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Cellulase and Other Polymer-Hydrolyzing Activities of *Trichomitopsis termopsidis*, a Symbiotic Protozoan from Termites†

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Crude extracts of the anaerobic, cellulolytic protozoan *Trichomitopsis termopsidis* possessed endo-β-1,4-glucanase and cellobiase activities, as evidenced by hydrolytic action on carboxymethyl cellulose and cellobiose, respectively. Cell extracts also hydrolyzed microcrystalline cellulose. Hydrolysis of microcrystalline cellulose displayed optima at pH 5 and at 30°C, and glucose was the sole product liberated. Cellulolytic activities of *T. termopsidis* appeared to be entirely cell associated. Hydrolytic activity was also detected against Douglas fir wood powder, xylan, starch, and protein, but not chitin. The importance of these enzymes in the nutrition of *T. termopsidis* is discussed in terms of the natural habitat of this protozoan (the hindgut of wood-eating termites).

Phylogenetically lower termites depend on dense and diverse populations of hindgut protozoa and bacteria to thrive by xylophagy, and our current understanding of this nutritional symbiosis has been recently reviewed (4–6, 19). Among this heterogeneous microbial community, it appears that anaerobic, flagellate protozoa are the major, if not sole, agents of wood cellulose hydrolysis. They are not only abundant in the hindgut, but they also have the ability to endocytose, and thereby sequester, wood particles for intracellular degradation. Major products of cellulose fermentation by mixed and axenic suspensions of the protozoa include H_2, CO_2, and acetate (12, 13, 21, 29, 30), with the latter compound serving as a major oxidizable energy source for termites (20), as well as constituting an important biosynthetic precursor for the insect (2, 3, 17, 24).

Our knowledge of the cell biology and biochemistry of termite gut protozoa is still in its infancy, having been hampered for many years by the lack of pure cultures for detailed study. However, a major breakthrough was recently made by Yamin, who reported the axenic cultivation of two species (*Trichomitopsis termopsidis* [28] and *Trichonympha sphaerica* [30]) and who studied some of their metabolic activities (31).

In the companion paper (21) we reported our studies on the nutrition and growth characteristics of one of Yamin's isolates, *Trichomitopsis termopsidis*, and our methods for increasing the cell yields of *T. termopsidis* in vitro. In the present study, we capitalized on the improved growth characteristics of *T. termopsidis* to obtain cells for biochemical studies. Herein we report on cellulase and other polymer-hydrolyzing enzyme activities in cell extracts of *T. termopsidis*.

MATERIALS AND METHODS

Organisms. *T. termopsidis* 6057C was used throughout this study and was obtained by bromoethanesulfonate treatment of *T. termopsidis* 6057, as previously described (21). The sources of, and cultivation methods for, *Bacteroides* sp. strain JW20 and *Methanospirillum hungatii* JF1 are given in the companion paper (21).

Cultivation of protozoa. *T. termopsidis* was grown under anaerobic conditions in modified Yamin medium containing heat-killed cells of *Bacteroides* sp. strain JW20 (final concentration, 0.1 mg [dry weight]/ml) in place of heat-killed rumen bacteria, and in co-cultivation with *M. hungatii* JF1 as previously described (21). For large volumes, bottles (Wheaton borosilicate glass, Type 400) were used. They contained 500 ml of medium and were sealed with a butyl rubber stopper. The medium for bottle cultures was prepared in a similar fashion to that used for tube cultures (21), except that glutathione was added to the sterile medium from a separate heat-sterilized stock solution. Growth of co-cultures of *T. termopsidis* and *M. hungatii* was monitored by periodic analysis of headspace gas for methane by using gas chromatography (21). Agitation of bottle cultures was completely avoided, or kept to a minimum, because shaking retards the growth of *T. termopsidis* (21).

Preparation of cell extracts. Bottle cultures of *T. termopsidis* were harvested when protozoan densities reached 2×10^4 to 3×10^4 cells per ml. Generally, the contents of three bottles were pooled (ca. 1.5-liter total volume), and cells were collected by centrifugation in airtight centrifuge bottles (Sorvall, catalog no. 03256; equipped with caps, catalog no. 03278; Ivan Sorvall, Inc. Norwalk, Conn.) at $1200 \times g$ for 20 min at 10°C. The resulting pellets, consisting mainly of *T. termopsidis* and unused cellulose particles, were suspended in a small volume of phosphate-buffered salts solution (see below), pooled, and then layered onto 100 ml of a 20% (wt/vol) solution of Ficoll prepared in phosphate-buffered salts. After 30 min, most of the undigested cellulose particles descended into the Ficoll layer. The layer above the Ficoll was then collected by aspiration and recentrifuged at $1200 \times g$ for 20 min at 10°C. The resulting pellet was again suspended in phosphate-buffered salts solution and centrifuged as described above, and the final pellet was suspended in 1 to 2 ml of phosphate-buffered salts solution at 4°C. Cell densities were then determined by direct microscopic counts (21). By using this procedure, recovery of *T. termopsidis* ranged from 30 to 50%. However, the final suspension was highly enriched in *T. termopsidis*, with only minor contamination from cellulose particles or bacterial cells.

Cell extracts were prepared either by the addition of Triton X-100 (final concentration, 2% [vol/vol]) to the cell suspension or by freezing and thawing. The latter was done

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TABLE 1. Cellulolytic enzyme activities of *T. termopsidis*

Extract prepn	Extract fraction	Enzyme activity (mU/10^6 cell equivalents) against the following substrates[a]:		
		Microcrystalline cellulose	Carboxymethyl cellulose	Cellobiose
Triton X-100	Soluble	1.60 ± 0.73[b]	22.8 ± 10.5[c]	17.5 ± 1.1
	Particulate	0.88 ± 0.22	5.8 ± 0.8	2.9 ± 0.6
Freeze-thaw	Soluble	1.71 ± 0.22	3.7 ± 0.6	3.5 ± 0.8[d]
	Particulate	1.54 ± 0.28	5.3 ± 1.9	8.7 ± 0.1

[a] Minimum level of activity detectable (in milliunits): microcrystalline cellulose (0.02); carboxymethylcellulose (0.2); and cellobiose (0.2). Values are the mean ± standard error of the mean for three to six determinations on a single extract (n = 1), except where indicated otherwise.
[b] n = 4.
[c] n = 2.
[d] n = 3.

by immersion of the cell suspension in a dry ice-acetone bath for 3 min, followed by incubation at room temperature until cell extracts were completely thawed. Both procedures resulted in virtually 100% lysis of protozoa, as determined microscopically. Cell extracts were then centrifuged at 12,000 × g at 4°C for 20 min and separated into a soluble (i.e., supernatant) and particulate (i.e., pellet) fraction.

All manipulations of cells and cell extracts were performed under O_2-free N_2, by using strict anaerobic techniques (11, 23). The phosphate-buffered salts solution used in the procedures described above was prepared under anaerobic conditions and contained (in millimolar) K_2HPO_4 (10.8); KH_2PO_4 (6.9); NaCl (24.5); KCl (21.5); and dithiothreitol (1.0). The final pH of this solution was 7.0.

Enzyme assays. Unless indicated otherwise, all assays were performed at 30°C, and determinations of cellulase activities generally followed the recommendations of Ghose et al. (10). Cellulase (i.e., the complete enzyme system [10, 16]) and endo-β-1,4-glucanase (EC 3.2.1.4) were assayed by measuring release of reducing sugar from microcrystalline cellulose (final concentration, 5.0% [wt/vol]; Type 20; Sigma Chemical Co., St. Louis, Mo.) and carboxymethylcellulose (final concentration, 0.5% [wt/vol]; Type 7MF; Hercules Inc.), respectively, in 0.2 M acetate buffer (pH 5). Reaction mixtures (final volume, 0.1 ml) were run in triplicate in Eppendorf conical centrifuge tubes (1.5 ml capacity) and were terminated by the addition of 0.025 ml of 1 N HCl. After neutralization with 0.025 ml of 1 N NaOH, samples were centrifuged at 11,310 × g for 5 min, and the supernatants were analyzed for reducing sugar by the method of Park and Johnson (22) or Bernfeld (1). In addition, reaction mixtures for the complete cellulase enzyme system were screened for specific products and intermediates of cellulose hydrolysis (i.e., glucose, cellobiose, and cellodextrins) by subjecting supernatants to high-pressure liquid chromatography (15) and by performing a specific assay for glucose with glucose oxidase (26). To determine the optimum pH for hydrolysis of microcrystalline cellulose by cell extracts, reaction mixtures were buffered with 0.2 M acetate (pH range, 3.0 to 6.0) or 50 mM Tris-hydrochloride (pH range, 7.0 to 8.0). The extent of saccharification of cellulose was estimated by using the procedures described above, but with less microcrystalline cellulose in reaction mixtures (final concentration, 0.04% [wt/vol]), as suggested by Johnson et al. (14). Cellobiase (EC 3.2.1.21) was assayed by using reaction mixtures similar to those described above, but by measuring release of glucose from cellobiose (final concentration, 1% [wt/vol]) with glucose oxidase (26). In general, reaction mixtures were incubated for (in hours): the complete cellulase enzyme system (24); endo-β-1,4-glucanase (0.5 to 1); and cellobiase (1 to 3). One unit of activity was defined as the release of 1 μmol of glucose equivalent per minute.

To determine other hydrolytic activities of *T. termopsidis*, ball-milled Douglas fir wood powder (20), xylan (catalog no. X-3875; Sigma), or ball-milled chitin (21) was substituted for microcrystalline cellulose, and the reaction mixtures were assayed for reducing sugar as described above. Protease was assayed by using azocasein as substrate, as described by Brock et al. (7), and β-amylase was assayed by using potato starch as substrate by the method of Bernfeld (1).

Estimation of protozoan cell protein. It was virtually impossible to separate *T. termopsidis* cells completely from bacterial cells (see above), yet still recover enough protozoa to prepare a suitable cell extract. Consequently, enzyme activities are reported herein as milliunits per 10^6 *T. termopsidis* cell equivalents, as determined by direct cell counts of suspensions before extract preparation. However, a numerical factor could be calculated for converting protozoan cell equivalents to milligrams of protozoan specific protein by the knowledge that *T. termopsidis* cells were approximately spheres of 40 ± 5 μm in diameter (see Fig. 4 of reference 21), and by the assumption that their density was 1.0, their water content was 80%, and protein constituted 50% of cell dry weight. By using these assumptions, it was estimated that 10^6 cells of *T. termopsidis* were equivalent to 3.4 mg of protein.

Chemicals. Cellodextrins (cellobiose through cellohexaose) were prepared by the acid hydrolysis of cellulose followed by column chromatography of the hydrolysate (18). All other chemicals were of reagent grade and were obtained from commercial sources.

RESULTS

Cell extracts of *T. termopsidis* possessed enzyme activity against carboxymethylcellulose and cellobiose, which indicated the presence of endo-β-1,4-glucanase and cellobiase, respectively (Table 1). Compared with freeze-thaw preparations, extracts prepared by treatment with Triton X-100 had greater total amounts of endoglucanase and cellobiase activities, as well as a greater percentage of these activities associated with the soluble fraction of extracts, although both procedures resulted in lysis of protozoan cells. This observation suggests that an appreciable amount of enzyme activity in freeze-thaw preparations remained masked, perhaps because it was associated with membranous subcellular organelles and was inaccessible to the substrates. Cellobiase activity was linear for at least 24 h (the maximum time tested), however endoglucanase activity decreased significantly after 1 h, at which time about 2% of the glycosidic bonds had been hydrolyzed.

Cell extracts of *T. termopsidis* also possessed hydrolytic activity against microcrystalline cellulose, although the rate of hydrolysis of cellulose was 3- to 10-fold less than that of carboxymethylcellulose or cellobiose (Table 1). The rate of hydrolysis of microcrystalline cellulose was linear for up to 24 h but declined slowly from 24 to 72 h. When low amounts of microcrystalline cellulose were used in reaction mixtures (i.e., final concentration, 0.04%), a maximum of 19.4% saccharification of the substrate was observed during a 64-h incubation period. However, most of this saccharification took place during the first 24 h.

All cellulolytic enzyme activities measured were proportional to the amount of cell extract used in the reaction mixture, were dependent on the presence of substrate, and were abolished by heating extracts to 100°C for 10 min. Moreover, control experiments showed that cellulolytic activity was associated only with the protozoa. No cellulolytic activities were detected in separate extracts of heat-killed *Bacteroides* sp. strain JW20 or viable *M. hungatii*, which were used in media to culture *T. termopsidis*.

Inasmuch as protozoan extracts contained small, but significant and variable, amounts of bacterial cell protein (and probably fetal bovine serum protein as well), the values reported in Table 1 were normalized to 10^6 protozoan cell equivalents. However, the total (soluble plus particulate) activity of any of the enzymes could be normalized to protozoan cell protein by assuming that 10^6 *T. termopsidis* cells were equivalent to 3.4 mg of protein (see above). Thus, for extracts prepared by treatment with, Triton X-100, the specific rates of substrate hydrolysis were approximately as follows (in milliunits per milligram of protein): microcrystalline cellulose (0.73); carboxymethylcellulose (8.4); and cellobiose (6.0).

Closer examination of the hydrolysis of microcrystalline cellulose by *T. termopsidis* extracts revealed apparent optima at pH 5 (Fig. 1) and at a temperature of 30°C (Fig. 2). Moreover, analysis of products formed from microcrystalline cellulose during a 72-h incubation period revealed that glucose (measured by reaction with glucose oxidase enzyme) was the only product, accounting for virtually all of the reducing sugar liberated (Fig. 3). During the course of cellulose hydrolysis, no cellobiose or other soluble cellodextrins were detected by high-pressure liquid chromatography (minimum detection limit, 0.25 mM glucosyl equivalents; data not shown).

Other substrates hydrolyzed by extracts of *T. termopsidis* included Douglas-fir wood powder, starch, and xylan (Table 2). Protease activity, as determined by azocasein hydrolysis, was also detected, although no activity against powdered chitin could be demonstrated (Table 2).

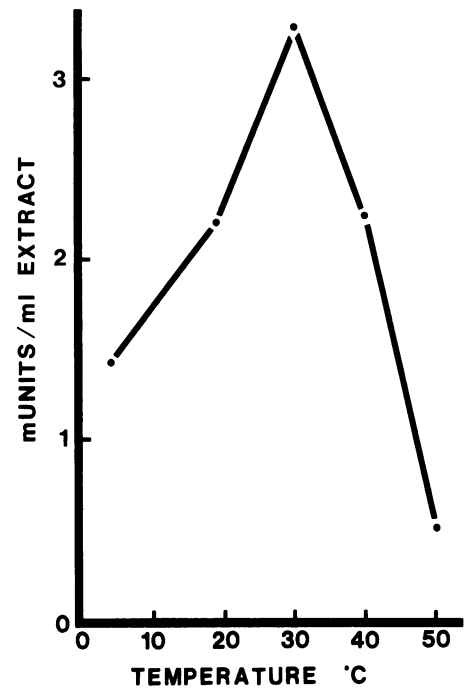

FIG. 2. Effect of temperature on the hydrolysis of microcrystalline cellulose at pH 5.0 by soluble (Triton X-100) extracts of *T. termopsidis* (equivalent to 4.0×10^6 cells per ml).

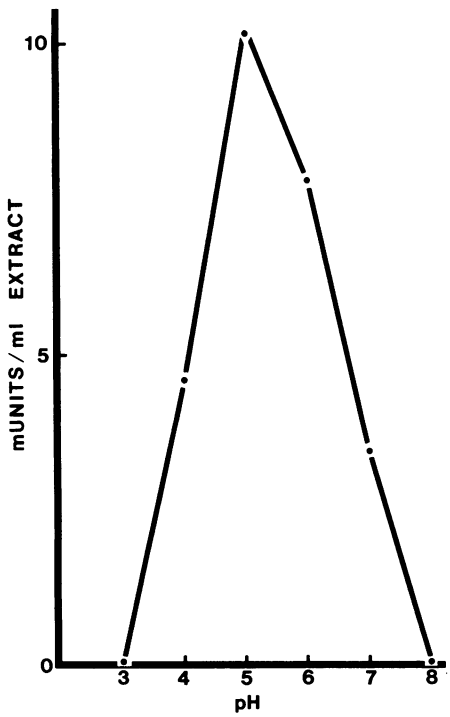

FIG. 1. Effect of pH on the hydrolysis of microcrystalline cellulose at 30°C by soluble (Triton X-100) extracts of *T. termopsidis* (equivalent to 7.3×10^6 cells per ml of extract).

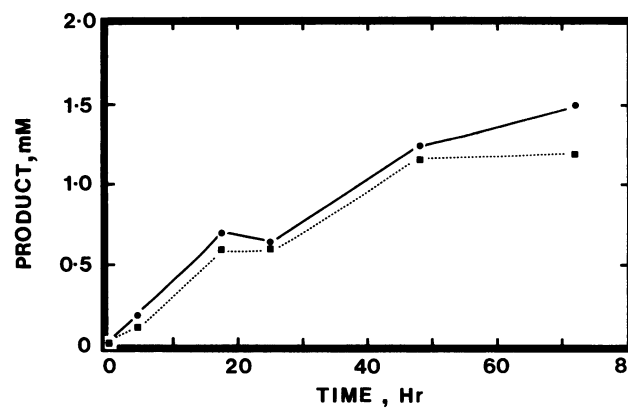

FIG. 3. Production of glucose (■) and reducing sugar (●) from microcrystalline cellulose by soluble (Triton X-100) extracts of *T. termopsidis* (1.1×10^6 cell equivalents per ml of reaction mixture).

TABLE 2. Hydrolytic activities of *T. termopsidis*

Substrate	Activity[a]
Potato starch	13.8 ± 4.8
Xylan	8.1 ± 1.2
Azocasein	5.3 ± 0.2
Douglas-fir powder	1.4 ± 0.2
Chitin	0

[a] Activity expressed as milliunits per 10^6 cell equivalents of soluble (Triton X-100) extract, except for azocasein which is micrograms hydrolyzed per minute × 10^6 cell equivalents of soluble (Triton X-100) extract. Values are the mean ± standard error of the mean of six determinations for $n = 1$.

DISCUSSION

Results presented herein indicate that *T. termopsidis* possesses at least two enzymes of the cellulase repertoire, endo-β-1,4-glucanase and cellobiase, as well as activity capable of hydrolyzing microcrystalline cellulose. Whether the latter activity reflects the presence of additional cellulolytic enzymes, such as cellobiohydrolase (EC 3.2.1.91), cannot be judged at present. Although no cellobiose was detected during or after extended (72 h) incubation of cell extracts with microcrystalline cellulose (Fig. 3), the cellobiase activity of such extracts (Table 1) could have obscured accumulation of the disaccharide to detectable levels.

Inasmuch as microcrystalline cellulose is endocytosed by *T. termopsidis* and dissimilated during growth of the protozoa in vitro (21, 28, 29), it was anticipated that cell extracts would effect a complete saccharification of the polymer. However, this was not the case. Only about 20% saccharification was observed. It may be that saccharification was limited by the intrinsic instability of one or more enzymes of the *T. termopsidis* cellulase system or by feedback inhibition of enzyme activity from products of cellulose hydrolysis. Nevertheless, our results are consistent with the hypothesis that *T. termopsidis* possesses enzymes capable of converting microcrystalline cellulose to glucose, a sugar that is used as a fermentable energy source for the cells. In fact, based on gas production rates of *T. termopsidis* growing in vitro (e.g., see Fig. 2 and 3 of reference 21), it can be estimated that cellulose hydrolysis must occur at a rate of at least 1.1 to 1.5 nmol of glucose units liberated per min × 10^6 cells (assuming that the gases are derived exclusively from cellulose decomposition). It was revealed in the present study that cell extracts hydrolyzed cellulose at rates of about 3 nmol of glucose units liberated per min × 10^6 cells, implying that we accounted for all of the cellulase activity of the protozoa. This observation also indicates that cellulase activity of *T. termopsidis* is entirely cell associated and is probably localized in intracellular food vacuoles. This notion is buttressed by the fact that no cellulase activity (25) or reducing sugars (21) are detectable in extracellular fluids of actively growing cultures, *T. termopsidis* endocytoses cellulose particles into food vacuoles (21, 28), and *T. termopsidis* will not grow on large disks of cellulose filter paper, which they are incapable of endocytosing (21).

Our results confirm and extend the work of Yamin and Trager (31), who also demonstrated endoglucanase and a β-glucosidase activity in crude extracts of *T. termopsidis*, but who did not report on the rates and characteristics of hydrolysis of microcrystalline cellulose by such preparations. In addition, our present studies reveal hydrolytic activity against wood particles (the natural substrate of the protozoa in vivo) and noncellulosic polymers that are also present in wood (xylan, starch, and protein). Protease activity may also assist in the digestion of bacterial cells, which are required for in vitro growth of *T. termopsidis* (21, 28) and which are probably grazed upon by these protozoa in vivo.

Although this study has focused on hydrolytic activities of *T. termopsidis*, it is not meant to obscure the probable importance of the termites themselves in cellulose decomposition in vivo. Indeed, pretreatment of cellulosic material by termites very likely enhances its degradability by hindgut protozoa. The grinding and chewing action of termite mouthparts reduces the initial substrate particle size, increases substrate surface area, and also probably disrupts the crystalline order of cellulose in much the same way as does ball milling (a treatment known to enhance the rate of enzymatic hydrolysis [9]). Secretion of cellulase from the salivary glands of some termites (26) could also augment the action of microbial enzymes (26). Thus, it is probably the combined action of both the termites and their gut microbiota which accounts for the fact that wood glucan dissimilation in these insects can be greater than 90% (8, 27).

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