantly in the midgut, and that the resultant glucose is absorbed through the midgut wall into the tissues to be used as important energy and carbon sources.

Chapter 3 PARTIAL PURIFICATION OF CELLULASE COMPONENTS FROM *COPTOTERMES FORMOSANUS* SHIRAKI AND CHARACTERIZATION OF HYDROLYSIS PRODUCTS FROM CRYSTALLINE CELLULOSE BY THE CELLULASE COMPONENTS

3.1 Introduction

As described in Chapter 2, the natural cellulose ingested by *C. formosanus* is hydrolyzed to glucose at a higher rate in the region of foregut and midgut than in the hindgut and the resultant glucose is absorbed through the midgut wall into the tissue of the termite.

However, cellulase components of the termite have not been isolated and characterized. It is noteworthy to compare cellulase components in the termite tissue (devoid of hindgut) with those in the hindgut.

The object of this Chapter is to determine if CBH (EC 3.2.1.91) activity assayed by using *p*-nitrophenyl β-D-cellobioside as a substrate is proportional to a activity of hydrolysis of natural cellulose by cellulolytic system in *C. formosanus* (Itakura *et al.*, 1998).

3.2 Materials and Methods

3.2.1 Termites

Worker caste termites of *C. formosanus* were collected from the laboratory colony as described in Chapter 1. Termites were fed at 28°C on Japanese red pine, placed on moist dental plaster, for about a week before use.

3.2.2 Preparation of enzyme extracts

Three thousand worker cast termites were immobilized and dissected. The gut was pulled out through the anus using fine-tipped forceps and divided into midgut and hindgut. The midgut was combined with the gutted-body. This preparation is hereafter referred as to hindgutted-body preparation. The hindgutted-body and hindgut were homogenized in distilled water (10 ml). The homogenates were centrifuged at 19500 x *g* for 20 min. The supernatants were obtained as crude extracts of the hindgutted-body and hindgut. These crude extracts were adjusted to 30% saturation with ammonium sulfate, left overnight, and centrifuged at 19500 x *g* for 30 min. The supernatants were adjusted to 60% saturation with ammonium sulfate, left overnight, and centrifuged at 19500 x *g* for 30 min again. The pellets were dissolved in MilliQ water (3 ml). The enzyme solutions were desalted on a column of Sephadex G-25, lyophilized, and redissolved in 3 ml of 20 mM tris(hydroxymethyl)aminomethane buffer (TB), pH 8.5. The resultant solutions were obtained as ammonium sulfate fractions of the hindgutted-body and hindgut. All operations were performed at 0 - 4°C. The protein content of each enzyme solution was measured by the method of Lowry *et al.* (1951), using bovine serum albumin as the standard.

3.2.3 Enzyme assays

One unit of cellulase activity is defined as the amount of enzyme that produces 1 μmol of reducing sugar (expressed as glucose) or *p*-nitrophenol per minute.

3.2.3.1 Exo-1,4-ß-glucanase assay

Each crude extract (20 μl) or ammonium sulfate fraction (5 μl) was incubated with 1 ml of 2% (w/v) Avicel (Funacel SF for thin-layer chromatography, Funakoshi Co., Ltd.) in

0.1 M SAB, pH 5.0, at 37°C for 3 h, whereas each fraction separated by anion exchange chromatography (100 µl) was incubated with 2% Avicel in 0.1 M SAB (400 µl) at 37°C for 6 h. The mixtures were centrifuged. The reducing sugar in each of the supernatants was determined by the method of Somogyi (1951).

3.2.3.2 Endo-1,4-ß-glucanase assay

Each crude extract (20 µl) or ammonium sulfate fraction (5 µl) was incubated with 1 ml of 2% (w/v) carboxymethylcellulose (CMC) (CMC sodium salt, Nacalai Tesque, Inc.) in 0.1 M SAB at 37°C for 30 min, whereas each fraction separated by anion exchange chromatography (20 µl) was incubated with 2% CMC in 0.1 M SAB (400 µl) at 37°C for 1 h. Determination of reducing sugar formed in the incubation mixtures was carried out in the same manner as described for **3.2.3.1 exo-1,4-β-glucanase.**

3.2.3.3 β-Glucosidase assay

Each crude extract (10 µl) or ammonium sulfate fraction (2.5 µl) was incubated with 500 µl of 20 mM *p*-nitrophenyl β-D-glucopyranoside (*p*NPG) (Sigma Chemical Co., Missouri) in 0.1 M SAB at 37°C for 30 min, whereas each fraction separated by anion exchange chromatography (20 µl) was incubated with 6.25 mM *p*NPG in 0.1 M SAB (80 µl) at 37°C for 1 h. *p*-Nitrophenol in each of the incubation mixtures was determined by the method of Deshpande *et al.* (1984).

3.2.3.4 Exo-cellobiohydrolase (CBH) assay

Assay of CBH was carried out in the same manner as described for **3.2.3.3 β-glucosidase** except that *p*-nitrophenyl β-D-cellobioside (*p*NPC) (Sigma Chemical Co.,

Missouri) replaced *p*NPG as a substrate. Assay also was performed in the presence of 5 or 20 mM D-glucono-1,5-δ-lactone (Wako Pure Chemical Industries, Ltd.) to remove the activity of β-glucosidase contaminating each of enzyme solutions.

3.2.4 Anion exchange chromatography

Each of ammonium sulfate fractions (500 μl) of the hindgutted-body and hindgut was fractionated by anion exchange chromatography on a TSK gel SuperQ-5PW (Tosoh). The mobile phase was 20 mM TB, pH 8.5, in a linear gradient of sodium chloride (Figures 3-1 and 3-2), of 0 M to 0.5 M at a flow rate of 1 ml/min. All operations were performed at 0 - 4°C.

3.2.5 Hydrolysis of crystalline cellulose by fractionated cellulase components

Each of fractions having significant extents of exo-1,4-ß-glucanase activity (fractions 3, 15, 24, and 27 of the hindgutted-body, and fractions 4, 13, 16, 24, and 27 of the hindgut) was incubated with 1% (w/v) Avicel in 0.1 M SAB (total volume of reaction mixture; 1.1 ml) at 37°C for 1 h. Volume of each fraction in the reaction mixture (Table 3-2) was determined to produce 200 μg (1.11 μmol) of reducing sugar (expressed as glucose) from crystalline cellulose during incubation of 3 h on the basis of exo-1,4-ß-glucanase activity of each fraction. When exo-1,4-ß-glucanase activity of a fraction was too weak to produce 200 μg of the reducing sugar from cellulose, volume of the fraction that produced 100 μg (0.56 μmol) of the reducing sugar was added to the reaction mixture (fraction 27 of the hindgutted-body, and fractions 4, 24, and 27 of the hindgut). The mixtures were centrifuged and each supernatant was recovered.

3.2.5.1 Glucose and cellooligosaccharides assay

Glucose and cellooligosaccharides in each supernatant from the reaction mixtures were measured by thin-layer chromatography (TLC) and gas-liquid chromatography (GLC). Aliquots (40 μl) of the supernatants were spotted on a thin-layer chromatoplate on silica gel 60 (Merck KGaA, Darmstadt), developed in chloroform-methanol-water (18:13:3), sprayed with 2% copper(II) sulfate-13% sulfuric acid, and heated to detect glucose and cellooligosaccharides (cellobiose, cellotriose, cellotetraose, cellopentaose, and cellohexaose). Glucose and cellooligosaccharides in each supernatant were converted to their trimethylsilyl (TMS) derivatives, and the TMS derivatives of glucose, cellobiose, and cellotriose were measured by GLC with a GC-14A gas chromatograph (Shimadzu) on a column (2.1 m x 3 mm id) packed with Silicone SE-30 on chromosorb WAW DMCS 80/100. The oven temperature was programmed from 150°C to 300°C at 10°C/min.

3.3 Results

Exo-1,4-ß-glucanase, endo-1,4-ß-glucanase, ß-glucosidase, and CBH activities in extracts of *C. formosanus* are compared in Table 3-1. Exo-1,4-ß-glucanase, endo-1,4-ß-glucanase, and ß-glucosidase activities in the hindgutted-body extract were significantly higher than those in the hindgut extract, while most of CBH activity in the whole termite was detected in the hindgut extract. D-Glucono-1,5-δ-lactone is a powerful inhibitor on ß-glucosidase. In the presence of D-glucono-1,5-δ-lactone, CBH activities in crude extract and ammonium sulfate fraction of the hindgutted-body were inhibited by 92% and 86% respectively, whereas those of the hindgut were inhibited by 56% and 36% respectively.

The results of anion exchange chromatography of the hindgutted-body and hindgut extracts are shown in Figures 3-1 and 3-2. Four cellulase components were separated from the extract from the hindgutted-body, while five components were separated from the hindgut extract. Among the four cellulase components isolated from the hindgutted-body, a

Table 3-1. Cellulase activities of cellulase components of *C. formosanus*.

	Protein (mg)	Total activity (mU)[1]				Specific activity (mU/mg)				Ratio of CMC : Avicel[3]
		Avicel[2]	CMC[2]	*p*NPG[2]	*p*NPC[2]	Avicel	CMC	*p*NPG	*p*NPC	
Hindgutted-body[4]										
Crude extract	214.3	2001.6	76598.4	2576.4	49.7[5] (591.6)[6]	9.3	357.4	12.0	0.2[5] (2.8)[6]	38.3 : 1
(NH4)2SO4 (30 to 60%)	25.0	761.4	28939.8	1163.1	18.2 (129.3)	30.5	1157.6	46.5	0.7 (5.2)	38.0 : 1
TSK gel SuperQ-5 PW										
Fraction 3	0.4	11.7	1423.2	0.2	0.1 (0.2)	29.3	3558.0	0.5	0.0 (0.5)	121.6 : 1
Fraction 15	0.5	5.2	95.5	7.9	0.7 (1.7)	10.4	191.0	15.8	1.4 (3.4)	18.4 : 1
Fraction 24	0.3	6.6	1252.5	7.1	0.1 (0.8)	22.0	4175.0	23.7	0.3 (2.7)	189.8 : 1
Fraction 27	0.4	2.4	35.7	41.6	0.0 (26.2)	6.0	89.3	104.0	0.0 (65.5)	14.9 : 1
Hindgut										
Crude extract	85.0	1057.2	27133.2	498.0	103.1(234.0)	12.4	319.2	5.9	1.2 (2.8)	25.7 : 1
(NH4)2SO4 (30 to 60%)	16.5	581.7	15238.2	393.6	87.4 (136.5)	35.3	923.5	23.9	5.3 (8.3)	26.2 : 1
TSK gel SuperQ-5 PW										
Fraction 4	0.2	1.9	84.1	0.1	0.1 (0.3)	9.5	420.5	0.5	0.5 (1.5)	44.3 : 1
Fraction 13	0.1	3.3	31.6	0.1	0.2 (0.3)	33.0	316.0	1.0	2.0 (3.0)	9.6 : 1
Fraction 16	0.3	7.0	673.2	0.8	1.8 (5.1)	23.3	2244.0	2.7	6.0 (17.0)	96.2 : 1
Fraction 24	0.2	1.7	30.7	2.3	0.3 (0.6)	8.5	153.5	11.5	1.5 (3.0)	18.1 : 1
Fraction 27	0.1	1.9	123.1	0.8	0.3 (0.6)	19.0	1231.0	8.0	3.0 (6.0)	64.8 : 1

1) One unit of cellulase activity is defined as the amount of enzyme that produces 1 μmol of reducing sugar (expressed as glucose) or *p*-nitrophenol per minute.

2) Exo-1,4-ß-glucanase, endo-1,4-ß-glucanase, ß-glucosidase, and CBH activities are expressed as Avicel, CMC, *p*NPG (*p*-nitrophenyl ß-D-glucopyranoside), and *p*NPC (*p*-nitrophenyl ß-D-cellobioside) respectively.

3) Ratio of endo-1,4-ß-glucanase activity to exo-1,4-ß-glucanase activity.

4) Hindgut was removed from whole termite.

5) CBH activity determined in the presence of 20 mM D-glucono-1,5-δ-lactone in the reaction mixture.

6) Values in parentheses are apparent CBH activity determined in the absence of D-glucono-1,5-δ-lactone in the reaction mixture.

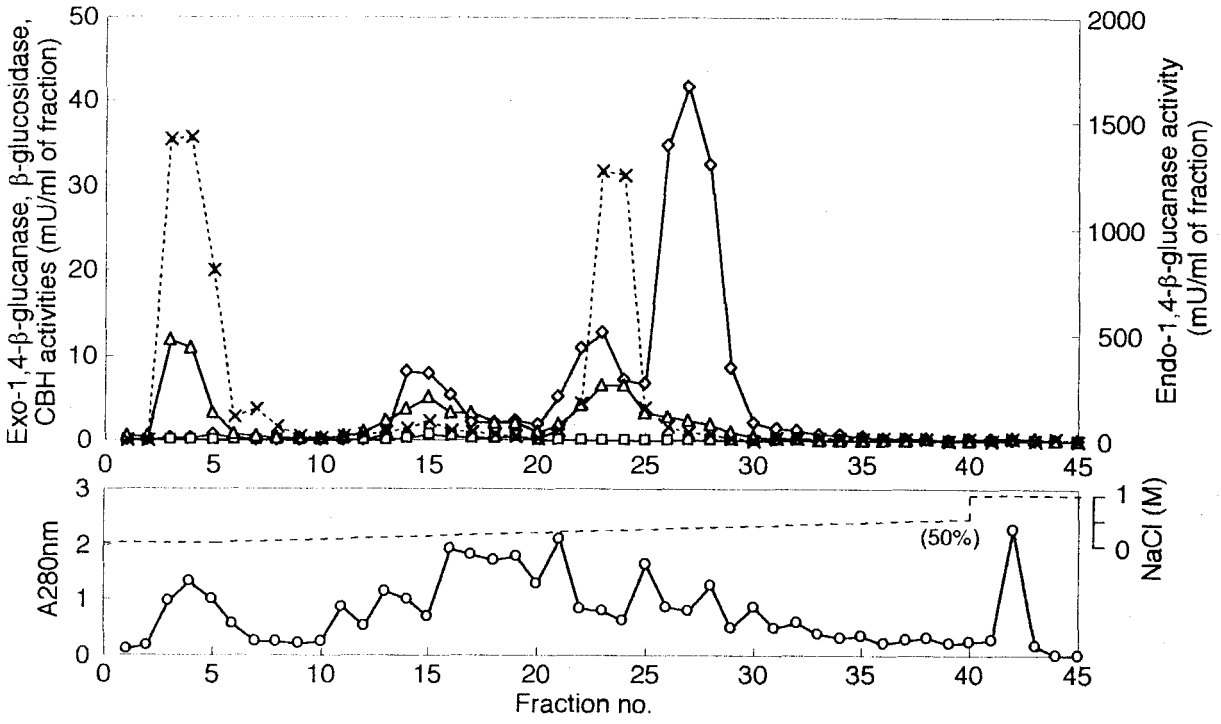

Fig. 3-1. Partial purification of cellulase components from the hindgutted-body extract on a TSK gel SuperQ-5PW column. A 500 μl of the extract (a precipitate with 30-60% $(NH_4)_2SO_4$) dissolved in 20 mM TB, pH 8.5, was applied to the column and eluted at 1 ml/min using a 0 - 0.5 M NaCl gradient in the same buffer. △, Exo-1,4-β-glucanase; ×, endo-1,4-β-glucanase; ◇, β-glucosidase; □, CBH activities (in the presence of 5 mM D-glucono-1,5-δ-lactone in reaction mixture); ○, Absorbance at 280nm.

cellulase component had exo-1,4-ß-glucanase and endo-1,4-ß-glucanase activities (fractions 3-4), two components had exo-1,4-ß-glucanase, endo-1,4-ß-glucanase, and ß-glucosidase activities (fractions 23 - 24 and 26 - 28), and one component had significant extents of exo-1,4-ß-glucanase, endo-1,4-ß-glucanase, ß-glucosidase, and CBH activities (fractions 14 - 15). Among the five cellulase components isolated from the hindgut extract, two cellulase components significantly showed exo-1,4-ß-glucanase and endo-1,4-ß-glucanase activities (fractions 3 - 4 and 13), and three cellulase components exo-1,4-ß-glucanase, endo-1,4-ß-glucanase, ß-glucosidase, and CBH activities (fractions 15 - 17, 24 - 25, and 27). Exo-1,4-ß-

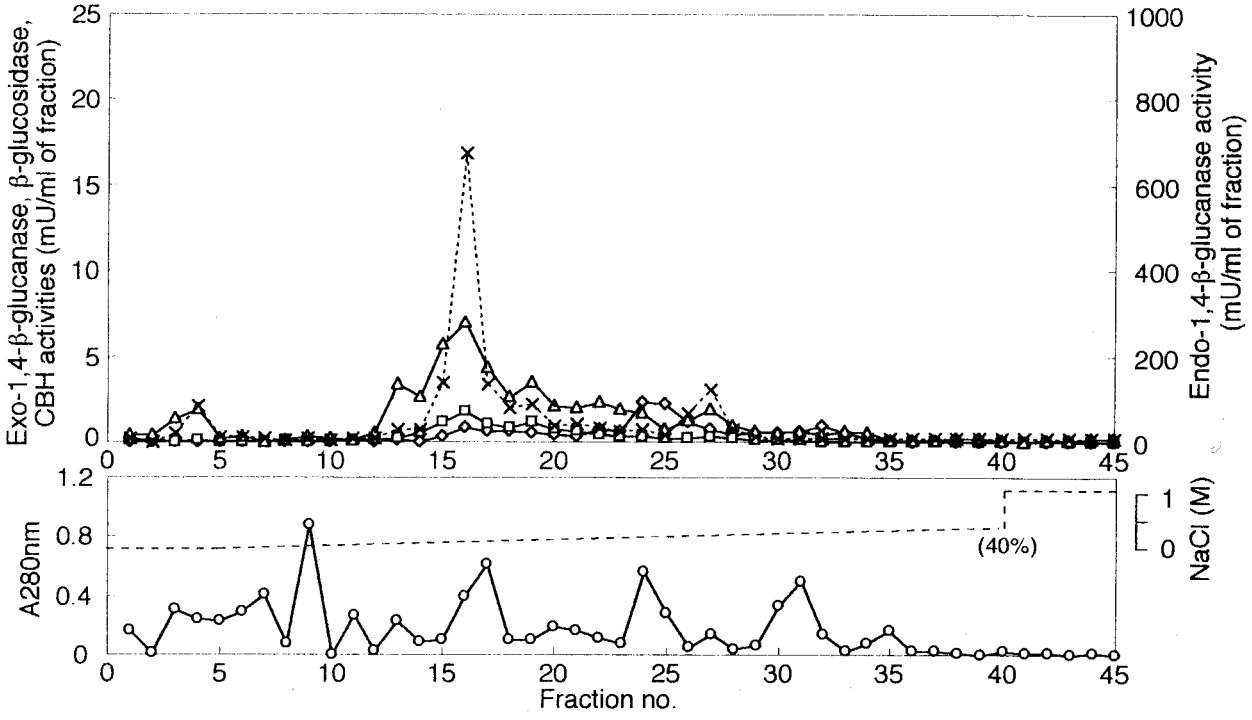

Fig. 3-2. Partial purification of cellulase components from the hindgut extract on a TSK gel SuperQ-5PW column. A 500 μl of the extract (a precipitate with 30-60% $(NH_4)_2SO_4$) dissolved in 20 mM TB, pH 8.5, was applied to the column and eluted at 1 ml/min using a 0 - 0.4 M NaCl gradient in the same buffer. △, Exo-1,4-β-glucanase; ×, endo-1,4-β-glucanase; ◇, β-glucosidase; □, CBH activities (in the presence of 5 mM D-glucono-1,5-δ-lactone in reaction mixture); ○, Absorbance at 280 nm.

glucanase, endo-1,4-ß-glucanase, ß-glucosidase, and CBH activities of the cellulase components separated from the hindgutted-body and hindgut extracts are shown in Table 3-1. The ratio of endo-1,4-ß-glucanase activity of each fraction to exo-1,4-ß-glucanase activity also is shown in Table 3-1. The ratios of two cellulase components of the hindgutted-body (fractions 3 and 24) and one component of the hindgut (fraction 16) were significantly higher than those of others, while the ratios were low in two components of the hindgutted-body (fractions 15 and 27) and two of the hindgut (fractions 13 and 24). Each of CBH activities of fractions 3, 15, 24, and 27 from the hindgutted-body extract was

inhibited by 50%, 59%, 88%, and 100% in the presence of D-glucono-1,5-δ-lactone respectively, while that of fractions 4, 13, 16, 24, and 27 from the hindgut extract was inhibited by 67%, 33%, 65%, 50%, and 50% respectively.

Table 3-2. Hydrolysis of crystalline cellulose by cellulase components isolated from *C. formosanus*.

	Volume of fraction in the reaction mixture (ml)	Amount formed from crystalline cellulose (μmol/ml of fraction)		
		Glucose	Cellobiose	Cellotriose
Hindgutted-body[1)]				
Fraction 3	0.264[2)]	2.86	1.30	ND[4)]
Fraction 15	0.593[2)]	1.00	0.47	ND
Fraction 24	0.467[2)]	1.95	0.09	ND
Fraction 27	0.642[3)]	0.76	0.08	ND
Hindgut				
Fraction 4	0.811[3)]	0.36	0.32	ND
Fraction 13	0.934[2)]	0.55	0.66	ND
Fraction 16	0.441[2)]	1.47	1.09	ND
Fraction 24	0.907[3)]	0.32	0.30	ND
Fraction 27	0.811[3)]	0.35	0.01	ND

1) Hindgut was removed from whole termite.

2) Volume of fraction in the reaction mixture was determined to produce 200 μg (1.11 μmol) of reducing sugar (expressed as glucose) from crystalline cellulose during incubation of 3 h on the basis of exo-1,4-ß-glucanase activity of each fraction.

3) Volume of fraction producing 100 μg (0.56 μmol) of the reducing sugar from crystalline cellulose was added to the reaction mixture when exo-1,4-ß-glucanase activity of a fraction was too weak to produce 200 μg of the reducing sugar as mentioned above (2).

4) ND, not detected.

Amounts of glucose, cellobiose, and cellotriose formed from Avicel by each of fractions isolated from the hindgutted-body and hindgut extracts are presented in Table 3-2.

A significant amount of glucose was formed from Avicel by each of fractions 3 and 24 of the hindgutted-body and fraction 16 of the hindgut. Neither of fractions of the hindgutted-body nor fractions of the hindgut produced cellotriose from Avicel. Cellotriose, cellotetraose, cellopentaose, and cellohexaose were not detected on the TLC plates on which the supernatants of the mixtures of Avicel and each of fractions isolated from both extracts were spotted.

3.4 Discussion

The IUBMB (International Union of Biochemistry and Molecular Biology) Enzyme Nomenclature (1992), which is based on the type of reaction that enzymes catalyze and on their substrate-specificity, classifies cellulases into three categories; endo-1,4-ß-glucanase, CBH, and exo-1,4-ß-glucosidase (EC 3.2.1.74). Recently classification of glycosyl hydrolases by Hydrophobic Cluster Analysis based on amino-acid-sequence similarities of their catalytic domains is proposed (Henrissat and Bairoch, 1996). Cellulases are classified into twelve families (families 5, 6, 7, 8, 9, 10, 12, 44, 45, 48, 51, and 56). Among these cellulase families, families 6, 7, 9, and 48 consist of both endo-1,4-ß-glucanase and CBH that have been considered to act on cellulose by completely different mechanisms, thus it is conceivable that mechanism of action of endo-1,4-ß-glucanase is similar to that of CBH. These results indicate that amino-acid-sequences of active sites in the catalytic domains of endo-1,4-ß-glucanase and CBH classified into families 6, 7, 9, and 48 are very similar to each other and these enzymes could effectively hydrolyze both the crystalline and amorphous regions of natural cellulose.

All cellulase components isolated from the hindgutted-body and hindgut of the termite hydrolyzed Avicel to glucose and cellobiose to significant extents although the ratio of endo-1,4-ß-glucanase activity of each component to exo-1,4-ß-glucanase activity of the component varied from about 10:1 to 190:1. Any cellulase component having

independently either endo-1,4-ß-glucanase activity or exo-1,4-ß-glucanase activity could not be separated by anion exchange chromatography and gel filtration chromatography. It is assumed that cellulase components in the termite are the enzymes hydrolyzing both crystalline and amorphous regions of cellulose as mentioned above. *p*NPC was used as a substrate to assay CBH activity in this study. In general, cellulolytic enzymes splitting off cellobiose units from Avicel or cellulose swollen in phosphoric acid act on the agluconic bond of *p*NPC to produce cellobiose and *p*-nitrophenol, whereas cellulolytic enzymes unable to act on *p*NPC to produce cellobiose do not produce cellobiose from natural cellulose such as Avicel. Some cellulases splitting off cellobiose from *p*NPC but not from Avicel are known also. Thus cellulases unable to hydrolyze *p*NPC can not produce cellobiose from Avicel. These facts lead to the conclusion that a cellulase component unable to split off the agluconic bond of *p*NPC does not have true CBH activity. Some cellulase components having no or little CBH activity, isolated from the hindgutted-body extract (fractions 3, 24, and 27) and the hindgut extract (fractions 4 and 13), hydrolyzed Avicel to glucose and cellobiose to significant extents. These results show that CBH activity is not essential for cellulolytic system in the termite to hydrolyze natural cellulose up to glucose. It has been reported that natural cellulose is hydrolyzed significantly by cellulolytic systems of *Bacillus circulans* (Kim, 1995), *Bacteroides succinogenes* (Groleau and Forsberg, 1981), *Cytophaga* sp. WHTC 2421 (Chang and Thayer, 1977), and *Pseudomonas fluorescens* subsp. *cellulosa* (Gilbert *et al.*, 1987) although any CBH has not been detected in cellulolytic systems of these bacteria. Endo-1,4-ß-glucanases isolated from *Irpex lacteus* (Kanda *et al.*, 1976) and *Trichoderma viride* (Beldman *et al.*, 1985) also have been reported to considerably hydrolyze natural cellulose in the absence of CBH.

Glucose was major product formed from Avicel during incubation of it with each of fractions 3 and 24 isolated from the hindgutted-body extract. The ratio of endo-1,4-ß-glucanase activity to exo-1,4-ß-glucanase activity in fractions 3 or 24 was very high and about 122 or 190. These fractions showed no and little CBH activities. Therefore, Avicel

could be hydrolyzed to glucose by cellulase components having mainly endo-1,4-ß-glucanase activity in these fractions. These results are consistent with the result by Klesov (1991), reporting that endo-1,4-ß-glucanase forming appreciable amounts of glucose in the hydrolysis of cellulose is necessary in reactions of solubilization of crystalline cellulose.

Ratios of endo-1,4-ß-glucanase activity of two endo-1,4-ß-glucanase components isolated from the other lower termite *R. speratus* Kolbe to exo-1,4-ß-glucanase activity of the components have been reported as 3100:1 and 7600:1 (Watanabe *et al.*, 1997). These results show that cellulase components isolated from the hindgutted-body extract of *C. formosanus* have a higher ability of hydrolyzing natural cellulose than endo-1,4-ß-glucanase isolated from *R. speratus*.

The termite *C. formosanus* digests over 70% of the cellulose in the wood-cell walls ingested by the termite (Chapter 1), although the ability of hydrolyzing natural cellulose by the cellulase components isolated from the termite is much lower than that by CBH isolated from the fungus *Trichoderma reesei* (Wood, 1991). This can be accounted for by the following mechanical actions that are characteristic of termites' digestion: termites grind wood to be less than 50 μm in size by trituration (Chapter 1); cellulose-exposed area in the cell walls of the ground wood particles, where the cellulase components are smoothly accessible to the exposed cellulose, tremendously increases; and the ground wood particles are continuously mixed and stirred with the cellulase components by peristaltic movement in the digestive system of termites until they have been excluded from anus of termites. The mechanical actions such as trituration, mixing, and stirring can significantly promote the hydrolysis of natural cellulose to glucose in termites' digestive system. These mechanical actions do not occur in the hydrolysis of the cellulose component in wood by the cellulases produced by wood decay fungi.

A considerable amount of ß-glucosidase activity (42 mU), as well as slight activities of exo-1,4-ß-glucanase (2 mU) and endo-1,4-ß-glucanase (36 mU) ,was detected in fraction 27 separated from the hindgutted-body extract. Among these activities, exo-1,4-ß-glucanase

and endo-1,4-ß-glucanase activities should be due to contamination with cellulase component mainly detected in fractions 22 - 23 (Fig. 3-1). Glucose (0.76 μmol/ml) was a major product in Avicel hydrolysis by fraction 27. Most part of the glucose could be produced by the ß-glucosidase of fraction 27 from the cellooligosaccharides which had been produced from Avicel by the contaminant from fractions 22 - 23 having exo-1,4-ß-glucanase and endo-1,4-ß-glucanase activities. Thus main cellulase component existing in the fraction 27 could be ß-glucosidase.

A significant CBH activity (1.8 mU) and a little ß-glucosidase activity (0.8 mU) were detected in fraction 16 separated from the hindgut extract. The CBH activity was inhibited by about 65% when D-glucono-1,5-δ-lactone was added to the reaction mixture. This result is conflict with the results with CBHs of fungi *T. reesei* and *Sporotrichum pulverulentum* of which activities are hardly inhibited by D-glucono-1,5-δ-lactone (Deshpande *et al.*, 1984). These results indicate that the action mechanism of a termite's cellulase component having CBH activity differs significantly from that of CBH of the fungi.

Significant amounts of glucose (1.47 μmol/ml) and cellobiose (1.09 μmol/ml) were produced from Avicel by fraction 16 from the hindgut extract. The glucose and cellobiose produced from Avicel by fraction 16 could be produced in the following mechanism caused by high endo-1,4-ß-glucanase (673 mU) and exo-1,4-ß-glucanase (7 mU) activities. Cellooligosaccharides are significantly produced from Avicel by endo-1,4-ß-glucanase; the cellooligosaccharides and cellulose are hydrolyzed to cellobiose by CBH and to glucose by exo-1,4-ß-glucosidase that degrades cellulose by splitting off glucose from the non-reducing end of the cellulose chain, but hardly hydrolyzes cellobiose (Enzyme Nomenclature, 1992). Cellobiose should have been the greatest product if the substrates had been hydrolyzed by CBH alone. Exo-1,4-ß-glucosidase should mainly act on the substrates because glucose was the greatest product in the hydrolysis by fraction 16.

The ratios of endo-1,4-ß-glucanase activity in fractions 13 and 16 separated from the hindgut extract to exo-1,4-ß-glucanase activity in the fractions were about 10:1 and 96:1. The ability in hydrolysis of natural cellulose by the cellulase components isolated from the hindgut extract as well as the hindgutted-body extract of *C. formosanus* was higher than that of endo-1,4-ß-glucanases of *R. speratus* (Watanabe *et al.*, 1997). The ratio of endo-1,4-ß-glucanase activity in fraction 13 to exo-1,4-ß-glucanase activity in the fraction was about a tenth of that in fraction 16. The specific activity of exo-1,4-ß-glucanase of fraction 13 was much higher than that of fraction 16, whereas the CBH activity in fraction 13 was a ninth of that in fraction 16. Thus the ability of natural cellulose hydrolysis by the cellulolytic system existing in the hindgut is not necessarily proportional to it's CBH activity.

The specific activity of exo-1,4-ß-glucanase in each fraction isolated from the hindgutted-body and hindgut extracts decreased by purification by anion exchange chromatography, while the specific activities of endo-1,4-ß-glucanase, ß-glucosidase, and CBH in certain fractions increased by the purification (Table 3-1). These results suggest that natural cellulose may be hydrolyzed more efficiently by the combined action of cellulase components in the termite than by solo cellulase component. These synergistic actions in hydrolysis of crystalline cellulose as described above have been reported in various fungal and bacterial cellulolytic system (Wood, 1991; Rapp and Beermann, 1991).

3.5 Summary

Four cellulase components were separated from an extract from hindgutted-body by anion exchange chromatography, while five components were separated from hindgut (with symbiotic protozoa) extract. Among the four cellulase components isolated from the hindgutted-body extract, a cellulase component had exo-1,4-ß-glucanase and endo-1,4-ß-glucanase activities, two components had exo-1,4-ß-glucanase, endo-1,4-ß-glucanase, and β-glucosidase activities, and one component had significant extents of exo-1,4-ß-glucanase,

endo-1,4-ß-glucanase, β-glucosidase, and CBH activities. Among the five cellulase components isolated from the hindgut extract, two cellulase components significantly showed exo-1,4-ß-glucanase and endo-1,4-ß-glucanase activities, and three cellulase components exo-1,4-ß-glucanase, endo-1,4-ß-glucanase, β-glucosidase, and CBH activities. All the cellulase components separated from *C. formosanus* hydrolyzed both crystalline and amorphous celluloses with significant rates, while the CBH activity of each of the cellulase components was not proportional to the activity of hydrolysis of crystalline cellulose by the cellulase component. It was apparent that certain cellulase components isolated from the termite were able to significantly hydrolyze crystalline cellulose to glucose in spite of lacking CBH activity.

Chapter 4 OCCURRENCE AND METABOLIC ROLE OF THE PYRUVATE DEHYDROGENASE COMPLEX IN *COPTOTERMES FORMOSANUS* SHIRAKI

4.1 Introduction

In Chapter 2, it was apparent that crystalline cellulose digested by *C. formosanus* is more efficiently hydrolyzed to glucose in the region anterior to the hindgut, and the glucose produced in this region is absorbed through the midgut cell wall. However, termites have been reported to be unable to use glucose directly as a source of carbon and energy, since they were believed to lack the pyruvate dehydrogenase complex as mentioned in INTRODUCTION. If termites possess the pyruvate dehydrogenase complex, they can oxidize glucose completely.

The objective of this Chapter is to determine whether the pyruvate dehydrogenase complex is active in termite tissue, in the contents of the hindgut, or in both (Itakura *et al.*, 1997a, 1999).

4.2 Material and Methods

4.2.1 Termites

Worker caste termites of *C. formosanus* were collected from laboratory colony as described in Chapter 1 and dissected within 6 h.

4.2.2 Preparation of enzyme extracts

Three hundred worker caste termites were immobilized and decapitated. The entire guts were removed using fine-tipped forceps. For the gutted termite tissue preparation, the gutted bodies were combined with the heads. The other preparations consisted of gutted bodies, head sections, and separated foregut/midgut and hindgut sections. The tissues were homogenized in 50 mM 3-morpholinopropanesulfonic acid (MOPS) buffer (pH 7.0), containing 2.7 mM ethylenediaminetetraacetic acid (EDTA), 0.1 mM dithiothreitol, 1 mM phenylmethanesulfonyl (PMSF), 1 mM benzamidine hydrochloride, and 3% (v/v) Triton X-100 (Stanley and Perham, 1980). The homogenates were centrifuged at 8300 x *g* for 15 min. The supernatants were dialyzed for 12 or 36 h against the MOPS buffer solution and filtered through membrane filters (Dismic-25, Advantec Toyo). The protein content of each filtered crude extract (6 - 9 ml) was measured by the method of Lowry *et al.* (1951), using bovine serum albumin as the standard. All operations were performed at 0 - 4°C.

4.2.3 Assay of pyruvate dehydrogenase complex activity using [2-^{14}C]-pyruvate

The complete assay solution for pyruvate dehydrogenase complex consisted of 2.9 mM NAD^+, 1.0 mM $MgCl_2$, 0.20 mM thiamine pyrophosphate, 0.0026 mM CoA, 2.6 mM cysteine hydrochloride, and 1 mM sodium pyruvate in 50 mM phosphate buffer (pH 8.0). Assay solutions (1.2 ml), containing sodium [2-^{14}C]-pyruvate (0.39 mM, 110 or 185 kBq, American Radiolabeled Chemicals, Missouri) and 0 - 8 mM acetyl-CoA, were maintained at 30°C. Reactions were initiated by the addition of crude extract (0.3 ml). Assays also were performed in the absence of NAD^+ and in the presence of NADH (3 mM). Control reactions contained crude extracts that had been heated for 5 min at 100°C or MilliQ water in place of the crude extract. After 1 h at 30°C, the acetyl-CoA in samples (150 µl) was analyzed by reverse-phase high-pressure liquid chromatography (HPLC) on a TSK gel ODS-80Ts column (150 x 4.6 mm) (Tosoh). The mobile phase was 10 mM potassium dihydrogen phosphate in a linear gradient of acetonitrile, of 5% to 20% in 20

min at a flow rate of 1 ml/min. The absorbance at 210 nm and the radioactivity of the eluate were monitored with an ultraviolet (UV) wavelength detector (UV-8020, Tosoh) and a radiation detector (RS-8020, Tosoh). Radioactive fractions were collected and the radioactivity of each fraction was assayed with a liquid scintillation counter (LSC-5100, Aloka). The total acetyl-CoA, produced by each of the crude extracts, was calculated by multiplying the amount of detected [^{14}C]-acetyl-CoA by the factor 3.56, the ratio of the total pyruvate to the [2-^{14}C]-pyruvate added to each assay.

4.2.4 Identification of acetyl-CoA

The reverse-phase HPLC fractions (1.0 ml) corresponding to compound b in Figure 4-1 were hydrolyzed with 0.1 N NaOH (100 µl). The hydrolysates were concentrated to one-eleventh of the original volumes with a vacuum drying apparatus (Automatic Environmental Speedvac with vapernet, ASE 1000, Savant Instruments, New York). 10-µl portions of the concentrated hydrolysates were analyzed by ion-exchange HPLC on a TSK gel DEAE-5PW column (75 x 7.5 mm, Tosoh). Isocratic elution was carried out with 200 mM ammonium dihydrogen phosphate, pH 5.0, containing 20% acetonitrile (v/v), for 50 min at a flow rate of 0.75 ml/min.

4.2.5 Assays of NADH production

The NADH produced from NAD^+ by each crude extract was determined by the method of Stanley (1971), with modifications. The complete assay solution was as described above, except that phosphate buffer (pH 7.5), containing 0.1 mM ß-mercaptoethanol, replaced phosphate buffer (pH 8.0). The assay solution (1.8 ml) was incubated at 30°C for 10 min, in a polypropylene tube (50 x 16 mm) inside a 20-ml scintillation vial containing 10 ml of distilled water. Flavin mononucleotide (10 µl of a 0.2

mM solution in the phosphate buffer), bacterial luciferase from *Photobacterium fischeri* (Sigma Chemical Co., Missouri; 20 µl of a 0.1% solution [w/v] in the phosphate buffer), and 200 µl of crude extract were added successively. The reaction mixture was incubated for 10 min at 30°C, followed by the addition of 10 µl of tetradecanal (saturated solution in ethanol). The bioluminescence generated by luciferase was counted for 1 min with a liquid scintillation counter. Assays also were conducted in the absence of NAD^+, in the absence of CoA, and in the absence of pyruvate. For control reactions, the crude extracts were inactivated by heating at 100°C for 5 min. The spectrophotometric assay of Perham and Lowe (1988) also was used to measure NADH production by the pyruvate dehydrogenase complex in each crude extract.

4.2.6 Rates of NADH oxidation

The complete assay solution was as described above except with the addition of NADH (50 µM). Crude extract (0.2 ml) was added to the solution (0.8 ml) at 30°C, and the absorbance of the mixture at 338 nm was monitored with an UV wavelength detector (UV-2400PC, Shimadzu) at 5 or 10 min intervals for 1h.

4.2.7 Assay of pyruvate dehydrogenase activity using [1-^{14}C]-pyruvate

The activity of pyruvate dehydrogenase (EC 1.2.4.1) in each extract was assayed by measuring $^{14}CO_2$ production from [1-^{14}C]-pyruvate, as described previously (Zhou *et al.*, 1995), with modifications. Each crude extract (45 µl) was incubated for 10 min at 30°C with the complete assay solution described above (150 µl). Reactions were initiated by the addition of 5 µl of sodium [1-^{14}C]-pyruvate (8.1 nmol, 3.76 kBq, American Radiolabeled Chemicals, Missouri). Reactions also were conducted in the absence of NAD^+, in the absence of CoA, in the presence of ATP (3 mM) and in the presence of acetyl-CoA (2.4

mM). Reaction mixtures were incubated for 20 min at 30°C and the reactions were stopped by the addition of 0.25 N H_3PO_4 (100 µl). The reactions were carried out in 1.5-ml uncapped microcentrifuge tubes (Treff AG, Degersheim), inside capped 20-ml scintillation vials, containing monoethanolamine (0.4 ml), methanol (0.4 ml), and liquid scintillator (3.2 ml) (Scintisol 500, Wako). The generated $^{14}CO_2$ was absorbed by the monoethanolamine, during a further incubation of 2 h at 30°C, and counted in a liquid scintillation counter. Control reactions contained crude extracts that had been heated for 5 min at 100°C. The total CO_2 produced by the extracts was calculated by multiplying the detected $^{14}CO_2$ by 19.3, the ratio of the total pyruvate in the reaction mixture to the amount of [1-^{14}C]-pyruvate.

4.2.8 Other enzyme assays

$_L$-Lactate dehydrogenase (EC 1.1.1.27), acetyl-CoA synthetase (EC 6.2.1.1), and malate dehydrogenase (decarboxylating) (EC 1.1.1.40) activities were assayed in each crude extract as described previously (Jones and Lipmann, 1955; Murai *et al.*, 1972; Vassault, 1983).

4.3 Results

4.3.1 Activity of the pyruvate dehydrogenase complex

The results of reverse-phase HPLC of the reaction mixture containing the complete assay solution, [2-^{14}C]-pyruvate, 4 mM acetyl-CoA, and the crude extract of the gutted termite tissue preparation from *C. formosanus*, are shown in Figure 4-1. Pyruvate had a retention time of 1.9 min (Fig. 4-1, compound a). Authentic acetyl-CoA eluted in the exactly the same position as the "b" peak in Figure 4-1. The fraction corresponding to

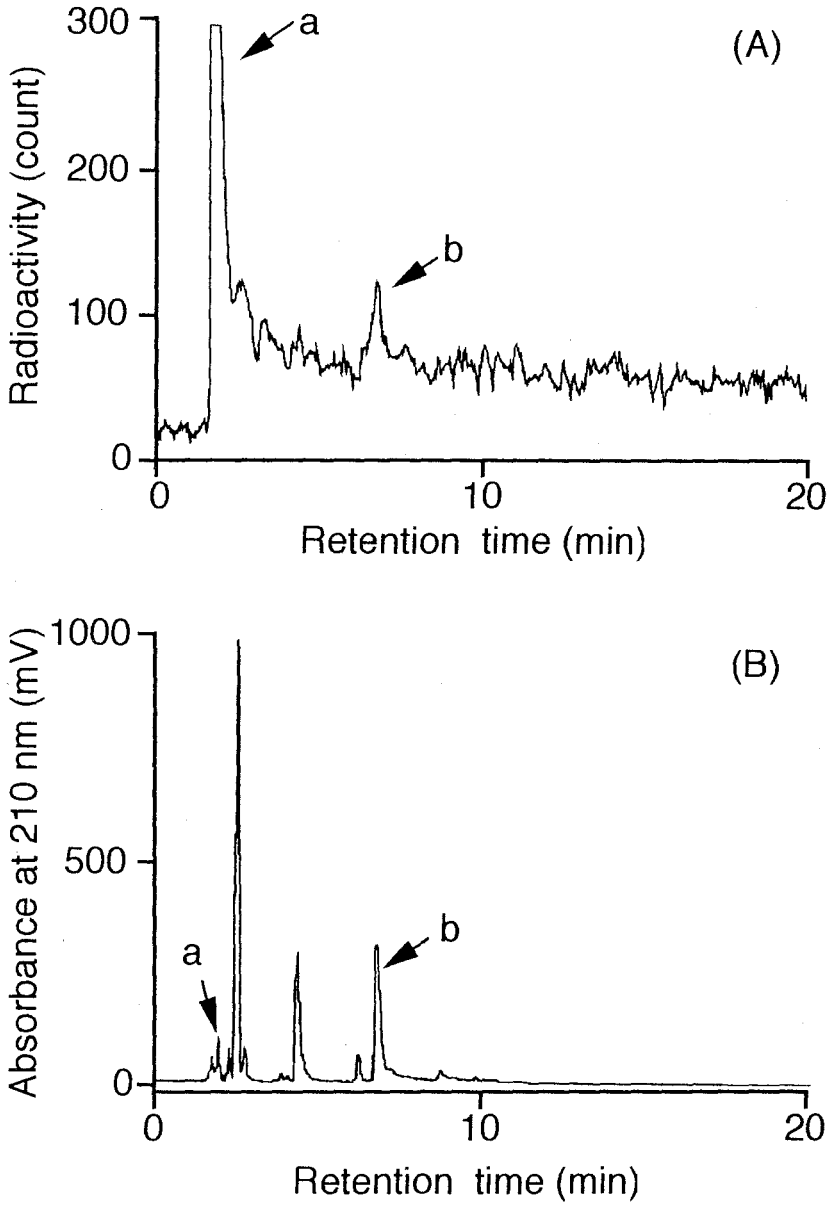

Fig. 4-1. Reverse-phase HPLC of crude extract of gutted termite tissue preparation from *C. formosanus* in assay solution containing sodium [2-^{14}C]-pyruvate (0.39 mM; 185 kBq) and acetyl-CoA (4 mM). Crude extract preparation, assay solution and HPLC were as described in the text. The radioactivity (A) and absorbance at 210 nm (B) of the eluate were monitored simultaneously as described in the text: a, pyruvate; b, acetyl-CoA.

compound b in Figure 4-1, with a retention time of 6.8 min, was hydrolyzed and analyzed by ion-exchange HPLC. [^{14}C]-Acetate was detected in the hydrolysate with a yield of 46.3% (data not shown), indicating that compound b in Figure 4-1 was [^{14}C]-acetyl-CoA.

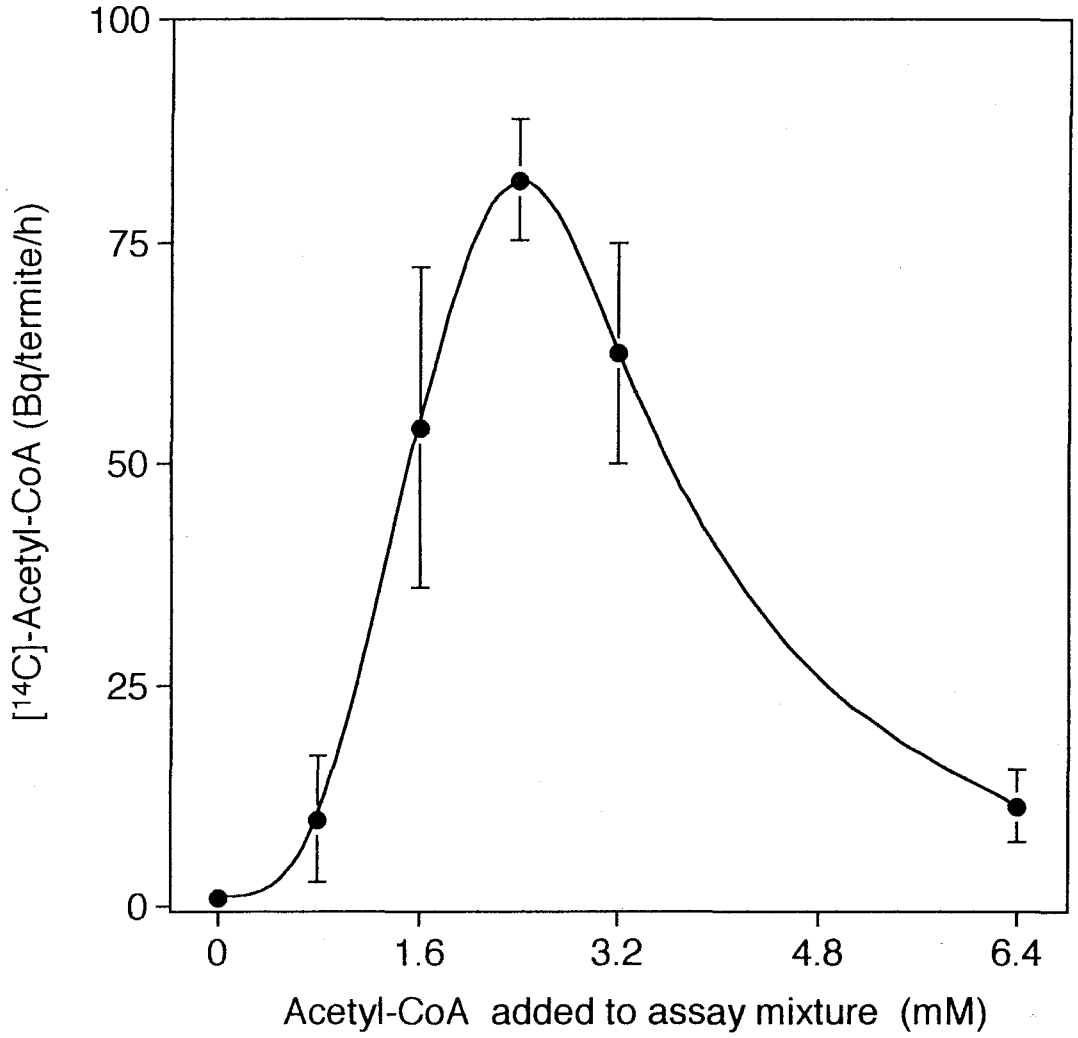

Fig. 4-2. Effect of acetyl-CoA concentration on [^{14}C]-acetyl-CoA production from [2-^{14}C]- pyruvate by a crude extract of gutted termite tissue from *C. formosanus*. Assays were as described in the text. The assay mixture was incubated for 1 h at 30°C. Results are the mean of 3 assays (95% confidence limit).

The extent of production of [^{14}C]-acetyl-CoA, by the crude extract of gutted termite tissue of *C. formosanus*, was dependent upon the amount of unlabeled acetyl-CoA added to the assay mixture (Fig. 4-2). Production of [^{14}C]-acetyl-CoA was greatest when 2.4 mM was added to the reaction. Little or no [^{14}C]-acetyl-CoA formation was detected with the extract of gutted termite tissue in the absence of unlabeled acetyl-CoA. Only a small amount of [^{14}C]-acetyl-CoA was detected when 6.4 mM was added to the assay (Fig. 4-2). Therefore, 2.4 mM unlabeled acetyl-CoA was used in the assays measuring the activity of the pyruvate dehydrogenase complex in crude extracts.

The detected [^{14}C]-acetyl-CoA and the calculated total acetyl-CoA for each of the assays are shown in Table 4-1. With the complete reaction mixture, the extent of acetyl-CoA production was approximately three times greater with the foregut/midgut extract than with each of the other extracts. The removal of NAD^+ or the addition of NADH (3 mM) to the reaction mixture decreased the extent of acetyl-CoA production by 80% or more, with all of the crude extracts. No significant production of [^{14}C]-acetyl-CoA was detected when the extracts were heated at 100°C for 5 min (Table 4-1), or when MilliQ water was used in place of the crude extract (data not shown). No carbon exchange was detected between [2-^{14}C]-pyruvate and the added acetyl-CoA.

The extent of acetyl-CoA consumption by each of the crude extracts in the complete reaction mixture was determined by the UV absorbance of acetyl-CoA on HPLC (Table 4-1). About 38% (1.35 μmol/1.2 ml) of the acetyl-CoA added to the reaction mixture was consumed by the gutted body extract during the 1 h incubation period; whereas about 5% of the added acetyl-CoA was consumed by the foregut/midgut extract (Table 4-1). The activities of the pyruvate dehydrogenase complex in the crude extracts, corrected for the extents of acetyl-CoA consumption, are shown in Table 4-1. The total activity of pyruvate dehydrogenase complex (TA) was calculated according to the following formula: TA = (activity determined by subtracting the rate of acetyl-CoA production in the reaction with heat-inactivated extract from that in the complete reaction mixture) / (1 - the ratio of

Table 4-1. Pyruvate dehydrogenase complex activity in crude extracts from *C. formosanus*[1] assayed by using [2-^{14}C]-pyruvate.

	Gutted body	Head	Foregut/midgut	Hindgut
Assay mixture	Total acetyl-CoA[4] (nmol/termite/h)			
Complete[2]	1.18 ± 0.18	1.22 ± 0.25	3.76 ± 0.36	1.25 ± 0.17
Minus NAD^+	0.09 ± 0.01	0.08 ± 0.02	0.27 ± 0.04	0.26 ± 0.02
Plus 3mM NADH	0.13 ± 0.03	0.08 ± 0.01	0.30 ± 0.02	0.22 ± 0.03
Heated extract[3]	0.07 ± 0.01	0.06 ± 0.01	0.02 ± 0.01	0.02 ± 0.01
Activity (nmol/termite/h)	1.11 ± 0.18	1.16 ± 0.25	3.74 ± 0.36	1.23 ± 0.17
Acetyl-CoA consumption (nmol/termite/h)[5]	90.0 (0.375)	30.7 (0.128)	12.7 (0.053)	18.7 (0.078)
Total activity[6] of pyruvate dehydrogenase complex (nmol/termite/h)	1.78 ± 0.29	1.33 ± 0.29	3.95 ± 0.38	1.33 ± 0.18
Protein (mg/termite)	0.166	0.081	0.039	0.062
Specific activity (mU/mg of protein)[7]	0.18 ± 0.03	0.27 ± 0.06	1.67 ± 0.16	0.36 ± 0.05

1) Crude extracts were prepared as described in the text and dialyzed for 36 h against 50 mM MOPS buffer.

2) The complete reaction mixture contained in 1.5 ml was as described in the text except for the amount of acetyl-CoA (2.4 mM) and crude extracts of gutted body, head, foregut/midgut, and hindgut containing 2.49, 1.21, 0.59, and 0.93 mg of protein, respectively. After 1 h at 30°C, [^{14}C]-acetyl-CoA was measured as described in the text. Values are the mean of 3 assays ± SEM.

3) Crude extracts were heated for 5 min at 100°C before use.

4) Total acetyl-CoA was determined as described in the text.

5) The rate of acetyl-CoA consumption by each of the crude extracts in the complete reaction mixture was determined by the UV absorbance of acetyl-CoA on HPLC. Values in parentheses represent the ratio of the acetyl-CoA consumed by each extract during 1 h incubation period to the amount of the acetyl-CoA added to the mixture.

6) Activities were corrected for acetyl-CoA consumption by the crude extracts during incubation.

7) One unit of pyruvate dehydrogenase complex activity is defined as the amount of enzyme that produces 1 μmol of acetyl-CoA per minute.

the acetyl-CoA consumed by each extract to the amount of the acetyl-CoA added to the reaction mixture).

In the assays utilizing bacterial luciferase under an air atmosphere, although NADH was detected in reactions containing extracts from the gutted body and hindgut, the bioluminescence measured in reactions containing extracts from the head and foregut/midgut was less than that measured in control reactions lacking extract. Furthermore, with each of the crude extracts, the measured bioluminescence was similar in reactions with and without CoA, and was substantially higher in reactions lacking pyruvate than in reactions containing pyruvate (data not shown). Thus, the reduction of NAD^+ to NADH by the pyruvate dehydrogenase complex could not be detected by assays using bacterial luciferase. Likewise, no NADH production was detected with any of the crude extracts, using the spectrophotometric assay of Perham and Lowe (1988) to measure the activity of the pyruvate dehydrogenase complex (data not shown).

4.3.2 Rates of NADH oxidation

When NADH (40 μM) was added to the reaction mixture, the concentrations of NADH after 1 h, with crude extracts from the gutted body, head, foregut/midgut, and hindgut had decreased to 0, 17.8, 36.7, and 9.2 μM, respectively. Thus, the rates of NADH production by the pyruvate dehydrogenase complex in each of the extracts were less than the rates of NADH oxidation by contaminants in the extracts. The rates of NADH consumption in reaction mixtures containing extracts from the gutted body, head, foregut/midgut, and hindgut were 4.0, 2.2, 0.33, and 3.1 nmol/termite/h, respectively.

4.3.3 Pyruvate dehydrogenase activity

The pyruvate dehydrogenase activity of each of the crude extracts is shown in Table 4-2. In the absence of either NAD^+ or CoA, CO_2 production decreased significantly. Likewise, the addition of acetyl-CoA or ATP, which promote the phosphorylation of pyruvate dehydrogenase and inactivation of the complex (Patel and Roche, 1990), significantly decreased CO_2 production (Table 4-2). These results indicate that the decarboxylation of pyruvate was catalyzed, to a large extent, by the pyruvate dehydrogenase complex in the crude extracts. Reaction mixtures were acidified to pH 4 with 0.25 N H_3PO_4 instead of 1 M trichloroacetic acid (Zhou *et al.*, 1995) to stop the reactions because trichloroacetic acid promotes the decarboxylation of pyruvate to significant extent. Under these acidic conditions (pH 4) most of the CO_2 was dissolved in the reaction mixtures. Since only gaseous $^{14}CO_2$, and not dissolved $^{14}CO_2$, was measured, the activities obtained for pyruvate dehydrogenase in the crude extracts generally were lower than those obtained for the pyruvate dehydrogenase complex (Tables 4-1, 4-2).

4.3.4 $_L$-Lactate dehydrogenase activity

Significant amounts of $_L$-lactate dehydrogenase activity, which catalyzes the reduction of pyruvate by NADH to form lactate, were detected in all of the extracts (Table 4-3). These activities were approximately four- to twenty-fold higher than the corresponding activities of the pyruvate dehydrogenase complex. Thus, the NADH produced by the pyruvate dehydrogenase complex would be oxidized immediately to NAD^+ by the $_L$-lactate dehydrogenase present in the crude extracts.

4.3.5 Acetyl-CoA synthetase activity

Acetyl-CoA synthetase activity was assayed in each of the crude extracts by measuring the formation of acetohydroxamate. However, in the presence of excess

Table 4-2. Pyruvate dehydrogenase activity in crude extracts from *C. formosanus*[1] assayed by using [1-^{14}C]-pyruvate.

	Gutted body	Head	Foregut/midgut	Hindgut
Assay mixture	Total CO_2 produced from pyruvate[4] (nmol/termite/h)			
Complete[2]	0.171 ± 0.012 (100.0)[5]	0.079± 0.009 (100.0)	0.190± 0.006 (100.0)	0.081± 0.007 (100.0)
Minus NAD^+	0.080 ± 0.006 (46.8)	0.008± 0.004 (10.1)	0.026± 0.001 (13.7)	0.017± 0.003 (21.0)
Minus CoA	0.062 ± 0.010 (36.3)	0.030± 0.006 (38.0)	0.006± 0.002 (3.2)	0.025± 0.007 (30.9)
Plus 3 mM ATP	0.086 ± 0.003 (50.3)	0.067± 0.004 (84.0)	0.046± 0.010 (24.2)	0.023± 0.007 (28.4)
Plus 2.4 mM acetyl-CoA	0.061 ± 0.005 (35.7)	0.014± 0.004 (17.7)	0.039± 0.004 (20.5)	0.025± 0.006 (30.9)
Heated extract[3]	0.023 ± 0.002 (13.5)	0.012± 0.002 (15.2)	0.010± 0.002 (5.3)	0.050± 0.007 (61.7)
Protein (mg/termite)	0.089	0.061	0.024	0.068
Activity (nmol/termite/h)	0.148 ± 0.012	0.067± 0.009	0.180± 0.006	0.031± 0.007
Specific activity (mU/mg of protein)[6]	0.028 ± 0.002	0.018± 0.002	0.125± 0.004	0.008± 0.002

1) Crude extracts were prepared and dialyzed for 36 h, as described in the text.

2) The complete reaction mixture (0.2 ml) was as described in the text and crude extracts of gutted body, head, foregut/midgut, and hindgut containing 0.200, 0.137, 0.054, and 0.153 mg of protein, respectively. The assay mixture was incubated for 20 min at 30°C.

3) Each extract was heated for 5 min at 100°C before use.

4) The total CO_2 and $^{14}CO_2$, generated from sodium pyruvate and sodium [1-^{14}C]-pyruvate, was measured as described in the text. Results are expressed as the mean of 3 assays ± SEM.

5) Values in parentheses are the fractions relative to the activity in the complete reaction mixture (100.0).

6) One unit of pyruvate dehydrogenase activity is defined as the amount of enzyme that produces 1 μmol of CO_2 per minute.

Table 4-3. L-Lactate dehydrogenase activity in extracts from *C. formosanus*.[1)]

	Protein (mg/termite)	Activity[2)] (nmol/termite/h)	Specific activity[3)] (mU/mg of protein)
Gutted body	0.096	20.8 ± 1.7	3.62 ± 0.30
Head	0.031	17.9 ± 3.4	9.60 ± 1.84
Foregut/midgut	0.032	15.9 ± 2.0	8.27 ± 1.03
Hindgut	0.060	25.8 ± 2.0	7.16 ± 0.55

1) Crude extracts were prepared and dialyzed for 36 h, as described in the text.

2) Assays were conducted as described in Vassault, 1983. Results are the mean of 3 assays ± SEM.

3) One unit of L-lactate dehydrogenase activity is defined as the amount of enzyme that oxidizes 1 μmol of NADH per minute.

hydroxylamine, acetohydroxamate is formed readily, both from acetyl-CoA produced by acetyl-CoA synthetase and from acyl phosphate produced by acetate kinase (EC 2.7.2.1) which catalyzes the phosphorylation of acetate using ATP (Rose, 1955). Although large amounts of acetohydroxamate were produced in the assays containing the hindgut extract, these amounts were almost identical in the reactions with and without CoA (Table 4-4). Furthermore, in the absence of ATP, acetohydroxamate formation with the hindgut extract was negligible. Thus, the acetohydroxamate production must have been due to acetate kinase present in the hindgut crude extract. The crude extracts from the gutted body and foregut/midgut also appeared to be contaminated with acetate kinase, since similar amounts of acetohydroxamate were detected in the reactions, with or without CoA, and these were approximately twice the amounts detected in the reactions with heat-inactivated extracts. Furthermore, the amounts of acetohydroxamate detected with the gutted body and foregut/midgut extracts in the reactions lacking CoA were approximately 3.5- and 1.5-fold higher, respectively, than in the reactions lacking ATP (Table 4-4).

Table 4-4. Acetyl-CoA synthetase activity in crude extracts from *C. formosanus.*[1)]

	Gutted body	Head	Foregut/midgut	Hindgut
Assay mixture	Acetohydroxamate (nmol/termite/h)			
Complete[2)]	13.95± 0.26	7.17±0.31	7.85± 0.76	139.31± 0.78
Minus acetate	3.74± 0.91	6.41±0.72	5.01± 1.12	6.01± 2.46
Minus ATP	2.86± 2.40	6.34±0.59	4.23± 0.71	0.17± 0.31
Minus CoA	10.05± 0.06	6.91±0.62	6.41± 1.18	139.37± 1.62
Minus Mg^{2+}	2.51± 1.08	4.22±0.61	6.44± 0.69	12.28± 0.20
Heated extract[3)]	5.66± 0.56	6.51±0.80	3.22± 1.39	2.19± 0.36
Protein (mg/termite)	0.132	0.058	0.040	0.047
Activity[4)] (nmol/termite/h)	8.29± 0.26	0.66±0.31	4.63± 0.76	137.12± 0.78
Activity[5)] (nmol/termite/h)	3.90± 0.26	0.26±0.31	1.44± 0.76	0.0
Specific activity (mU/mg of protein)[6)]	0.49± 0.03	0.07±0.09	0.60± 0.32	0.0

1) Crude extracts were prepared and dialyzed for 36 h as described in the text.

2) The complete reaction mixture (in mM) contained, in 1.2 ml: tris(hydroxymethyl) aminomethane buffer, pH 8.2 (100); potassium acetate (20); ATP (10); CoA (0.081); $MgCl_2$ (10); and hydroxyamine solution, pH 7.4 (200). Crude extracts of the gutted body, head, foregut/midgut, and hindgut contained 0.32, 0.14, 0.10, and 0.11 mg of protein, respectively. The reaction mixture was incubated for 20 min at 30°C, and assayed as described in Jones and Lipmann, 1955. Results are the mean of 3 assays ±SEM.

3) Extracts were heated for 5 min at 100°C before use.

4) Rate of acetohydroxamate production in the complete reaction mixture, corrected for the rate obtained with the heated extract.

5) Acetyl-CoA synthetase activity was determined by subtracting the rate of acetohydroxamate production in the reaction mixture lacking CoA from that in the complete reaction mixture, since the activity in the absence of CoA is comparable to the acetate kinase activity in the crude extracts.

6) One unit of acetyl-CoA synthetase activity is defined as the amount of enzyme that produces 1 μmol of acetohydroxamate per minute, corrected by the activity in the absence of CoA.

4.3.6 Malate dehydrogenase activity

The activity of malate dehydrogenase, which catalyzes the oxidative decarboxylation of malate to pyruvate, was about ten times higher in the hindgut extract than in the gutted body and head extracts (Table 4-5). The activity in the foregut/midgut extract was much lower than in the other extracts.

Table 4-5. Malate dehydrogenase (decarboxylating) activity in crude extracts from *C. formosanus*.[1)]

	Protein (mg/termite)	Activity[2)] (nmol/termite/h)	Specific activity[3)] (mU/mg of protein)
Gutted body	0.166	5.45 ± 0.97	0.55 ± 0.10
Head	0.081	4.25 ± 0.48	0.87 ± 0.10
Foregut/midgut	0.040	0.09 ± 0.05	0.04 ± 0.02
Hindgut	0.062	47.51 ± 1.24	12.77 ± 0.33

1) Crude extractrs were prepared and dialyzed for 36 h, as described in the text.

2) Assays were performed and activity measured, as described in Murai, *et al.*, 1972. Results are the mean of 3 assays ± SEM.

3) One unit of malate dehydrogenase (decarboxylating) activity is defined as the amount of enzyme that produces 1 μmol of NADPH per minute.

4.4 Discussion

The activities of the pyruvate dehydrogenase complex, which consists of the enzymes pyruvate dehydrogenase, dihydrolipoamide acetyltransferase (EC 2.3.1.12), and dihydrolipoamide dehydrogenase (EC 1.8.1.4), have been determined in extracts from the gutted body, head, foregut/midgut, and hindgut of *C. formosanus* by measuring directly the [^{14}C]-acetyl-CoA produced in the reactions between [2-^{14}C]-pyruvate, CoA, and NAD^+,

catalyzed by the enzyme complex. [2-^{14}C]-Pyruvate is converted to [^{14}C]-acetyl-CoA at a significant rate by the pyruvate dehydrogenase complex present in each of the extracts (Table 4-1). Since acetyl-CoA is being consumed by a contaminant, such as citrate synthase, which is present in each of the crude extracts (Table 4-1), the addition of unlabeled acetyl-CoA to the reaction is necessary for detecting [^{14}C]-acetyl-CoA formation. Since increasing the acetyl-CoA/CoA ratio promotes phosphorylation and deactivation of the pyruvate dehydrogenase component of the enzyme complex (Patel and Roche, 1990), the optimal amount of added acetyl-CoA has been determined (Fig. 4-2). This consumption of acetyl-CoA by the crude extracts leads to an underestimation of the pyruvate dehydrogenase complex activity in termite tissues. Therefore the activities have been corrected for the extent of acetyl-CoA consumption by each tissue extract. The sum of the pyruvate dehydrogenase complex activities in the gutted body, head, and foregut/midgut tissues are about 5-fold greater than that in the hindgut tissue (Table 4-1). Much of the activity in the hindgut may be due to microorganisms, as described for a higher termite (Slaytor *et al.*, 1997).

The rate of NADH production, catalyzed by the pyruvate dehydrogenase complex in each of extracts, is less than the rate of NADH consumption by the extract. The consumption of NADH by NADH oxidase (Reed and Mukherjee, 1969) and $_{L}$-lactate dehydrogenase (Chretien *et al.*, 1995) present in crude extracts has been reported previously. The activity of $_{L}$-lactate dehydrogenase in each crude extract (Table 4-3) is between four- and twenty-fold higher than the activity of the pyruvate dehydrogenase complex in each extract (Table 4-1). These results contrast with those of O'Brien and Breznak (1984) who reported negligible NADH oxidation, and little or no $_{L}$-lactate dehydrogenase activity, in extracts from the termites *Reticulitermes flavipes, Coptotermes lacteus,* and *Nasutitermes exitiosus.*

As shown in Table 4-2, the removal of NAD^+ or CoA or the addition of ATP or acetyl-CoA decrease the amount of $^{14}CO_2$ generated by the decarboxylation of [1-^{14}C]-

pyruvate. These results indicate that decarboxylation of pyruvate by the termite extracts, in the presence of NAD^+ and CoA, is catalyzed primarily by pyruvate dehydrogenase. These results also are consistent with the fact that increasing the ratios of $NADH/NAD^+$, acetyl-CoA/CoA, or ATP/ADP promote phosphorylation of the pyruvate dehydrogenase component and hence deactivation of the complex (Patel and Roche, 1990).

Significant acetyl-CoA synthetase activity is present in the gutted body, head, and foregut/midgut, but not in the hindgut, as measured by the formation of acetohydroxamate from acetyl-CoA and hydroxylamine (Table 4-4). Since acetyl-CoA synthetase and acetate kinase often are both present in crude enzyme extracts, and since acylphosphate, which is produced by acetate kinase in the presence of ATP and acetate, readily reacts with hydroxylamine to form acetohydroxamate, acetyl-CoA synthetase activity is determined by subtracting the activity measured in the absence of CoA from the total activity (Jones and Lipmann, 1955). The sum of the pyruvate dehydrogenase complex activities in the termite tissues other than the hindgut (7.1 nmol/termite/h) (Table 4-1) is somewhat greater than the acetyl-CoA synthetase activities in those tissues (5.6 nmol/termite/h) (Table 4-4), suggesting that the pyruvate dehydrogenase complex has an important role in acetyl-CoA formation in these tissues.

Natural cellulose ingested by the termite is hydrolyzed to glucose in the region anterior to the hindgut at a higher rate than in the hindgut (Chapter 2). Likewise, glucose is effectively converted to pyruvate by glycolysis in termite tissues other than the hindgut (Slaytor *et al.*, 1997). A large portion of the acetate present in the whole body of the lower termite is found in the midgut and hindgut (8.6 nmol/termite) and a significant amount is found in other tissues (2.8 nmol/termite) (Chapter 2). Taken together, these results indicate that: pyruvate is produced efficiently from glucose by glycolysis in termite tissue; significant amounts of acetyl-CoA are formed from pyruvate by the pyruvate dehydrogenase complex and from acetate by acetyl-CoA synthetase, in termite tissues other

than the hindgut; and both the pyruvate dehydrogenase complex and acetyl-CoA synthetase originate from the termite itself.

The results reported here on the localization of the pyruvate dehydrogenase complex and acetyl-CoA synthetase activities in *C. formosanus* tissue differ from results reported with the higher termite *N. walkeri*. In that report, acetyl-CoA synthetase activity in the hindgut was very high (213 nmol/termite/h) and about 3-fold higher than the pyruvate dehydrogenase complex activity in the hindgut (66 nmol/termite/h); whereas little or no acetyl-CoA synthetase or pyruvate dehydrogenase complex activities were found in other tissues (Slaytor *et al.*, 1997). These results indicate that, although pyruvate is produced from glucose by glycolysis in sufficient quantities, the higher termite does not convert pyruvate directly to acetyl-CoA.

Natural cellulose ingested by the termite is hydrolyzed to oligosaccharides in the foregut and midgut, as well as in the hindgut. The oligosaccharides are hydrolyzed to glucose, primarily in the midgut, and the glucose is absorbed through the midgut wall into the tissues, as an important source of carbon and energy (Chapter 2). Glucose is converted to pyruvate by the glycolytic enzymes in the cytosol of termite cells and in symbiotic protozoa inhabiting the hindgut. Pyruvate also may be produced from malate by malate dehydrogenase (decarboxylating) in hindgut microbiota and in the cytosol of termite cells. Some of the pyruvate is converted to lactate by $_{L}$-lactate dehydrogenase in hindgut microbiota and in the cytosol of termite cells. Malate and lactate dehydrogenases in the cytosol also may participate in the reverse reactions to a significant extent. Pyruvate is transported into the mitochondria, where it is converted to acetyl-CoA by the pyruvate dehydrogenase complex. The activated acetyl unit is oxidized completely to CO_2 by the enzymes of the TCA cycle in the mitochondria. Some pyruvate could be converted to oxaloacetate by pyruvate carboxylase in the mitochondria, to replenish TCA cycle intermediates, although very little pyruvate carboxylase activity has been detected in the termite *N. walkeri* (Slaytor *et al.*, 1997). Acetyl-CoA also is produced from acetate by

acetyl-CoA synthetase in termite tissues. Although, it is not clear whether this reaction occurs in the cytosol or the mitochondria, since the activity of the pyruvate dehydrogenase complex is higher than the acetyl-CoA synthetase activity (Tables 4-1 and 4-4), acetyl-CoA could be produced more efficiently from pyruvate than from acetate. Since the reactions catalyzed by malate dehydrogenase (decarboxylating) and $_L$-lactate dehydrogenase occur in the cytosol, these enzymes do not compete with the mitochondrial pyruvate dehydrogenase complex for available pyruvate. Therefore, most of the mitochondrial pyruvate is converted to acetyl-CoA by the pyruvate dehydrogenase complex. Acetate occurring in the hindgut would be converted to acetyl phosphate by acetate kinase, rather than to acetyl-CoA by acetyl-CoA synthetase, since the acetate kinase activity in the hindgut is very high and the acetyl-CoA synthetase activity is very low (Table 4-4). Acetyl phosphate could be converted to acetyl-CoA by microbial phosphate acetyltransferase in the hindgut, since the conversion of acetate to acetyl-CoA by acetate kinase and phosphate acetyltransferase is a well-known example of acyl group activation in bacteria (Abeles *et al.*, 1992).

In conclusion, results in the present Chapter indicate that pyruvate originating from glucose is converted to acetyl-CoA, at significant rates, by the pyruvate dehydrogenase complexes in both the tissue of *C. formosanus* and the hindgut microbiota. Thus, glucose produced from cellulose in the region anterior to the hindgut serves as an important source of carbon and energy for the termite.

4.5 Summary

The activities of the pyruvate dehydrogenase complex in extracts of the gutted body, head, foregut/midgut and hindgut (hindgut epithelium and microorganisms) tissues of the lower termite *C. formosanus* were determined by measuring the [^{14}C]-acetyl-CoA produced from [2-^{14}C]-pyruvate and the $^{14}CO_2$ produced from [1-^{14}C]-pyruvate. The activities of pyruvate dehydrogenase, $_L$-lactate dehydrogenase, acetyl-CoA synthetase,

malate dehydrogenase (decarboxylating), and acetate kinase in the termite tissues and the hindgut also were determined. The sum (7.1 nmol/termite/h) of the pyruvate dehydrogenase complex activities in the termite tissues other than the hindgut was 5 times higher than the activity in the hindgut. Significant amounts of $_{L}$-lactate dehydrogenase activity were found in all of the tissues. All of the tissues other than the hindgut had significant amounts of acetyl-CoA synthetase activity. Malate dehydrogenase (decarboxylating) activity was about ten times higher in the hindgut extract than in the gutted body and head extracts and the activity in the foregut/midgut extract was very low. These results indicate that acetyl-CoA for the TCA cycle is produced effectively in the tissues of the termite itself, both from pyruvate by the pyruvate dehydrogenase complex and from acetate by acetyl-CoA synthetase.

CONCLUSION

The lower termite *C. formosanus* ingested wood-pieces and grounded it to particles smaller than 50 μm in size. This mechanical action would increase the cellulose-exposed area to make cellulases more accessible to substrates. Thus the termite would be able to digest most preferentially the cellulose in wood in spite of having no or little ligninolytic enzyme in the digestive system.

Activities of all the necessary cellulases for hydrolysis of natural cellulose to glucose, that is, exo-1,4-ß-glucanase, endo-1,4-ß-glucanase, and ß-glucosidase, were present in the region from the salivary glands to the midgut in significant extents. About three fourths of the total ß-glucosidase activity in the termite were detected particularly in the midgut. Therefore natural cellulose ingested by the termite would be hydrolyzed to oligosaccharides in the foregut and midgut as well as in the hindgut, but the resultant oligosaccharides are hydrolyzed to glucose predominantly in the midgut. Moreover, most of the glucose and trehalose were detected in the termite tissue devoid of midgut and hindgut (refereed as to gutted body in Chapter 2), whereas most of the glucose in the gut was present in the midgut. These results suggest that the glucose produced from the ingested natural cellulose would be absorbed through the midgut wall into the tissues of the termite.

Certain cellulase components having no or little CBH activity, isolated from the extract of termite tissue devoid of hindgut (refereed as to hindgutted-body in Chapter 3) and the hindgut extract (hindgut epithelium and microorganisms), hydrolyzed crystalline cellulose to glucose and cellobiose to significant extents. The CBH activity of each cellulase component was not proportional to the ability of each component to produce glucose and cellobiose from crystalline cellulose.

The pyruvate dehydrogenase complex activity in the termite was determined by measuring the [^{14}C]-acetyl-CoA produced from [2-^{14}C]-pyruvate and $^{14}CO_2$ produced from [1-^{14}C]-pyruvate. A significant amount of the pyruvate dehydrogenase complex

activity was detected in the termite tissue devoid of hindgut (gutted body, head, and foregut/midgut) as well as in the hindgut. Deactivation of the complex by the phosphorylation of the pyruvate dehydrogenase component was also observed. Glucose absorbed through the midgut wall into the tissues is converted to pyruvate by the glycolytic enzymes in the cytosol of termite cells. Pyruvate is transported into the mitochondria, where it is converted to acetyl-CoA by the pyruvate dehydrogenase complex. The activated acetyl unit is oxidized completely to CO_2 by the enzymes of the TCA cycle in the mitochondria. Thus, glucose produced from cellulose in the region anterior to the hindgut serves as an important source of carbon and energy for the termite.

In conclusion, the metabolism of the cellulose in wood in the lower termite *C. formosanus* can be summarized as follows: (a) Wood-cell wall is ground to particles smaller than 50 μm in size by trituration; cellulose-exposed area in the cell walls of the ground wood particles greatly increases. (b) The ground wood particles are continuously mixed and stirred with the cellulase components, that are secreted from salivary glands into the digestive system of the termites, having endo- and exo-1,4-ß-glucanase activities by peristaltic movement; thus natural cellulose in wood is hydrolyzed to cellooligosaccharides in the foregut and midgut by the combined action of the peristaltic movement and the cellulase components. (c) The cellooligosaccharides are hydrolyzed to glucose by ß-glucosidase in the midgut and the resultant glucose is absorbed through the midgut wall into the tissue of the termite. (d) Some of the ground wood particles and cellooligosaccharides are transported into the hindgut to be used by the symbiotic microorganisms (Fig. 5-1). (e) A certain part of the glucose absorbed through midgut wall is converted to pyruvate by the glycolytic enzymes in the cytosol of termite cells. (f) Pyruvate is converted to acetyl-CoA by the pyruvate dehydrogenase complex in the mitochondrial matrix. (g) Acetyl-CoA is oxidized completely to CO_2 by the enzymes involved in the TCA cycle in the mitochondrial matrix (Fig. 5-2).

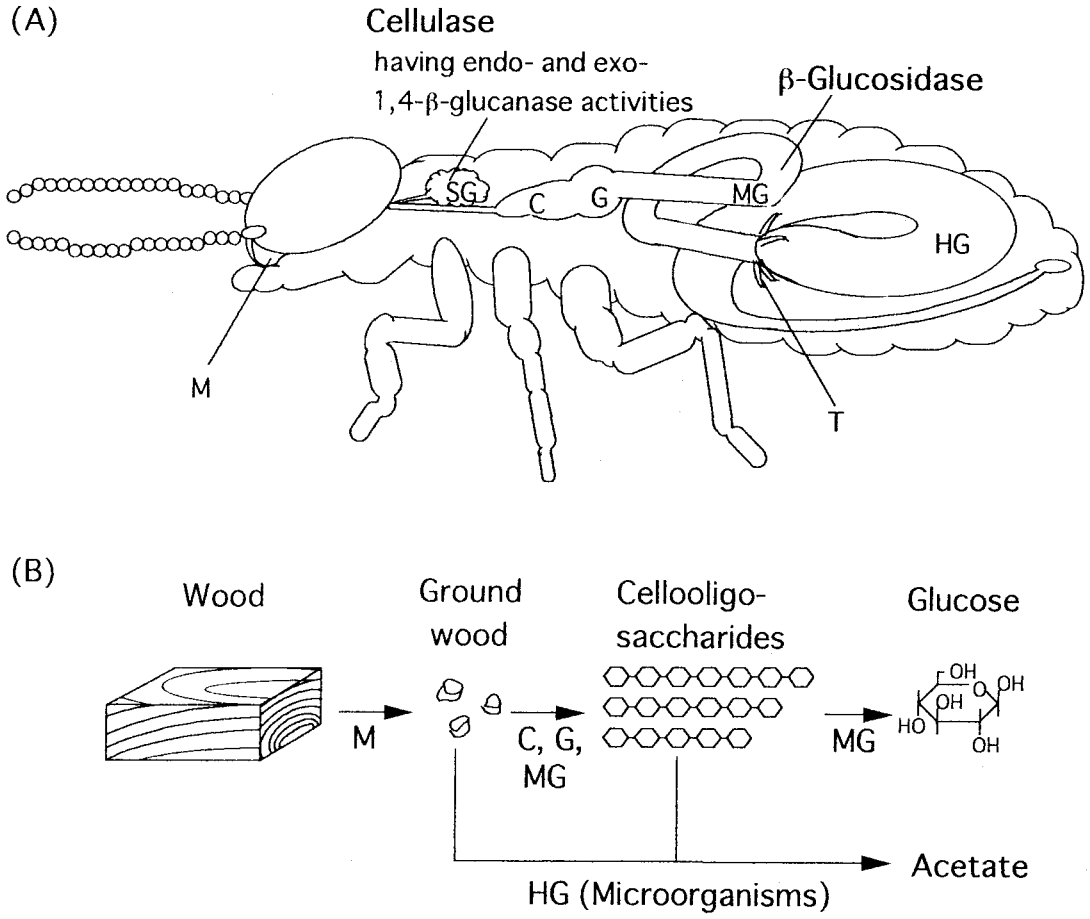

Fig. 5-1. Schematic representation of the digestive process of the cellulose in wood by *C. formosanus*. (A) digestive system and digestive glands secreting cellulase and β-glucosidase; (B) conversion of cellulose in wood into glucose in the digestive system of the termite. M, mandible; SG, salivary glands; C, crop; G, gizzard; MG, midgut; HG, hindgut; T, Malpighian tubules.

Most termite species of the over 2300 species in the world play an important role in the breakdown and recycling of dead wood and other plant debris. Less than two hundred species are known to damage buildings but only some fifty species cause serious damage. I hope the findings in this dissertation will contribute to a development of novel and specific methods to eliminate the termite's colonies established around buildings and other articles useful to humans.

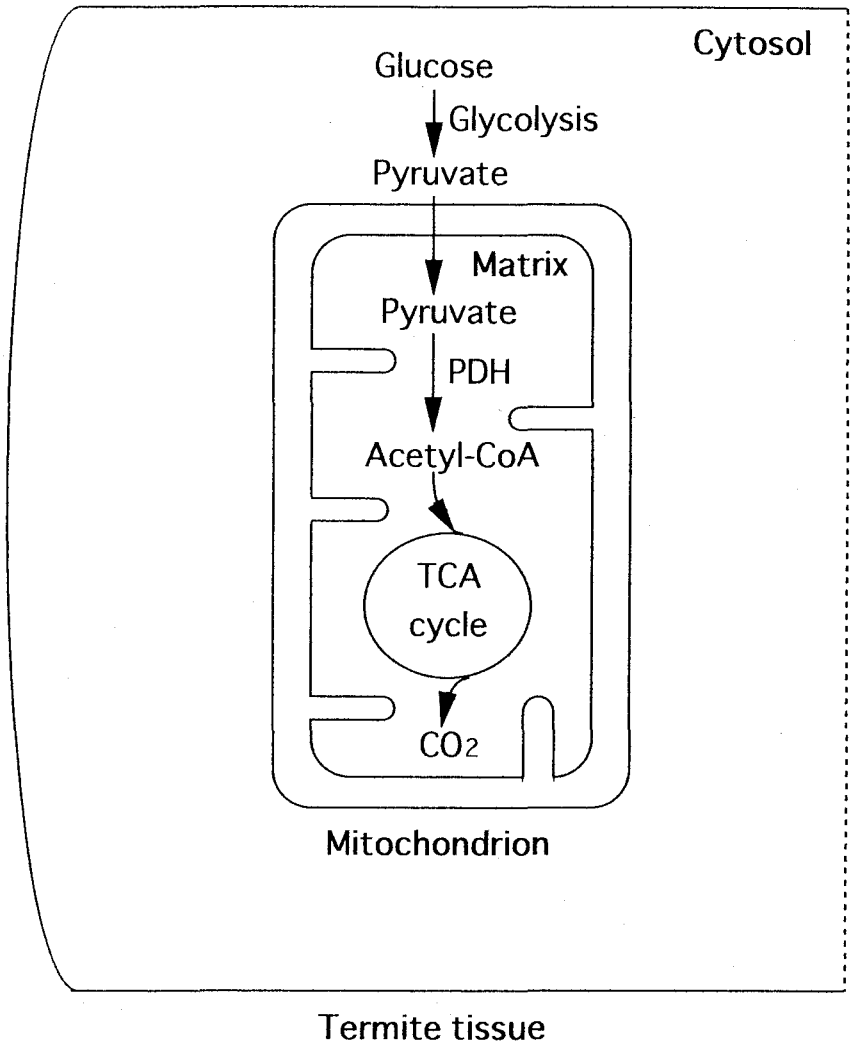

Fig. 5-2. Schematic diagram of a mitochondrion. Glucose is converted to pyruvate by the glycolytic enzymes in the cytosol of termite cells. Pyruvate is transported into the mitochondrion, where it is converted to acetyl-CoA by the pyruvate dehydrogenase complex. The activated acetyl unit is oxidized completely to CO_2 by the TCA cycle in the mitochondrion. PDH, pyruvate dehydrogenase complex.

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