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Inhibition of *Clostridium thermocellum* Cellulase by End Products of Cellulolysis¹

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Cellulase from *Clostridium thermocellum* was strongly inhibited by cellobiose, and to a much lesser extent by glucose, when acting on microcrystalline cellulose. This inhibition was partially relieved when β -glucosidase was added. The enzyme complex was much less susceptible to inhibition when acting on amorphous substrates such as phosphoric acid-swollen cellulose and resistant to inhibition when trinitrophenylcarboxy-methylcellulose was employed as the substrate.

INTRODUCTION

Our laboratory has recently shown (1) that the thermophilic, anaerobic bacterium *Clostridium thermocellum* secretes a true cellulase, capable of extensively saccharifying complex cellulosic substrates. This cellulase preferentially attacks the highly crystalline cotton and is less active on filter paper or Avicel, which contain higher proportions of $\beta + \gamma$ cellulose. On a broth volume basis, *C. thermocellum* extracellular cellulase degrades cotton more rapidly than reconstituted culture broth from *Trichoderma reesei* QM9414 or RUT-C30. When compared to *Trichoderma* on a crude protein basis, the superior activity of *C. thermocellum* is even more dramatic (Johnson and Demain, unpublished), which suggests that the bacterial cellulase is of relatively high specific activity when a low concentration of native cellulose is used as a substrate. On the other hand, the *T. reesei* preparations hydrolyze swollen cellulose or carboxymethylcellulose (CMC)⁴ at a faster rate than the clostridial preparation on a crude protein or broth volume basis.

Our early studies on the activity of the *C. thermocellum* filtrate on dyed-CMC (2) showed no inhibition by glucose or cellobiose. Ng and Zeikus (3) have reported lack of inhibition of activity on dyed-Avicel. Since cellobiose and glucose are the main products of cellulose hydrolysis by thermophilic clostridia under a variety of

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⁴ Abbreviations used: CMC, carboxymethylcellulose; FPU, filter paper units; DTT, dithiothreitol; TNP-CMC, trinitrophenylcarboxymethylcellulose.

conditions (1, 3-6), it was felt necessary to examine this apparent lack of inhibition using more realistic types of cellulose. In this report, we show that action on microcrystalline cellulose is strongly inhibited by cellobiose but only slightly by glucose.

MATERIALS AND METHODS

Sources of enzymes. Growth of *C. thermocellum* and preparation of cellulase was as described earlier (1). Briefly, *C. thermocellum* ATCC 27405 was grown anaerobically in fermentor culture at 60°C for 60-68 h using cellobiose or Solka-floc as substrate in CM-4 or GS-2 culture media (1). Cells were removed by centrifugation (18,000g for 15 min at room temperature) and the supernatant fluid (~0.2 mg protein ml⁻¹) was used as such or concentrated by ammonium sulfate precipitation and desalted by gel filtration on Biogel P-2.

A dried powder from *T. reesei* RUT-C30 (0.53 FPU mg⁻¹), supplied by M. Mandels, was prepared in 50 mM sodium citrate buffer, pH 4.8, at 9 FPU ml⁻¹ (7).

Measurement of cellulase activity. Cellulase activity was determined by decrease in turbidity (660 nm) of an Avicel suspension (type PH 105, 20- μ m particles, FMC Corp., Marcus Hook, Pa.) as described earlier (1). Avicel samples (3 mg) were suspended in Hungate tubes in 3 ml of 100 mM sodium succinate buffer, pH 5.8, 0.5 ml of 100 mM dithiothreitol (DTT), 0.5 ml of 1% CaCl₂ · 2H₂O, various volumes of enzyme and water to 5 ml. Activity was also determined by turbidity on phosphoric-acid swollen cellulose (prepared from Avicel according to Tansey (8)) suspended in 50 mM citrate buffer, pH 5.7. Activity on trinitrophenylcarboxymethyl-cellulose (TNP-CMC) and remalzol brilliant blue dyed cellulose was determined as described (2, 3). D(+)-Cellobiose was obtained from Sigma Chemical Company (St. Louis, Mo.) and anhydrous glucose from Anachemia (Champlain, N. Y.); when included in the incubations, these were dissolved in the appropriate buffer. β -Glucosidase (EC 3.2.1.21) from almonds was from Sigma.

For comparison of end product inhibition, *T. reesei* reconstituted broth (0.5 ml, 9 FPU ml⁻¹) was incubated in 3 ml of 50 mM sodium citrate buffer, 3 mg of Avicel, and water to 5 ml.

RESULTS

For effective breakdown of native cellulose, the clostridial cellulase requires calcium ion and a thiol reducing agent, of which DTT is the best of those tested (1). Under these conditions, 1 ml of broth supernatant fluid solubilizes low concentrations of cotton, Avicel, or filter paper at rates comparable to 1 ml of the *T. reesei* supernatant fluid (1). The use of a turbidity assay with powdered cellulose as substrate allows the determination of cellulase activity in the presence of reducing sugars and thiols, and measurement of enzyme activity by the decrease in turbidity of Avicel directly correlates with enzyme activity measured by loss in weight of cotton or filter paper (1).

When 50 μ g of crude, desalted extracellular *C. thermocellum* protein was incubated with phosphoric acid-swollen Avicel and various concentrations of cellobiose, no more than 50% inhibition was observed even at high (50 mg/ml) concentrations of cellobiose (Fig. 1). Much less inhibition was seen with glucose. These results agree with those found by Shinmyo *et al.* (2). However, when microcrystalline Avicel was used as the substrate, significant (35%) inhibition was observed

Avicel
0.6 mg/ml

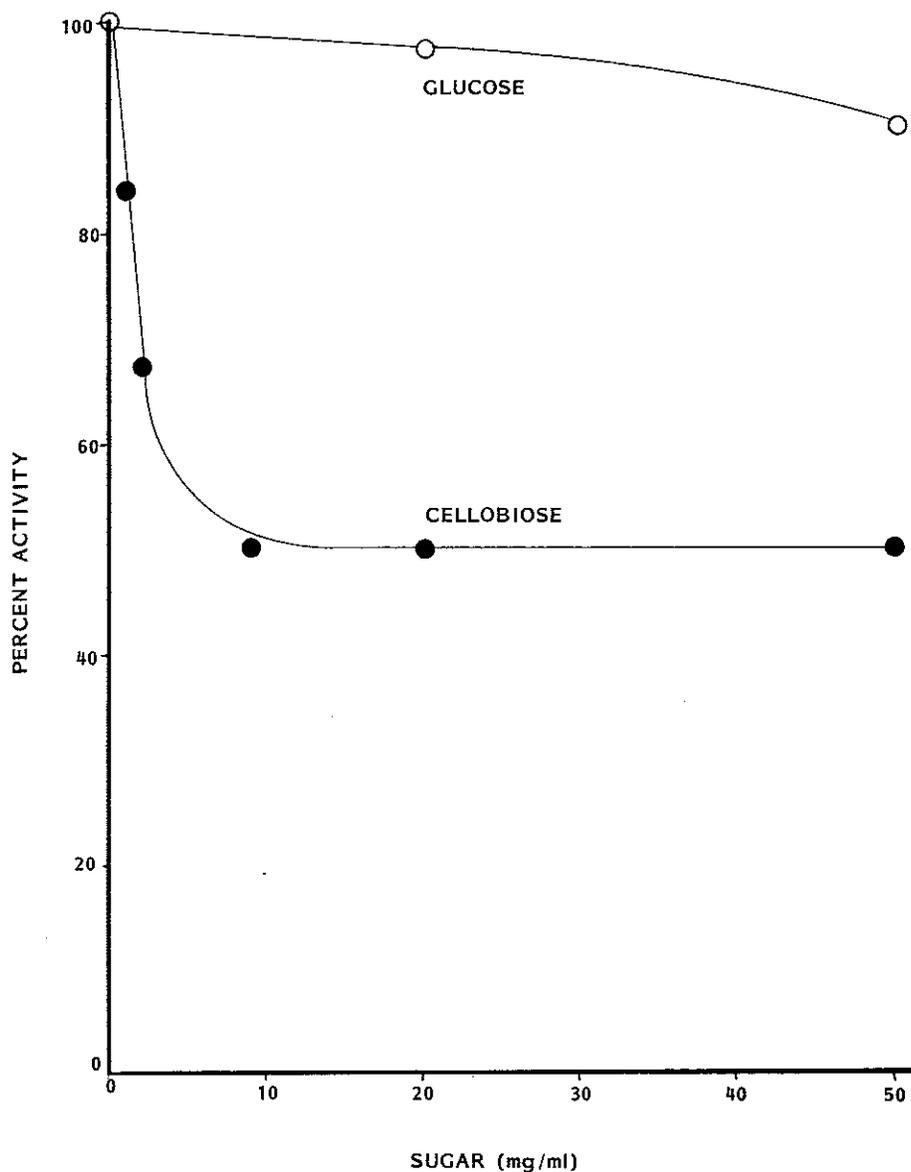


FIG. 1. Inhibition of *C. thermocellum* cellulase by cellobiose (●) or glucose (○) on phosphoric acid-swollen Avicel. Activity was measured by decrease in turbidity of a swollen cellulose suspension after 3 h incubation at 60°C. Fifty micrograms of crude desalted enzyme was used in this experiment.

at 1 mg/ml cellobiose (Fig. 2) and complete inhibition occurred at concentrations of 20 mg/ml cellobiose. Glucose produced only 35% inhibition even at 60 mg/ml. These experiments were also done using various volumes of untreated *C. thermocellum* culture broth as the enzyme source and similar inhibition patterns were observed (Fig. 3). In this experiment, the *T. reesei* RUT-C30 cellulase was also studied; it was less inhibited by cellobiose than the clostridial enzyme at equal broth volumes. The *T. reesei* cellulase, however, was more strongly inhibited by glucose than was the *C. thermocellum* enzyme.

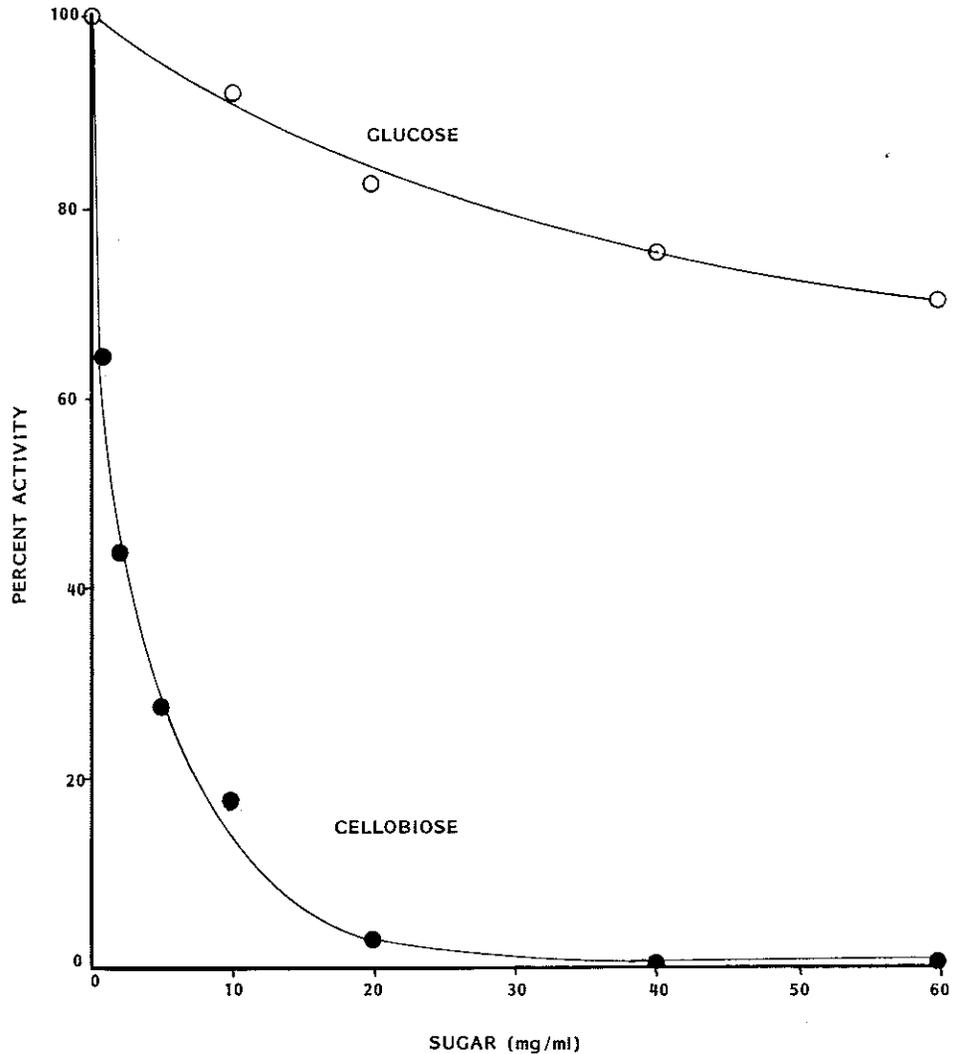


FIG. 2. Inhibition of *C. thermocellum* cellulase (50 μ g of crude desalted enzyme) by cellobiose (●) or glucose (○) acting on microcrystalline Avicel. Activity was measured after 18 h incubation at 60°C; maximal (100%) activity corresponds to the degradation of 0.9 mg Avicel.

As shown above, cellobiose but not glucose is an inhibitor of *C. thermocellum* cellulase activity. To determine whether β -glucosidase would benefit clostridial cellulase activity by destroying cellobiose, a β -glucosidase preparation was incubated (10 units/ml) with the bacterial cellulase at 45°C (Table I). The addition of β -glucosidase promoted a 16% increase in cellulose hydrolysis in the absence of added cellobiose and increased the activity fivefold in the presence of cellobiose.⁵ Glucose did not affect activity in the presence or absence of added β -glucosidase.

⁵ The comparatively small increase in the absence of added cellobiose is not unexpected since the total amount of cellobiose released over 24 h in the experiment without added cellobiose (variation 1, Table I) is less than a quarter of that present initially in the experiment with added cellobiose (variation 2, Table I). Thus, there is very much less inhibition in variation 1 than in variation 2 and consequently much less room for the stimulating effect of β -glucosidase in variation 3 than in variation 4.

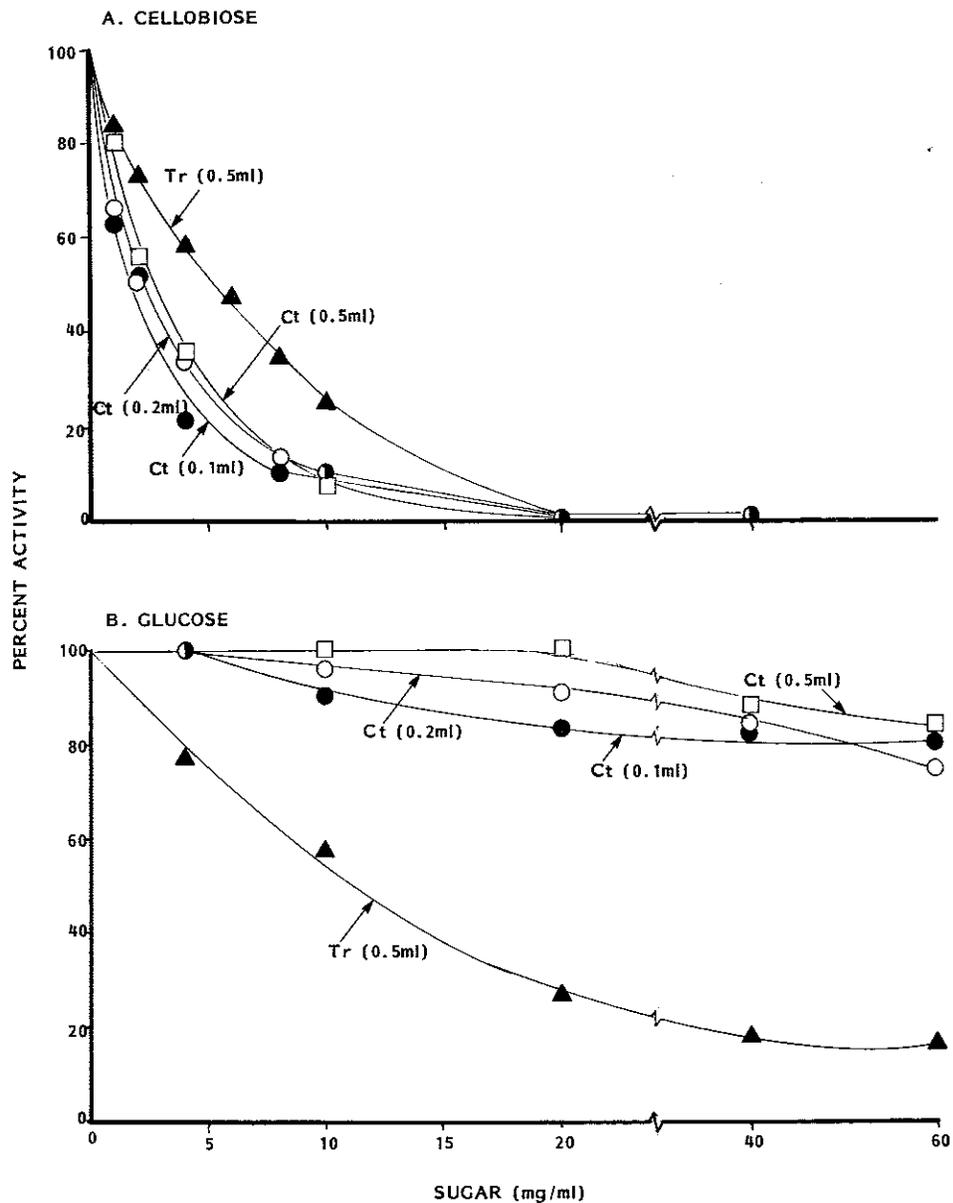


FIG. 3. Inhibition of cellulase activity in untreated culture broths of *C. thermocellum* (Ct) and *T. reesei* (Tr) by cellobiose (A) or glucose (B). Various volumes of culture broth were used. Avicel was the substrate and the duration of the experiment was 20 h. Maximal (100%) activity for *C. thermocellum* corresponds to the degradation of 0.73 mg Avicel (0.1 ml broth containing 20 μ g protein), 0.93 mg Avicel (0.2 ml broth; 40 μ g protein), and 1.7 mg Avicel (0.5 ml broth; 100 μ g protein). For *T. reesei*, 100% corresponds to 1.7 mg Avicel (0.5 ml broth containing 6 mg protein).

A number of sugars other than cellobiose and glucose were tested to see whether they inhibited *C. thermocellum* cellulase (Table II). Certain glucosides and galactosides, e.g., salicin, lactose, and arbutin, were fairly inhibitory, but none were as inhibitory as cellobiose.

TABLE I

Influence of β -Glucosidase on Cellulose Hydrolysis by *C. thermocellum* Cellulase^a

Variation	Additions				Activity (mg-Avicel hydrolyzed in 24 h)
	β -Glucosidase (10 units/ml)	Cellobiose (10 mg/ml)	Glucose (10 mg/ml)	Cellulase	
1	-	-	-	+	1.16
2	-	+	-	+	0.24
3	+	-	-	+	1.35
4	+	+	-	+	1.29
5	+	+	+	+	1.29
6	+	-	+	+	1.29
7	-	-	+	+	1.16
8	+	-	-	-	0

^a Reactions were done at 45°C in succinate buffer with Avicel as substrate as described under Materials and Methods.

DISCUSSION

Despite earlier observations that hydrolysis of dyed-CMC and dyed-Avicel by *C. thermocellum* cellulase was not inhibited by cellobiose or glucose (2, 3), we have found in the present work that hydrolysis of crystalline cellulose is inhibited by cellobiose. While this manuscript was in preparation, Petre *et al.* (9) reported that purified *endo*- β -glucanase of *C. thermocellum* is relatively insensitive to cellobiose using TNP-CMC as substrate. The effect of cellobiose is therefore dependent on the nature of the substrate. Reese *et al.* (10) first showed that cellobiose may have anomalous effects on cellulase activity depending on the substrate used and the incubation conditions; inhibition or even stimulation may occur with CMC or derived celluloses. Similar results have been obtained by Klyosov and Rabinovich (11).

An important limitation in cellulose hydrolysis is enzyme inhibition by cellobiose, especially when the substrate is highly crystalline and the enzyme preparation is low in β -glucosidase (12). With respect to cellobiose inhibition, our studies point to a similarity between *C. thermocellum* and *Trichoderma*. The inhibition of *Trichoderma* cellulase is competitive (12-14), and increases with resistance of the cellulose to breakdown (12). Addition of a β -glucosidase preparation of high specific activity to cellulose saccharification mixtures leads to cellobiose hydrolysis and thus alleviates the inhibition of the cellulase by its product (15, 16).

The sensitivities of the fungal and bacterial cellulases to glucose inhibition are strikingly different (Fig. 3); this may be the result of different β -glucosidase concentrations in the broths. β -Glucosidase is known to greatly enhance cellulose hydrolysis (15) and is present in low concentrations in *T. reesei* RUT-C30 (7). On the other hand, *C. thermocellum* is not known to produce an extracellular β -glucosidase although it possesses a periplasmic β -glucosidase (17) and a periplasmic cellobiose phosphorylase (18), which together convert cellobiose to glucose and glucose-1-phosphate. The improvement in cellulose saccharification observed in the presence of added β -glucosidase (Table I) suggests that it would be useful to develop strains which secrete cellobiase.

A potentially important finding in this study is that cellobiose analogs such as

TABLE II
Inhibition of *C. thermocellum* Cellulase by Various Carbohydrates^a

Inhibitor	Concentration (mg/ml)	Activity (%)
None	—	100
Glucose	40	76
2-Deoxyglucose	40	67
Glucose 1-phosphate	3	100
Methyl- β -D-glucoside	40	33
Gentiobiose	40	42
Maltose	40	62
Trehalose	40	83
Sucrose	40	100
Cellobiose	10	10
	20	0
	40	0
Lactose	10	67
	20	50
	40	17
	60	12
Arbutin	10	67
	20	51
	40	18
	60	12
Salicin	10	45
	20	36
	40	17
Xylan	3	100
Laminarin	3	100

^a Assays were done with Avicel as substrate. Maximum (100%) activity corresponds to 1.21 mg Avicel solubilization in 24 h.

salicin, arbutin, and lactose also inhibit *C. thermocellum* cellulase (Table II). It is interesting that the cellulase of *C. thermocellulaseum* is inhibited by cellobiose and lactose (19). Since salicin, arbutin, and lactose are not carbon sources for growth of *C. thermocellum* (2, 3, 20), our finding suggests that these compounds may be useful as selective agents for isolation of improved strains of *C. thermocellum*, i.e., inclusion of such a nonmetabolizable inhibitor in a growth medium containing crystalline cellulose as the major carbon source should select for mutants which are hypercellulase producers or producers of a modified cellulase less sensitive to cellobiose inhibition.

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