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Details of the digestive system in the midgut of *Coptotermes formosanus* Shiraki

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Abstract Wood-feeding termites have evolved an efficient cellulose-decomposing system. The termite has two independent cellulose-digesting systems: one in the midgut and the other in the hindgut. Because the digestion system in the midgut should be the sole source of soluble sugars for the host termite, the details of the decomposition of wood particles in the midgut were clarified in one of the most common pest species, *Coptotermes formosanus*. The spatial distribution of cellulase in the midgut was found by immunohistochemistry, and the amount of endogenous cellulases and the volume of the endoperitrophic space were determined. The size of wood particles in the foregut and the midgut were compared. The results showed that one of the characteristics of wood degradation by termites is the mechanical grinding of food by the mandibles and the cuticular apparatus of the foregut. This process greatly increases the surface area of the substrates. Extremely high concentrations of cellulase attack the ground-up wood in the midgut, and the glucose produced is removed quickly through the peritrophic membrane.

Key words Termite · Cellulase · Peritrophic membrane · Wood degradation

Introduction

Termites are considered one of the major cellulose-decomposing animals. A termite has two independent cellulose-digesting systems: one in the midgut where cellulose digestion is accomplished by endogenous cellulases, and the other in the hindgut, which makes use of other cellulases from symbiotic flagellates[1] and/or bacteria.[2] The flagellates are harbored only by the termite species referred to as "lower termites,"[3] which have both decomposing systems. Nakashima et al. confirmed that the midgut endogenous cellulase and the hindgut protozoan cellulase are confined in the lumen of the midgut and hindgut, respectively: thus, they denied synergistic action between the endogenous and protozoan cellulases,[1] although the termite cellulases show a synergistic effect with cellulases of fungal origin.[4] As the xylophagous protozoa encapsulate wood particles and digest them inside their cells,[5,6] the protozoan digestive system is not considered to deliver free sugar to the host termites. Therefore, the midgut cellulose digestion system should be the sole source of soluble sugars (mainly glucose) for the host termites, and this would be the reason that the ingested wood particles are processed by the midgut system ahead of the hindgut protozoan system in lower termites. The midgut system processes lignocellulose rapidly, and thus the midgut systems are of general interest.

The cellulose in wood is coated with hemicellulose and lignin, which makes it difficult for cellulases to attack it directly. Therefore, for termite endogenous cellulase to function efficiently, mastication is one of the most important steps, allowing the termite cellulases to easily access the cellulose fiber by breaking down the lignin matrix and increasing the exposure to cellulose fibers. A termite masticates wood materials in two steps: using the mandibles and using the cuticular structures of the proventiculus.

To analyze the contribution of the masticating organs to the preconditioning of the wood particles, we measured the size of wood particles in the foregut and the midgut of termites. In addition, the distribution and activity of termite cellulase and the volume of the termite midgut were measured to determine the concentration of endogenous cellulase, which directly affects the efficiency of enzymatic digestion of the ingested wood particles. Finally, the spatial distribution of termite cellulase was determined by immunohistochemistry to precisely elucidate enzymatic digestion in the midgut.

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Materials and methods

Termites

Workers of *Coptotermes formosanus* Shiraki (Isoptera: Rhinotermitidae) were collected from a laboratory-maintained colony, kept under constant darkness with blocks of *Pinus densiflora* as the food source.

Cellulase activity in the gut of termites

Crude enzyme extract was prepared as described by Tokuda et al.[7] from the worker termites of *C. formosanus*. Endo-β-1,4-glucanase activity was assayed according to Tokuda et al.,[7] using 1% sodium carboxymethylcellulose (CMC) in 0.1 M sodium acetate buffer (pH 5.5). When the absorbance was more than 1.0 in the detection of reducing sugars with tetrazolium blue reagent as described by Tokuda et al.,[8] enzyme activity was again determined by incubating with diluted crude enzymes. One unit of enzyme activity was defined as the amount of enzyme that produced 1 μmol of reducing sugar per minute.

Immunoblotting

Five sets of salivary glands and whole guts were dissected from workers. The guts were divided into foregut, midgut, and hindgut. The salivary glands and the guts were collected in microtubes and homogenized with a sterilized pestle in 100 μl protein inhibitor cocktail (Complete Mini EDTA-free; Roche Diagnostics, Mannheim, Germany, twofold concentration) on ice. Then, the microtubes were centrifuged at 20000 *g* for 15 min at 4°C. The amount of total protein in the supernatant was determined by Quick Start Bradford Protein Assay (Bio-Rad Laboratories, Hercules, CA, USA), using bovine serum albumin as a standard. The supernatants were diluted to equalize the protein concentration. Samples of 10 μl of diluted supernatants were mixed with the same volume of Laemmli Sample Buffer (Bio-Rad) with 2.5% (v/v) 2-mercaptoethanol and heat denatured for 5 min. Then, 10 μl of diluted supernatant was applied to precast gradient polyacrylamide slab separating gels (5%–20%; ATTO, Osaka, Japan); 5 μl XL-Ladder Broad (APRO Life Science Institute, Tokushima, Japan) was also run simultaneously.

To conduct immunoblotting, the proteins separated in the gel were transferred to a nitrocellulose membrane (Sequi-Blot PVDF Membrane; Bio-Rad) in transfer buffer [39 mM Tris, 48 mM glycine, 0.0375% sodium dodecyl sulfate (SDS)] using a semidry-type electroblotting apparatus (Trans-Blot Semidry Transfercell; Bio-Rad) at 2 mA/cm^2 for 60 min. The antiserum raised against A18, a chimeric protein of four termite species endoglucanases[9] in rabbit, was employed for the experiment. The membrane was incubated in 5% skimmed milk (Nacalai Tesque, Kyoto, Japan) for 2 h to block nonspecific binding of the antibodies, washed with phosphate-buffered saline (PBS) containing 0.05% Tween-20 (PBST: 137 mM NaCl, 8.1 mM Na_2HPO_4, 2.68 mM KCl, 1.47 mM KH_2PO_4) five times, and reacted with anti-A18 rabbit serum diluted (1:1000) in PBST overnight. After being washed thoroughly with PBST, the membrane was incubated with diluted (1:1000) secondary antibody [goat antirabbit IgG (H + L), horseradish peroxidase (HRP) conjugated] in PBST for 2 h, and washed with PBST five times. Immunodetection was performed using tetramethyl benzidine (3,3′,5,5′-TMB) Liquid Substrate System for Membranes (Sigma-Aldrich, St. Louis, MO, USA).

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

Ten pairs of salivary glands and whole guts were dissected from the workers of *C. formosanus*. The guts were divided into foregut, midgut, and hindgut. The peritrophic membrane and its contents were squeezed out together by touching the outside of the midgut wall with forceps. The salivary glands, guts, midgut contents, and midgut walls were collected in microtubes and homogenized with sterilized pestles in 100 μl protein inhibitor cocktail (Complete Mini EDTA-free; twofold concentration) on ice. Then, the microtubes were centrifuged at 20000 *g* for 15 min at 4°C. The amount of total protein in the supernatant was determined by Quick Start Bradford Protein Assay (Bio-Rad).

For the supernatants prepared from the foregut and the midgut contents, 20 μl of supernatants was mixed with the 10 μl of Laemmli Sample Buffer (Bio-Rad) containing 2-mercaptoethanol, and all the mixture was subjected to SDS-PAGE using precast gradient polyacrylamide slab separating gels (5%–20%; ATTO) after 5 min heat denaturing. For the other supernatants, 10 μl supernatant was mixed with the same volume of sample buffer and heat denatured for 5 min to prepare the sample. Volumes of 15 μl of prepared samples were subjected to SDS-PAGE.

To visualize the total proteins separated, the gel was stained with SeePico CBB Stain Kit (Benebiosis, Seoul, Korea). The ratio of the separated proteins was calculated by one-dimensional (1D) densitometry using the free software ImageJ (ver. 1.38).

Measurement of midgut volume

To determine the cellulase concentration inside the peritrophic membrane, the volume of endoperitrophic area was measured with a digital microscope (VH-8000; Keyence, Osaka, Japan). To make the contrast clear between the inside and the outside of the peritrophic membrane, worker individuals were fed filter papers moistened with red food coloring (Kyoritsu Foods, Tokyo, Japan) for 1 h. The *C. formosanus* gut was then pulled out and placed on agarose gel (1%) to keep the gut wet. The diameters of ten evenly distanced points and the length of the midgut were measured. Eight repetitions were made, and the average volume was calculated by considering the midgut as a uniform cylinder.

Immunohistochemistry

The whole gut was fixed with 4% paraformaldehyde phosphate buffer solution (Wako, Osaka, Japan) at 4°C overnight and dehydrated in a water:ethanol series with an increasing proportion of ethanol and absolute xylene. The dehydrated samples were embedded in paraffin. The embedded sample was sectioned to produce successive slices 5–7 μm thick, which were placed on a glass slide coated with MAS-GP (Matsunami, Osaka, Japan). The sample slides were subjected to immunoreaction after paraffin was removed, by dipping in absolute xylene, and the slides had been rehydrated in a water:ethanol series with an increasing proportion of water. The sectioned samples on the slides were incubated with 2% skimmed milk for 1 h to block nonspecific binding of antibodies and incubated overnight with anti-A18 rabbit antiserum diluted (1:1000) in PBS. After washing with PBS, the samples were incubated with secondary antibody in PBS for 2 h, and then washed thoroughly again with PBS. Finally, the samples were incubated with AEC Peroxidase Substrate Kit (Vector Laboratories, Burlingame, CA, USA), which stained bound peroxidase on the tissue violet. For control experiments, anti-A18 rabbit serum was replaced with normal rabbit serum.

The measurement of wood particles

According to Sleaford et al.,[10] phloroglucinol/HCl solution was used to detect the lignified wood materials. Five g of phloroglucinol (Nacalai Tesque, Kyoto, Japan) was mixed with 50 ml ethanol and 25 ml concentrated (35%) HCl was added. Gut contents of the foregut and the midgut of *C. formosanus* were diluted with 10 μl phloroglucinol/HCl solution on glass slides. A glass coverslip was put on the sample and its periphery was sealed with clear nail varnish to prevent drying. Wood particles stained in red were distinguished from nonwood materials under a microscope. Ten prominently larger wood particles were chosen, and the lengths of the major axes were measured using a digital microscope (VHX-1000; Keyence). Five worker termites of *C. formosanus* were used for the experiment.

Results

Distribution of endogenous cellulase in *C. formosanus*

Cellulase activity was found predominantly in the salivary glands of worker of *C. formosanus* (Fig. 1A), and 15% of the total activity was distributed in the midgut. The endoglucanase origins of the cellulase activities of the salivary glands and the midgut were clarified by Western blotting.

Separation of gut protein on SDS-PAGE

Total soluble proteins of the gut compartments from *C. formosanus* were separated on a polyacrylamide gel. The molecular mass of approximately 47 kDa was estimated

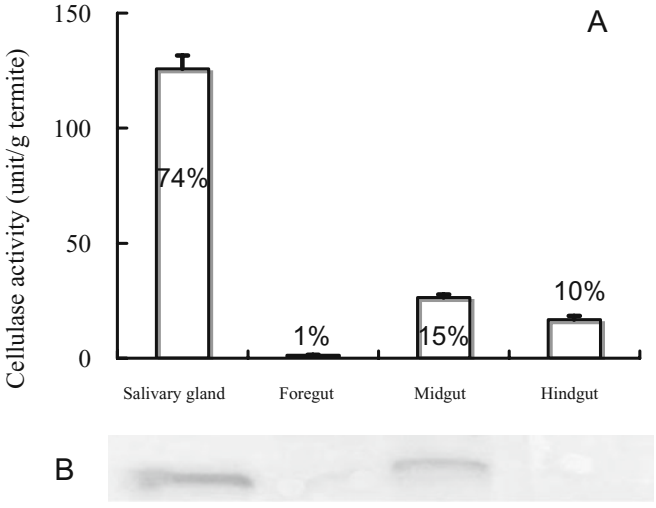

Fig. 1. A Distribution of cellulase activity in the salivary glands and the digestive tract of *Coptotermes formosanus*. Columns indicate the mean ± SD of three repetitions. **B** Western blotting using anti-A18 rabbit serum against protein extracts of the salivary glands, foregut, midgut, and hindgut. The clip of the membrane between 30 and 50 kDa is shown

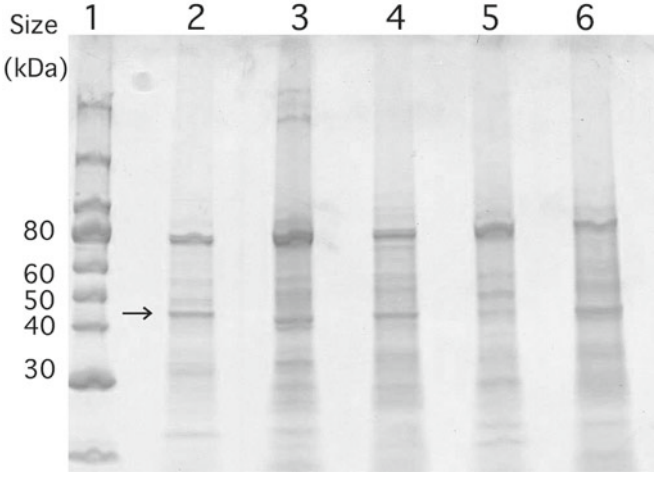

Fig. 2. SDS-PAGE of crude extracts of *C. formosanus*. *Lane 1*, Protein marker; *lane 2*, crude extract from the salivary glands; *lane 3*, crude extract from the foregut; *lane 4*, crude extract from the midgut wall; *lane 5*, crude extract from the midgut contents; *lane 6*, crude extract from the hindgut. The band assumed to be termite cellulase is indicated with an *arrow*

from the amino acid sequences of *C. formosanus* cellulase, as determined by Nakashima et al.,[1] and the Rf value of the protein immunoreacted with anti-A18 antibody (Fig. 1B). Cellulase was one of the main proteins in the salivary glands (Fig. 2), and its presence in the gel was confirmed by Western blotting using anti-A18 antibody to form immunoreacted bands. A weak band near the lower detection limit was found at 47 kDa in the lane of midgut contents. The protein amount of this band accounted for 5.7% of the total midgut proteins by 1D densitometry analysis. The total protein in the midgut content of a *C. formosanus* worker was

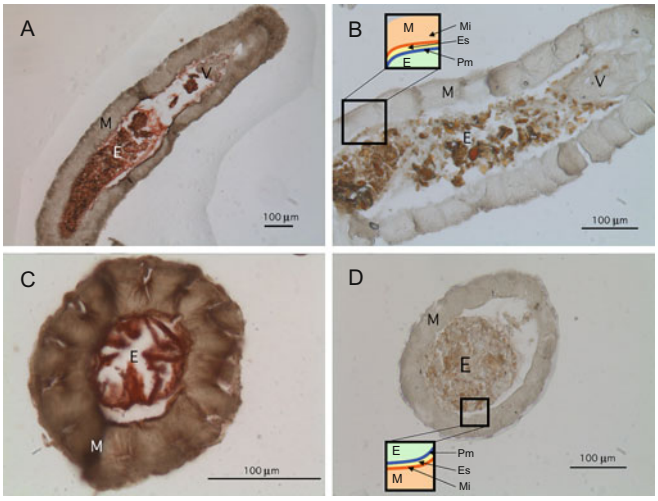

Fig. 3. Immunohistochemistry of endogenous cellulase activity in the midgut of *C. formosanus*. **A** and **C** were immunoreacted with anti-A18 clonal rabbit serum; **B** and **D** are the negative controls. *M*, Midgut wall; *V*, stomodaeal valve; *E*, endoperitrophic space; *Mi*, microvilli; *Es*, ectoperitrophic space; *Pm*, peritrophic membrane

3.22 ± 0.34 µg. The luminal volume of endoperitrophic space was 61.3 ± 13.9 nl.

Space distribution of endogenous cellulase in the midgut of *C. formosanus*

Western blotting demonstrated the presence of the endogenous cellulase in the midgut of workers of *C. formosanus* (Fig. 1A). To clarify the cellulase function site in the midgut of *C. formosanus* workers, immunohistochemistry was performed using rabbit serum anti-A18. In the longitudinal paraffin sections, the microvilli distributed on the ectoperitrophic space side of columnar cells were stained specifically (Fig. 3A). In addition, the peritrophic membrane and the contents of the endoperitrophic space were reacted with anti-A18 rabbit serum. The signal on the microvilli was weak in the anterior part of the midgut. The cross sections of the middle of the midgut (Fig. 3C,D) confirmed the results. The control experiments using normal rabbit serum did not show staining of either microvilli or endoperitrophic space (Fig. 3B,D).

The size of wood particles

The average sizes of ten distinctively larger wood particles in the foregut and the midgut of *C. formosanus* workers were 19.6 ± 1.9 µm and 9.5 ± 1.6 µm, respectively.

Discussion

The distributions of cellulase activity in the digestive tract were measured in the workers of *C. formosanus* (Fig. 1A). This distribution of enzyme activity is in accordance with previous reports.[7] The site where saliva acts is unknown. Saliva may spread on the surface of wood particles and contribute to predigestion, helping mastication by the mandibles and the gizzard.

Western blotting showed that the cellulase activities in the salivary glands and the midgut come from termite endogenous cellulase (see Fig. 1B). This result corresponds to the reverse transcript polymerase chain reaction (PCR) experiment carried out by Nakashima et al.,[1] confirming exclusive expression of the endogenous cellulase in the salivary glands and midgut tissue. Cellulase activity was also found in the hindgut of *C. formosanus*. The hindgut extract did not contain the protein that reacts with A18 rabbit antiserum, showing that the hindgut cellulase activity did not contain a detectable amount of endogenous cellulase. This result corresponds well with the results of the study by Tokuda et al.[7] The hindgut cellulase activity is considered to come from symbiotic protozoa because the removal of hindgut protozoa only reduces hindgut cellulase activity in *Neotermes koshunensis*.[11]

The proportion of cellulase protein in the total midgut soluble protein was estimated to be 5.7%; as the total soluble protein amount in a worker midgut was 3.22 µg, it was calculated that one worker midgut contains 0.18 µg cellulase. From the volume of endoperitrophic space, cellulase concentration in the worker midgut was calculated to be 2.94 mg/ml, and the activity was approximately 1.0×10^3 units/ml; this is about 100 times higher than the activity in the gastric fluid of crayfish.[12] This extremely high cellulase concentration in the small midgut is an important characteristic of the termite cellulose-degrading system.

The spatial distribution of termite cellulase in the midgut was clarified by immunohistochemistry (Fig. 3). It was shown that most of the termite cellulase was distributed in the endoperitrophic space and the rest was found on the microvilli (Fig. 3A,C). Cellulase seemed to be secreted from the inner side of the columnar cells of the midgut, and then transferred into the endoperitrophic space where it acts. The peritrophic membrane separates the endo- and ectoperitrophic space. The wood particles ingested by termites are degraded by endogenous cellulase into soluble sugars, mainly cellobiose and glucose, which could pass to the ectoperitrophic space and be absorbed through the midgut wall. The peritrophic membrane could keep the concentration of the soluble sugars low enough in the endoperitrophic space to prevent product inhibition against the cellulase and promote the production of sugars. Cellobiose could be broken down into glucose by β-glucosidase, which is another cellulolytic enzyme produced endogenously by termites,[13] and could be absorbed by possible glucose receptors distributed on the midgut walls. This membrane structure is the second main characteristic of the efficient wood degradation of termites.

At the end of the midgut, the termite has an enteric valve,[14] which would inhibit the passage of endogenous cellulase into the hindgut. Because wood-feeding termites have diets containing very little nitrogen, the C/N balance problem is one of the main subjects in termite physiology.[15,16] Endogenous cellulase secreted in the termite midgut would be kept inside the endoperitrophic space and then

back-flow through the gap between the midgut wall and the outer wall of the peritrophic membrane to be recycled.[17] The degenerated enzymes could also be a nutrient source for the host termites, rather than simply being passed into the hindgut. The system of protein digestion and amino acid absorption is not clear, but this assumption is reasonable because wood-feeding termites live on extremely nitrogen-poor resources. Protease activity in the midgut of termites has been reported previously.[18,19]

Another main characteristic of wood decomposition by termites is the presence of mechanical grinding systems (mandibles and the gizzard). The mandibles are generally masticating organs that cut and crush ingested food in many insects.[20] In addition to the mandibles, termites have a well-developed cuticular armature of a remarkably similar arrangement among different species[21] in the epithelium at the posterior terminus of the foregut (the gizzard). The wood particles collected from the gut of *C. formosanus* were reported to be smaller than 50 μm, although it is not clear from which gut compartment the wood particles were recovered.[22] Our measurements showed the average sizes of wood particles in the foregut were larger than those in the midgut. The results suggest that the gizzard plays a role in grinding the ingested wood particles into smaller pieces than those produced by the mandibles. A change in the surface structure of wood particles in the digestive tract of *C. formosanus* was observed previously with a scanning microscope.[23] It was reported that the size of wood particles from both the posterior part of the midgut and the foregut ranged from 20 to 100 μm and did not differ in size between the different parts of the gut. We observed thin cuticular-like structures of around 100 μm in length in the midgut of the workers, but saw no large wood particles comparable to those in their report.

We concluded that the morphological and functional characteristics and the cellulose distribution observed in the current study would sustain efficient cellulose digestion by the termites. Wood-feeding termites live solely on wood, in most cases, by producing all the energy and nutrients acquired from wood to enable their reproduction without the input of energy from other sources.

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