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Termitomyces: A New Source of Potent Cellulases

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ABSTRACT. The association of certain species of termites with fungi is a very complex phenomenon. Previously, it was demonstrated the importance of a basidiomycete fungus belonging to the genus *Termitomyces* as a source of potent cellulases. In this paper, the saccharifying activities of *Termitomyces* conidiophores against various oligo- and polysaccharides were compared. In addition, partial purification of the cellulase complex of conidiophores was achieved by using a Sephadex column G-100. The fungal cellulase activity was also compared with five commercial cellulases namely: *A. niger* cellulase (from Calbiochem comp.), Drisellase-N10 (Kyomo Hakko Comp.), Trichoderma-SP122 (The Novo Comp.), Trichoderma-4 (Natick, US Army) and Onozuka SSP1500 (Kink Yakult Comp.). Cellulase(s) activities were measured against cotton wool (C_1), filter paper (FP), carboxymethylcellulose (CMC) and cellobios (ß-glucosidase). Results showed that the activity of *Termitomyces* cellulase was far more superior than others when the substrates were filter paper and CMC. However, the cellulolytic activity on cotton wool was intermediate.

Introduction

Termites of the higher termite subfamily Macrotermitinae common in Africa and Asia, have long intrigued biologists because of their symbiotic association with a basidiomycete fungi that grow in their nests on structures known as "fungus gardens". Despite more than one hundred years ago since the discovery of fungus gardens, the true nature of these gardens and their symbiotic relationship to termites has not yet fully understood[1]. A biochemical approach has been undertaken to look into this phenomenon[2-4]. Thus, a thorough chemical analysis of fungus gardens was conducted, together with the role of some of the chemical constituents that were discovered in them[2,3]. Furthermore, two cellulases or ß-1, 4-glucan hydrolases enzymes were first reported to exist in *Termitomyces* conidiophores of the "fungus garden"[4].

This fact was also confirmed by Martin and Martin[5] while working independently on African termites. The findings of both studies demonstrated for the first time a new theory on the mechanism by which termites utilize cellulosic materials.

This paper presents some additional biochemical properties of *Termitomyces* cellulases, which are; the specificity of the enzymic system towards various substrates; the pH optimum; the constant values of active fractions obtained by using a molecular-sieve chromatography; and a comparison between the activity of the partially purified fungal cellulase system and that of five commercial enzymic preparations was carried out.

Material and Methods

Fresh fungus combs were obtained from termite mounds of *Macrotermes michaelseni* in Kajiado District, Rift Valley Province, Kenya. These combs are usually covered with numerous white conidiophores of different sizes (*Termitomyces* sp.). Conidiophores were hand-picked, then either crushed by a small glass tissue homogenizer, in 0.1 M Na-citrate buffer pH 4.8 and centrifuged at 1000 g at 4°C to remove debris, or suspended in the same buffer for few hours to release its enzymic contents. The supernatent was used for detection of enzymic activities, and for further purification by column chromatography.

Determination of Enzyme Activities

For the assay of C_1-cellulase, pure absorbent cotton (50 mg) was incubated (16 hr) with the enzyme preparation (0.4 ml in citrate buffer); total volume 4.0 ml, pH 4.6 at 50°C. FP-cellulase was measured by using a 50 mg strip (1 × 6 cm) Whatman No. 1 filter paper as a substrate. Buffered enzyme preparation (0.2 ml) was added to the filter paper; total volume 2.0 ml (made-up by dist. H_2O) for 1 hr at 50°C. For assay of CM-cellulase (C_x-cellulases), Na-salt carboxymethylcellulase (1% w/v of low viscosity, BDH) was used as a substrate. ß-glucosidase activity was measured by using cellobiose (1 mM). Enzyme preparation (0.2 ml) was added to 1.0 ml of each substrate (CMC or cellobiose), total volume 2.0 ml, 1 hr incubation at 50°C.

The increase in reducing sugars was determined by the dinitrosalicylic acid method[6], and activities of all enzymes are expressed as μ moles reducing sugar (as glucose) produced per hr at 50°C (pH 4.8) per mg protein.

Purification of the Enzyme Complex

Partial purification of the cellulase complex of *termitomyces* conidiophores was carried out by gel-filtration chromatography using a Sephadex G-100 column (3 × 30 cm; Pharmacia, Uppsala, Sweden) eluted with 0.1 *M* sodium citrate buffer (pH 5.2). An ultraviolet monitor with a 280-nm filter for detection of protein was coupled to the column, together with a fraction collector.

pH-activitiy Profile of Cellulase

pH-activity curves were obtained by incubating (12 hr) and assaying enzymic activities at different buffer solutions ranging from 3.0 to 5.0 (Na-citrate/HCl) and pH 5.0 to 8.9 (phosphate buffer).

Protein Estimation

Protein concentration was routinely determined by the biuret method[7], using bovine serum albumin as the standard. This allowed expression of enzyme activity in terms of μ moles glucose released per mg protein per hour.

Preparation of Cellulase Acetone Powder

For comparison between cellulase activity of *Termitomyces* conidiophores and five enzymic commercial cellulases, an acetone powder of the former enzyme was used. One gm of intact conidiophores in 10 ml distilled water was incubated for 24 hr at 30°C. To the clear liquid supernatent, a 50 ml chilled acetone cooled by dry-ice was added. The resulted suspension was centrifuged and the supernatent was decanted. Final acetone powder was dissolved in 5.0 ml distilled water and used directly for assay.

Sources of Commercial Cellulases

Five commercial cellulase powders were used in this study for comparison, namely: Calbiochem (lot 601710, Calif.); Drisellase N10 (Kyomo Hakko Comp., Japan); Onozuka SSP-1500 (Kinki Yakult Mfg. Co. Ltd., Nishinomiya, Japan); Trichoderma SP 122 (The Novo Company, Copenhagen) and Trichoderma Natick No. 4 (a gift from Dr. M. Mandels of the US Army, Natick, Massachusetts). All samples were assayed for reducing sugars by using the dinitrosalicylic acid method[6].

Results and Discussion

The cellulase system of *Termitomyces* conidiophores associated with the "fungus garden" of the higher termite *M. michaelseni* is very active and displays exceptionally high activity towards both soluble and insoluble forms of cellulose (Table 1). This finding confirms previous studies conducted under similar conditions[4,5,8,9]. Moreover, this cellulase system shows high specificity towards ß(1 → 4) glycosidic linkage. The basis for this specificity is not only due to the type of glucosidic linkage but also the type of the glycosyl units as well. In general, the most active microorganisms in degrading soluble cellulose are usually the most active toward insoluble cellulose as well[9].

Fractionation of the conidial cellulase system by a Sephadex G-100 column gave rise to two main peaks with FP-cellulase activity (Fig. 1). Most of the activity however, was concentrated in the second peak which corresponds with the main peak. Cellulase from conidiophores emerged also in two main peaks on CM-Sepharose, whereas on DEAE-Sepharose only one peak was observed[8]. These findings

TABLE 1. Hydrolitic activity of *Termitomyces* conidiophores against cellulose and other sugars.

Sugar[a]	Glycosidic linkage	Specific Activity[b]
Cellulose (insoluble)	ß(1→4)	13.04
Carboxymethyl cellulose	ß(1→4)	23.47
Chitin (insoluble)	ß(1→4)	3.10
Cellobiose	ß(1→4)	3.71
Maltose	α(1→4)	1.44
Melibiose	α(1→6)	1.30
Trehalose	α(1→1)	0.09
Sucrose	α(1→2)	1.07

[a]Cellulose (insoluble) = filter paper (1 × 6 cm strip of 50 mg Whatman No. 1); Carboxymethyl Cellulose (1%); Chitin (20 mg per incubation); all other sugars (1m *M*) 1.0 ml per incubation.
[b]Figures are means of 4 replicates expressed as μ moles glucose mg^{-1} protein hr^{-1} at 50°C (pH 4.8). Buffered enzyme preparations (0.2 ml in 0.05 *M* Na-citrate) were added to 1.0 ml of substrate solution.

suggests that the cellulase system of *Termitomyces* is composed of various kinds of components, probably of the endo- and exocellulase type[8]. Figure 1 shows also slight difference in the elution pattern of proteins and cellulases. This suggests that complexes between the enzymes and other components such as polysaccharides may account for these differences.

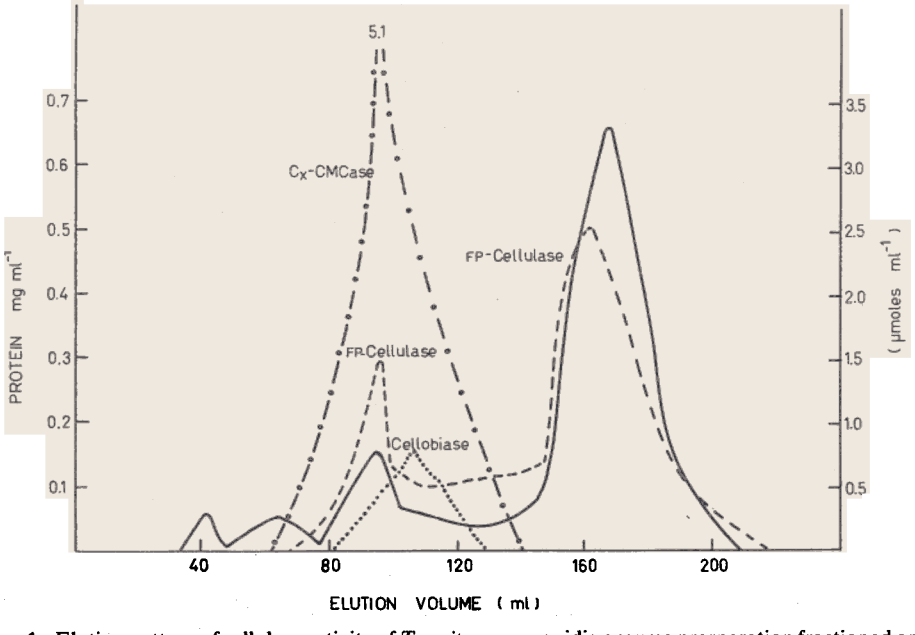


FIG. 1. Elution pattern of cellulase activity of *Termitomyces* conidia enzyme prerparation fractioned on a Sephadex G-100 column with 0.1 *M* Na-citrate buffer pH 5.2. The broken lines indicate the elution pattern of different cellulases, while the solid line represents the protein pattern. Column 3 × 30cm, flow rate 1.5 ml min^{-1}, sample volume 3.0 ml.

Partial purification of the cellulase complex of conidiophores was achieved by using a Sephadex G-100 column eluted with citrate buffer (pH 5.2). High yields were obtained for both FP- and C_x-cellulase, with purification factors ranging between 12.3-12.8 (Table 2). Since the ß-glucosidase fraction was less active than the other two enzymic fractions, it was difficult to assess both the purification factor and yield. We also calculated the constants of active fraction ($K_{av.}$) obtained from the column under one set of conditions (Table 3). There distinct fractions were obtained ($K_{av.}$ 031, 0.253, 0.663) when crushed extracts of conidiophores were used. Two of these peaks correspond to FP-cellulase ($K_{av.}$ 0.252). When conidiophores were allowed to release its enzymic content to the medium, only one active fraction (FP-cellulase) was obtained. Therefore, it is possible by using different extraction procedures, to obtain different enzymic pattern.

TABLE 2. Purification of cellulases of *Termitomyces* conidiophores by a Sephadex G-100 column chromotography[a].

Step	Enzyme[b] (ml)	Volume (ml)	Protein concn (mg/ml)[c]	Activity (U/ml)	Total activity (U)	Specific activity (U/mg)	Purification factor	Yield (%)
Crude extract			35.3	74.5	74.5	2.1		100
	FP-Cellulase							
After elution		50	0.05	1.3	65.0	26.0	12.3	87.2
Crude extract								
	C_x-Cellulase							
After elution		50	0.05	2.3	115.0	46.0	12.8	90.8
Crude extract		1						
	ß-glucosidase							
After elution		33	0.68	0.69	22.8	1.0		46.1

[a] 400 mg freshly collected conidiophores were suspended in 4.0 ml dist. H_2O for 48 hr at 30°C of clear solution was used for column chromatography. Eluant: Na-citrate buffer (pH 5.2).

[b] For measurements of FP and C_x cellulases, a 50 mg strip of Whatman No. 1 filter paper (1 × 6 cm) and 1.0 ml of CMC, Na-Salt of low viscosity were used respectively. For ß-glucosidase 1.0 ml of 1 mM cellobiose was used. Total volume 2.0 ml (pH 5.2).

[c] Protein conc. was calculated according to $A_{280}X$ correction factor = mg/ml protein.

TABLE 3. Elution of pattern *Termitomyces* proteins and cellulases using a Sephadex G-100 column

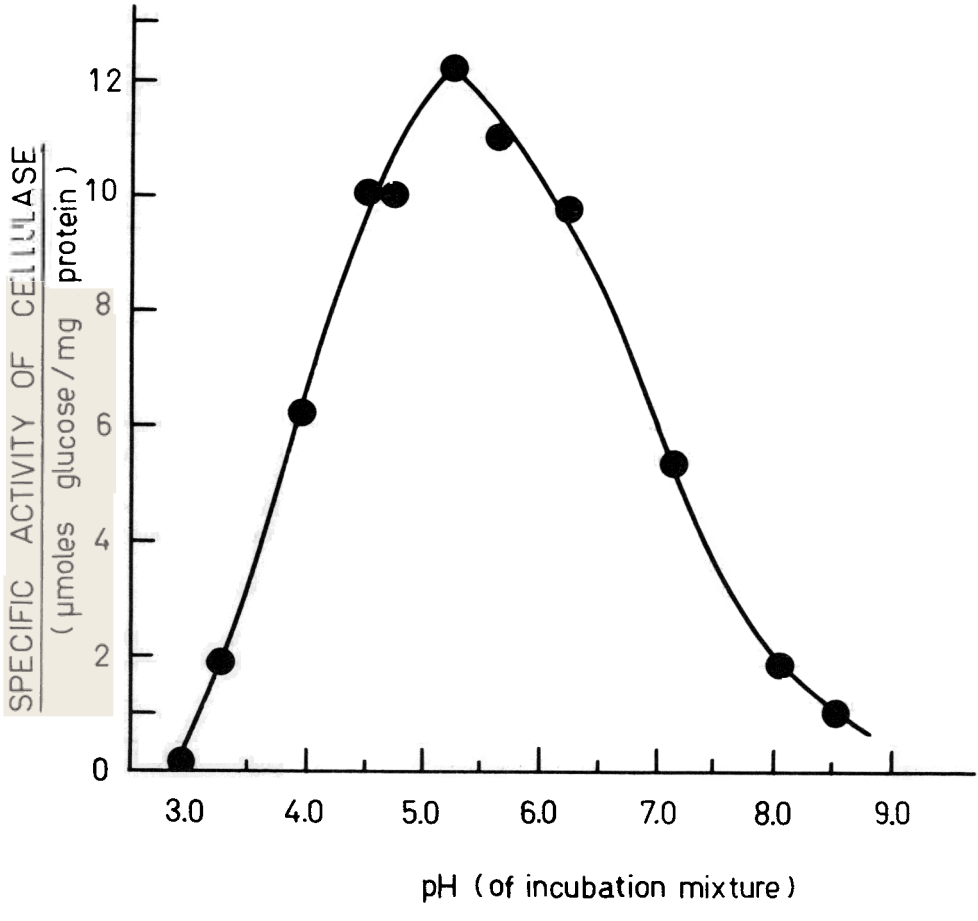
Active Fraction	K_{av} (constant of active fraction)[a]	
	Conidiophores[b]	Conidiophores[c]
1. Protein Pattern	(Homogenate method) 0.031±0.006 0.253±0.011 0.663±0.022	(Slow release method) 0.104±0.049 0.318±0.033 0.733±0.027
2. FP-Cellulase	0.252±0.011 0.639±0.016	0.325±0.016
3. C_x-Cellulase	0.252±0.011	0.329±0.014
4. Cellobiase	0.302±0.016	(v. low activity)

[a] $K_{av} = ve - vo/v - vo$; ve = elution volume of the (active material); vo = void volume (blue dextran); v_t = total volume of gel bed ($\simeq \pi r^2 h$; r = radius and h = height of the column); eluant; Sod citrate buffer pH 5.2 (value ± standard deviation of four different runs).

[b] 100 mg fresh conidiophores in 6 ml Na-Citrate buffer (pH 5.2) were homogenized, centrifuged at 7,000 g (10 min.) and 1.8 ml of the supernatent was used for the column (bed dimensions 3 × 3 cm).

[c] 200 mg fresh intact conidiophores were left in 10 ml Na-citrate buffer (pH 5.2) for a period of 12 hr at 30°C, then centrifuged and 2.0 ml of the clear solution was used.

It was important to determine the optimum condition for the enzymic system to operate. Determination of pH for optimum activity (Fig. 2) showed an optimum pH at 5.2. Therefore, assays were subsequently conducted at this optimal pH by using Na-citrate buffer.



2. The pH activity curve for *Termitomyces* conidia FP-cellulase. The circles represent points obtained by using two buffer systems; Na-citrate/HCl (pH 3.0-5.0) and a phosphate buffer (pH 5-8.9). pH optimum = 5.2.

The potency of *Termitomyces* cellulytic activity in comparison with some known commercial cellulases was determined. Five cellulase preparations were selected, some of them are products of microorganisms such as *Trichoderma* and *Irpex*. The acetone powder preparation of *Termitomyces* conidiophores showed superior FP- and C_x-cellulase activities over the other commercial enzymes (Table 4). In addition, *Termitomyces* cellulase showed greater potency against cotton wool (C_1-cellulase) when compared with "Calbiochem" or "Drisellase N 10". On the other hand, *Trichoderma* cellulases possess very active C_1-cellulase. It is important to note however, that some of the commercial cellulases contain sugar impurities exceeding, sometimes, 20% of the dry weight (Table 4).

TABLE 4. Comparison between cellulase activity of *Termitomyces* and five commercial cellulases.

Enzyme Preparation	Cellulase activity (per mg solid)[a]				Sugar content (%)
	Cotton wool (C_1-Cellulase)	Filter paper (FP-)	CMC (C_x-)	Cellobiose (ß-glucosidase)	
Conidiophores (acetone powder)	8.88 (±1.81)	12.71 (±1.88)	18.81 (±2.44)	1.30 (±0.34)	nil
"Calbiochem" cellulase	0.28 (±0.13)	0.93 (±0.38)	1.71 (±0.42)	2.0 (±0.88)	13.0
Drisellase N10	2.15 (±0.45)	2.42 (±0.44)	3.61 (±0.58)	0.35 (±0.2)	nil
Onozuka SSP-1500	6.86 (±1.20)	2.31 (±0.72)	3.76 (±0.45)	1.25 (±0.33)	22.0
Trichoderma SP-122	21.81 (±1.77)	10.86 (±1.05)	5.43 (±1.05)	0.49 (±0.12)	nil
Trichoderma Natick (No. 4)	22.45 (±1.88)	11.34 (±2.34)	5.40 (±1.0)	0.29 (±0.15)	nil

Values of cellulase activity are average of 9 determinations (± s.d.) expressed as μ moles glucose released per mg enzyme preparation. All reactions were carried out in Na-citrate buffer (pH 5.2).

These results together, indicate that the fungus *Termitomyces* is exceptionally good source for a cellulase system that is required for efficient saccharification of cellulose in biomass into glucose. This initial process is followed by fermentation to produce alcohol and biogas. Such renewable resources of fuel is currently receiving increasing attention worldwide[10]. The world production of waste cellulosic materials exceeds 4 billion tons per year[11]. It was suggested that the enzymatic process for converting this waste to fuel through the use of suitable microorganisms are preferred to alternative technologies[12].

Although some strains of fungi species such as those belonging to *Tricoderma, Fusarium* and *Penicillium* are good sources of saccharifying cellulases[12], the search for other microorganisms should be encouraged. It is hoped that with the successful cultivation of the *Termitomyces* fungi, it will be possible to produce these enzymes cheaply for production of such biofuels. It has been shown previously[3,4] that D-mannitol (3%) in agar medium can support the growth of monocultures of *Termitomyces*

under laboratory conditions. With genetic manipulation however, it should be possible to produce new strains of *Termitomyces* of high cellulose saccharifying activity for efficient biomass degradation.

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الفـــطر « ترميتومايسِـــس » : مصدر جديد وفعال للإنزيمات المحلـــلة للسليلوز

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إن العلاقة القائمة بين بعض أنواع النمل الأبيض وبين بعض الفطريات التي تنمو على أوكارها ، لهى علاقة معقدة للغاية . فلقد سجلنا من قبل أهمية نوع معين من الفطريات « البازيدية » التابعة للجنس « ترميتومايسِس » وهي التي تعيش معيشة تكافلية مع النمل الأبيض ، كمصدر هام لبعض الإنزيمات المحللة للسليلوز التي تستخدمها هذه الحشرات للاستفادة من المواد السليلوزية .

وفي هذا البحث تم عمل دراسة مقارنة للنشاط الإنزيمي المحلل للسكريات باستخدام عدة أنواع من السكريات الثنائية العديدة . كما تم بنجاح تنقية جزئية للإنزيمات المحللة للسليلوز الموجودة في الأجسام الكونيدية للفطر باستخدام عمود الكروماتوجرافيا (سيفاديكس - ١٠٠) . كذلك أجريت دراسة مقارنة بين درجة النشاط الإنزيمي للفطر ، وبين خمسة مستحضرات إنزيمية تجارية اتضح فيها بصفة عامة تفوق إنزيم هذا المصدر على المصادر الإنزيمية الأخرى .