Cellulase of fungus-growing termites: A new hypothesis on its origin

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Summary. Termites depend for cellulose digestion on cellulases produced by their symbionts (intraintestinal flagellates and bacteria). In this paper a new source of cellulase used by Macrotermitinae is described: ingested conidiophores of the symbiotic fungus Termitomyces.

The association of termites with fungi has reached a high degree of specialization in the sub-family Macrotermitinae[2]. These termites construct 'fungus-gardens' within their nests and cultivate a basidiomycete fungus belonging to the genus Termitomyces[2]. The fungus-gardens produce numerous pearly white nodules known as conidiophores. Several suggestions have been made concerning the role of fungus-gardens in termite colonies. They have been considered to be a source of food and vitamins[3], and also temperature[4] and humidity[5] regulating devices. It is generally agreed that the fungus attacks lignin-cellulose complexes making cellulose accessible to termite digestion[6]. However, the mechanism by which fungus-growing termites utilize cellulose has been a matter of speculation[7].

In this study, I found that *Termitomyces* conidiophores associated with the mound-building termite *Macrotermes subhyalinus* contain high levels of cellulase activity. The fungus comb substrate, a mixture of digested plant material and soil particles held together by dense mycelial growth, lacks this activity. As fungus conidiophores are considered to be an important part of the termite diet, I have examined the cellulase system of both conidiophores and termite digestive tracts. The results suggest that some of the cellulase components found in the digestive system of termites are obtained from *Termitomyces* conidiophores.

According to the multiple-component theory of cellulase[8], digestion of native cellulose is achieved by a sequential action of 3 cellulase components. The C_1-enzyme (E.C.3.2.1.4, β-1,4-glucanhydrolase) attacks native cellulose to produce a reactive cellulose which is subsequently hydrolysed by the C_x-enzyme (endo β-glucanase or CM-cellulase) to yield soluble low - mol. wt oligosaccharides. These are hydrolysed by cellobiase (E.C.3.2.1.21, β-glucosidase) to produce the sugar D-glucose. In this study, filter paper (FP)[9,10] and absorbent cotton were used to test for C_1 enzyme; carboxymethylcellulose (CMC) for C_x and cellobiose for cellobiase activity. The increase in reducing sugars was determined by the dinitrosalicylic acid method[11].

The cellulase system of *Termitomyces* conidiophores is very active and displays exceptionally high activities towards (FP) and (CMC) (table 1). About 80% of the total cellulase activity was retained after drying conidiophores at 60 °C for 16 h and storing them at room temperature for 6 months. By constrast, fungus comb substrate exhibited very low cellulase activity, and its contribution to cellulose breakdown is doubtful. The digestive system of the termite *M. subhyalinus* however, contained higher levels of cellulase activity than reported for other termite species[12,13].

When different sections of the intestinal tract were examined, most (FP) cellulase activity (75.3% of the total activity of the digestive system) was found in the midgut, while 52.5% of the cellobiase activity was in the hindgut (particularly in the 3 voluminous segment known as paunch)[7]. CM-cellulase activity was equally distributed between the midgut and the paunch.

In order to locate the origin of cellulase activity in the midgut, sections of midgut were opened and cleared of their contents, then washed in cold saline solution (0.9% NaCl). Both midgut contents and the washed sections of the midgut wall were examined for cellulase activity. Practically all (FP) cellulase (96.4% of the total activity of the midgut) and more than $\frac{2}{3}$ of CM-cellulase activities were found in the midgut contents. By contrast, the midgut wall contained about 54% of the total cellobiase activity. To examine the possible contribution of symbionts, samples of the midgut and paunch contents were examined for bac-

Table 1. Cellulase activity of *Termitomyces* conidiophores and its associated termite *M. subhyalinus**

Specimen	Per	(FP) cellulase	CM-cellulase	Cellobiase
Conidiophores	1 mg (fw)	3.77	6.15	0.34
	1 mg (dw)	3.00	4.75	0.32
	1 mg (protein)	13.04	21.27	1.18
Fungus comb substrate	10 mg (fw)	0.0	0.0	0.0
	40 mg (fw)	0.20	0.35	0.0
Major workers (wild)	1 whole digestive system	1.82	2.84	0.84
	1. midgut	75.3	41.8	47.5
(% of total activity)	a) contents	96.4	67.2	45.9
	b) walls	3.5	32.8	54.1
	2. paunch	19.0	43.3	52.2
	3. rectum and colon	5.7	14.8	0.0
Mixed workers (incipient)	2 whole digestive systems	0.0	0.53	0.02
Major workers (wild-starved)	1 whole digestive system	0.94	2.24	0.82

* Figures of cellulase activity are means of 6 replicates expressed as µmoles reducing sugar (as glucose) produced per h at 50 °C (pH 4.8) per unit sample. Buffered enzyme preparations (0.2 ml in 0.05 M citrate) were added to 1.0 ml of a substrate (1% CMC, Na-salt of low viscosity BDH or 1 mM cellobiose) for measurements of CM-cellulase and cellobiase activity respectively, or to 50 mg (a strip 1 × 6 cm) of Whatman No. 1 filter paper for measurements of (FP) cellulase[9,10]. Total volume was 2.0 ml. *Termitomyces* conidiophores collected from several termite mounds (located in Kajiado district near Nairobi, Kenya) were used fresh (fw) or after drying (dw) at 60 °C for 16 h. Pieces of fungus comb substrate (conidiophore-free) were homogenized in buffer, centrifuged and the supernatant was used as a source of enzyme. Major workers (wild) from the same termite mounds were dissected under cold solution (0.9% NaCl) and digestive tracts were removed, homogenized in buffer and centrifuged at 7,000 rpm (10 min). Sections of the digestive system (midgut, paunch, rectum, and colon) were homogenized separately. Midguts of termites were cleared of their contents[15] and the remaining clean midgut walls together with their contents were assayed separately. Mixed workers (minor and major, 30-40 days old) were obtained from incipient colonies that were maintained on a fungus-free diet (gras only) since hatching. Termites (wild) were starved for 6 days and kept at 30 °C.

Table 2. Comparison between cellulase of *Termitomyces* conidiophores and that of the digestive system of the termite *M. subhyalinus**

Property	Conidiophores	Termite
Ability to hydrolyse absorbent cotton, (FP), (CMC), cellulose powder, and cellobiose	+	+
Main end product of (FP) hydrolysis	Cellobiose	D-glucose
Ratio of (FP) cellulase: CM-cellulase activity	1:1.6	1:1.6
Ratio of (FP) cellulase: cellobiase activity	1:0.1	1:0.5
Molecular weight of (FP)/CM-cellulase component	16,000	16,000
Molecular weight of cellobiase	12,000	> 60,000
Activation energies of (FP) cellulase and CM-cellulase, respectively	7.5 and 2.2 kcal/mole	10.9 and 2.9 kcal/mole
pH-optima range (stability and activity)	4.8–5.2	4.8–5.2
Influence of papain on (FP) cellulase activity	–	–
Influence of zinc ions on (FP) cellulase activity	+	+

* Absorbent cotton (50 mg) was incubated with the enzyme preparation (total volume 4.0 ml pH 4.8) for 16 h at 50 °C. Means of cellulase activities of conidiophores and termite guts were 10.2 µmoles (glucose) per 100 mg (fw) and 1.1 µmoles per 100 guts respectively. Cellulose powder (Azure-Calbiochem) was incubated (30 mg/3.0 ml incubation mixture; pH 4.8) with enzyme for 1 h at 50 °C then centrifuged and the blue colour was determined spectrophotometrically at 595 nm. Conidiophores (3 mg) and termites (5 guts) produced mean values of 0.24 and 0.49 absorption units respectively. Reaction products were analysed by TLC (pre-coated cellulose plates) developed by ethyl acetate-pyridine-water (60:25:20 v/v) and sugar spots were visualised by silver nitrate reagent. Partial purification of cellulose was achieved by gel-filtration chromatography using a Sephadex G-100 column (40×2.5 cm; Pharmacia, Uppsala, Sweden) eluted with 0.1 M sodium citrate buffer (pH 5.2). For mol.wt determinations, the column was calibrated against 5 reference proteins[17]. Activation energies were calculated by using the Arrhenius equation for 5 incubation-temperatures ranging from 20 to 60 °C. pH-activity and -stability profiles were obtained by incubating (12 h) and assaying enzymic activities in different buffer solutions ranging from pH 3.0 to pH 8.9. Various concentrations of papain (1–40 µg/ml) were incubated with the enzyme at 40 °C for 1 h. Zinc sulphate solutions were added (3–100 µg zinc/ml) prior to incubation. Maximum activation of (FP) cellulase activity (about 25%) was observed at a zinc concn. 16 µg/ml of incubation mixture.

terial (gram-positive) and protozoan symbionts (Giemsa stain). Protozoa in this termite were never observed, confirming the results of earlier studies[14]. The midgut contained very low numbers of short chains of *Streptococcus* bacteria which were the only bacteria present. The lack of a large bacterial fauna in the midgut of other higher termite species, e.g. *Trinervitermes trinervoides*, has also been reported[15]. Unlike the midgut, the paunch did contain a large population of small rods of gram-positive bacteria (probably clostridia) in addition to large number of streptococci. These findings indicate that (FP) cellulase activities in the midgut of *M. subhyalinus* termite were not produced solely by termites themselves or by their symbiotic bacteria. This is further supported by the observation that no (FP) cellulase activity was found in termites raised throughout their life on a fungus-free diet (table 1), although some traces of CM-cellulase and cellobiase were detectable. Furthermore, starvation of termites caused significant reduction in (FP) cellulase (about 50% reduction) and CM-cellulase (20%) activities. Cellobiase activity however, remained practically unchanged.

Table 2 shows remarkable similarities between the cellulase systems of conidiophores and termites. Both enzymic systems are truly cellulolytic[16] since they were capable of attacking most forms of cellulose including cotton fibres. The main cellulolytic pathway in conidiophores releases cellobiose as the soluble product, while in termites a subsequent hydrolysis to glucose follows. It is likely that glucose is the form which is absorbed by the termite gut. The activity of cellobiase in the termite gut was 5 times greater than that of conidiophores. Further similarities between conidiophore and termite cellulase are shown in table 2. (FP)-and-CM-cellulase of both organisms had the same mol. wt, activity ratios, pH-optima, activation energies, insensitivity to papain, and similar activation by zinc ions. However, the cellobiase component showed marked differences in mol. wt and activity ratios.

I have occasionally observed the presence of one or several conidiophores inside the midgut of dissected termites. Although the cell walls were intact, the conidiophores were hollow and fragile. It seems that termites do not need to digest conidiophores in order to obtain cellulase, since a rapid release of cellulase (within 2–3 h at 30 °C) could be achieved when intact conidiophores were suspended in a saline medium.

I conclude that the fungus-growing termite *M. subhyalinus* utilizes *Termitomyces* conidiophores as a source of cellulase (mainly for C_1 and C_x) which aids them in the initial phases of cellulose digestion. Fungus conidiophores are thus capable of performing some of the metabolic functions of the missing protozoa in this termite species. Termites seem to produce their own cellobiase which enables them to utilize the end product cellobiose. Bacterial symbionts of the hindgut may contribute to some extent to the overall cellulolytic activity of the digestive system by providing some C_x enzyme. Conidiophores may also contribute substantially to the nitrogen requirement of this termite species[18].

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