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Efficient Cellulose Solubilization by a Combined Cellulosome-β-Glucosidase System

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ABSTRACT

The cellulosome, the multienzyme complex of the cellulase system of *Clostridium thermocellum,* that mediates the solubilization of insoluble cellulose, is strongly inhibited by the major end product, cellobiose. By combining a purified β-glucosidase from *Aspergillus niger* with the cellulosome, accumulated cellobiose was hydrolyzed thereby resulting in a dramatic enhancement (up to 10-fold) of cellulose degradation. The observed enhancement was expressed both in the rate and degree of solubilization of microcrystalline cellulose, compared with that observed for the unsupplemented cellulosome. Near-complete conversion of cellulose to glucose could be obtained from dense substrate suspensions (up to at least 200 g/L).

Index Entries: *Clostridium thermocellum;* cellulosome; cellulase; cellulose, solubilization of; *Aspergillus niger;* Novozym; β-glucosidase; cellobiase.

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INTRODUCTION

For many years, the cellulase system of *Clostridium thermocellum* has been promoted for the industrial conversion of cellulose to glucose or other potentially useful products (e.g., ethanol). As the most prevalent example of an anaerobic thermophilic cellulolytic strain, this organism is considered particularly attractive for industrial application (*1*). Several approaches have been attempted. Some groups have tried to employ intact cell systems for this purpose. Others have utilized crude cell-free extracts or partially purified cellulase preparations. Both strategies have essentially failed. On the one hand, cells of *C. thermocellum* are sensitive to relatively low concentrations of ethanol (one of the major and the preferred product). On the other hand, both crude and purified enzyme preparations are subject to potent inhibition that leads to ineffectual solubilization of cellulosic substrates.

An important measure of the efficacy of cellulose degradation has been described and termed "true" cellulase activity, or the capacity to completely solubilize microcrystalline cellulose (*2*). In *C. thermocellum*, the major portion of true cellulase activity resides in the cellulosome, a high-mol wt, multienzyme protein complex (*3–5*). However, the capacity of this complex to degrade high concentrations of cellulose is limited; in the past, only the solubilization of relatively dilute suspensions of cellulose has been reported (*2,6,7*).

In earlier works, Demain and colleagues have shown that a crude cellulase preparation can be inhibited by cellobiose (*8*). In a similar vein, we demonstrated (*6*) that the true cellulase activity of the purified cellulosome is also inhibited by cellobiose (the major end product). In this communication, we show that a purified fraction of a commercially available β-glucosidase from *A. niger* counteracts the observed cellobiose-induced inhibition of cellulose degradation by the cellulosome of *C. thermocellum*. This phenomenon results in the near complete conversion of microcrystalline cellulose to glucose at concentrations of at least 200 g/L employing 5–10 mg of the combined enzyme preparation/g of cellulose.

EXPERIMENTAL

Materials

Avicel PH 105 (microcrystalline cellulose) was a product of FMC (Philadelphia, PA). Crude β-glucosidase (Novozym 188) was kindly provided by Eilat Reisner, the local agent of Novo Industries a/s (Bagsvared, Denmark).

Purified cellulosome fractions were obtained from the cell-free broth of cellulose-grown cells of *C. thermocellum* strain YS as described previously (*9*). The 2-step procedure consisted of affinity chromatography on cellulose and gel filtration on a Sepharose 4B column.

DEAE-cellulose (DE 52) was purchased from Whatman Ltd., (Kent, UK). Cysteine-HCl, cellobiose, glucose, *p*-nitrophenyl β-glucopyranoside, and the glucose determination kit (No. 510) were obtained from the Sigma Chemical Co. (St. Louis, MO).

Enzymatic Activities

The PNPG Assay

This assay was used to rapidly assess the presence of β-glucosidase activity during the purification procedure for this enzyme. Samples were incubated for 30 min with a 1-mL solution containing *p*-nitrophenyl-β-D-glucopyranoside in 50 m*M* sodium acetate buffer (pH 5) at 60°C. The reaction was terminated upon addition of 30 μL of 2 *M* Na_2CO_3. The absorbance at 400 nm was measured. A unit of PNPGase activity is defined as the number of micromoles of *p*-nitrophenol ($\epsilon_{400} = 13901\ M^{-1}cm^{-1}$) formed/min.

Cellobiase Activity

The assay was performed under conditions coincident with those required for efficient solubilization of cellulose by the cellulosome. The assay was carried out at 60°C under nitrogen for 60 min. The assay mixture consisted of 2 m*M* EDTA, 10 m*M* $CaCl_2$, 5 m*M* cysteine and 2% cellobiose in 50 m*M* sodium acetate buffer, pH 5.0. The amount of glucose formed from cellobiose was determined using the combined glucose oxidase/peroxidase system. A unit of activity is defined as the number of micromoles of cellobiose converted to glucose/min.

Cellobiose Inhibition Assay

The assay is based on the capacity of cellobiose to inhibit "true cellulase" activity in the cellulosome (*6*). The solubilization of cellulose was determined turbidometrically by a procedure modified from Johnson et al. (*2*). The reaction was conducted with continuous shaking in horizontally oriented rubber bung-sealed 13-mm test tubes under nitrogen atmosphere. A suspension (3 mL) of Avicel (0.06%) in 50 m*M* sodium acetate buffer (pH 5.0), containing 2 m*M* EDTA, 10 m*M* $CaCl_2$, and 5 m*M* cysteine-HCl, was incubated for 26 h at 60°C. The suspension was brought to a concentration of 5 μg/mL of cellulosome. Turbidity was measured at the indicated times at 660 nm by inserting the incubation tube into a Spectronic 20 spectrophotometer (Milton Roy Analytical Products Div., Rochester, NY).

The degree of solubilization in a given sample was calculated as the ratio of turbidity decrease to the initial turbidity of the Avicel suspension. In inhibition experiments, cellobiose was added at the indicated concentrations.

Purification of β-Glucosidase

DEAE-Cellulose Chromatography

Novozym (600 mg) was diluted with 1 L of 10 m*M* potassium phosphate buffer, pH 6.4 (phosphate buffer) and DEAE-cellulose (50 mL resin preswollen in the same buffer) was added. The suspension was stirred for 1 h at 4°C. The supernatant fluids were decanted, and the DEAE-cellulose was packed in a column (1.5×28 cm). The column was then washed with 200 mL of the phosphate buffer. A linear gradient of increasing NaCl concentration was applied by means of two reservoirs, one containing 150 mL of phosphate buffer and the other containing 150 mL of 0.5 *M* NaCl in the same buffer. Fractions of 4.0 mL were collected at a rate of 24 mL/h. The peak of β-glucosidase (determined by the PNPG assay to elute at about 0.2 *M* NaCl) was pooled and concentrated to 1 mL by ultrafiltration (Omegacell™ unit membrane, 10 K nominal mol wt limit; Filtron Technology Corp., Clinton, MA).

Gel Filtration Chromatography

The DEAE-cellulose fraction was applied to a Sephacryl S200 column (1.5×82 cm), equilibrated in phosphate buffer, containing 0.05% sodium azide. Fractions (2 mL) were collected at a rate of 40 mL/h. The β-glucosidase-containing peak was pooled and dialyzed against 0.1 *M* sodium acetate buffer, pH 5.0. The specific cellobiase activity of the purified enzyme was determined to be 273 units/mg enzyme.

Miscellaneous Methods

Protein was determined by the Bradford method (*10*). Reducing sugars were determined by the DNS method (*11*) and calibrated using either glucose or cellobiose as indicated below. Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate was performed in 6 or 10% gels essentially as described previously (*12*).

Bulk Solubilization of Cellulose

A 6-mL suspension of microcrystalline cellulose at the desired concentration was prepared in 11-mL rubber bung-sealed serum bottles using 50 m*M* sodium acetate buffer (pH 5.0), containing 2 m*M* EDTA, 10 m*M* $CaCl_2$ and 5 m*M* cysteine-HCl. A stock solution of the cellulosome was introduced to give a ratio of 8 mg per g substrate. This ratio of cellulosome-to-substrate was previously determined to provide maximal levels of activity (*6*). When required, purified β-glucosidase was added at 86 PNPG U/g substrate (equivalent to 42 cellobiase U/g substrate). For experiments using

20% cellulose, a second dose of the combined enzyme system was applied at the desired time interval. The bottles were flushed with nitrogen gas, and the incubation was carried out with shaking at 60°C. The reaction bottles were sampled anaerobically, using a syringe equipped with an 18-gauge needle. Assay of reducing sugars was performed after removal of residual cellulose by centrifugation. For reaction mixtures containing β-glucosidase, calibration of the assay was performed with a glucose standard, since the final (and only) detectable product was glucose; for reaction mixtures lacking β-glucosidase, cellobiose (by far the predominant product of cellulose action) was used for calibration. The above-described assessment of cellulose degradation was complemented by turbidometric measurements of appropriate dilutions (see section on cellobiose inhibition assay) of the respective reaction mixtures.

RESULTS

Inhibition of Cellulosome by Cellobiose

In previous work (*6*), we have shown that the solubilization of cellulose by the purified cellulosome of *C. thermocellum* strain YS undergoes near-complete inhibition by 2% cellobiose. This study had been prompted by an earlier finding by Johnson et al. (*8*) who showed a similar inhibitory effect of cellobiose on the crude cellulase system in a different strain of *C. thermocellum*.

The phenomenon of cellulase inhibition by end products of cellulolysis was examined by analyzing the decrease in cellulose turbidity (Fig. 1A). This particular assay system is considered to be a measure of the "true cellulase activity" of the given enzyme preparation. In these experiments, the measurement of reducing sugar by the DNS procedure would be inapplicable because of the cellobiose or glucose (as potential inhibitors) added exogenously to the reaction mixture.

Using this system, half-maximal inhibition was reached at exogenous cellobiose concentrations of about 0.1% (Fig. 1B). Using the same assay system, only nominal levels of inhibition were observed upon addition of equivalent levels of glucose, similar to the results reported for the crude cellulose system of strain ATCC 27405 (*8*).

Purification of *A. niger* β-Glucosidase

Since cellobiose (the major product of cellulosome action on cellulose) appeared to be the major cause for inhibition, we considered converting the cellobiose produced to glucose using an appropriate enzyme. This would especially be important for industrial application where hydrolysis of high concentrations of substrate would be required. We therefore looked for a suitable source of enzyme.

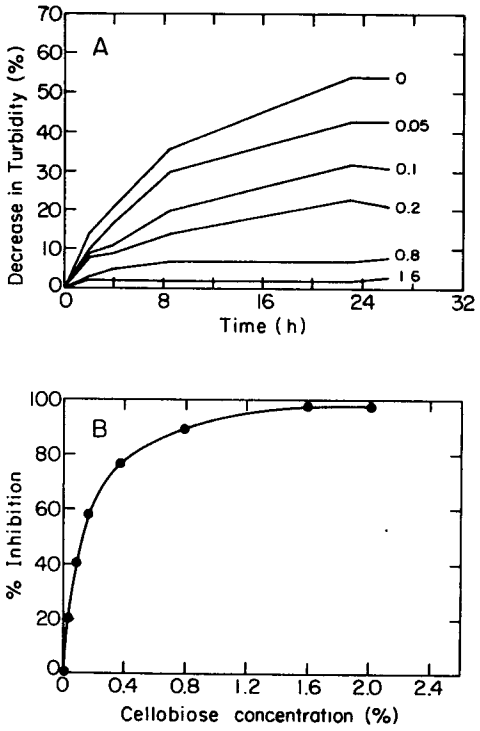

Fig. 1. Cellobiose-induced inhibition of true cellulase activity in the cellulosome. Panel A shows the decrease in turbidity generated by the action of the cellulosome either alone (0) or in the presence of the indicated concentrations (in %, wt/vol) of cellobiose. Note that both the initial rate and the degree of solubilization of the substrate are severely affected by the addition of cellobiose. Panel B shows the extent of inhibition of cellulose degradation after incubation at 24 h as a function of the concentration of cellobiose.

Novozyme, a crude preparation from culture filtrates of the fungus *A. niger,* is commercially available from Novo Industries, and a simple 2-step procedure was developed in this work for the purification of the β-glucosidase. A similar procedure was recently described by McCleary and Harrington (*13*).

The protocol consisted of DEAE-cellulose ion-exchange chromatography followed by gel filtration on a Sephacryl S-200 column (Table 1). The active fractions from each step and the crude enzyme were subjected to SDS-PAGE. As shown in Figure 2, the purified enzyme displayed a single polypeptide band. The mol wt, as estimated by SDS-PAGE, was 116,000.

In order to further assess the suitability of the purified enzyme to the thermophilic reaction conditions consistent with the cellulosome action on cellulose, various stability properties of the β-glucosidase were examined. Thus, the activity of the purified enzyme was determined after incubation

Table 1
Purification of the β-glucosidase from Novozym

Fraction	Total Activity (PNPG units)*	Total Protein (mg)	Specific Activity (units/mg)
Novozym	37650	600	65
DEAE-Cellulose	21800	78	286
Sephacryl S200	5600	10	554

* One PNPG unit is equivalent to 0.493 cellobiase U (micromole glucose/min, 60°C).

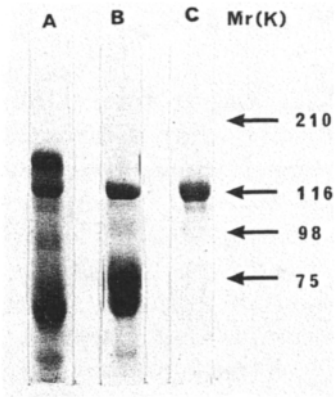

Fig. 2. Polypeptide profile of β-glucosidase preparations following the major steps of the purification procedure. Lane A, crude commercially obtained enzyme (Novozym); Lane B, DEAE-cellulose fraction; Lane C, purified enzyme.

for different time periods (up to 72 h) at 60°C (pH 5) in the presence or absence of 1% glucose (Fig. 3). More than 60% of the activity was retained after 72 h when the enzyme was incubated in the presence of glucose. In the absence of the sugar, a significant decrease in the stability of the enzyme was observed under the above-described conditions; after 24 h, only about 30% of the original activity remained.

Enhancement of Avicelase Activity by the Combined Enzyme System

The effect of β-glucosidase addition to the reaction mixture was tested by measuring reducing sugar either turbidometrically or using the DNS method (*11*). This was accomplished employing up to 200 mg Avicel/mL reaction mixture, the amount of cellulosome, and β-glucosidase applied was proportional to the concentration of Avicel.

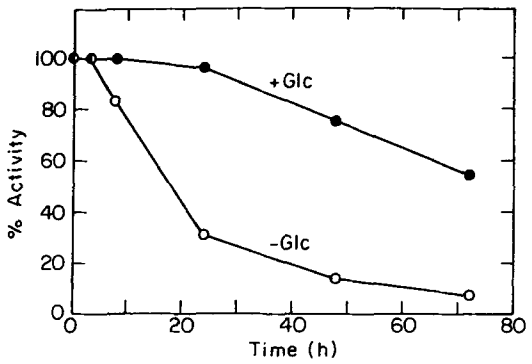

Fig. 3. Stability of purified β-glucosidase under conditions compatible with those required for efficient cellulosome action. The purified enzyme was incubated in the presence (+Glc) or absence (−Glc) of 1% glucose at 60°C (pH 5.0) in 0.1 *M* sodium acetate buffer, containing 2 m*M* EDTA, 10 m*M* $CaCl_2$ and 5 m*M* cysteine.

The addition of β-glucosidase to the reaction mixture increased the level of cellulose solubilization (Fig. 4). At 20 mg of substrate/mL, the addition of β-glucosidase enhanced the level of solubilization about 2-fold. A 3-fold enhancement was observed at 50 mg of substrate/mL. Complete solubilization in this case was evident after 96 h in the presence of β-glucosidase, compared to only about 30% in the absence of the enzyme. The most striking result was observed at 200 mg of substrate/mL reaction mixture; in this case, a 10-fold enhancement was observed. At such high concentrations of substrate, however, a fresh addition of the combined enzyme system was required at an intermediate stage of the process to achieve complete solubilization (*see* arrow, Fig. 4C). In most cases, the β-glucosidase caused the enhancement in both the initial rates and degree of solubilization. It is interesting to note that independent of the initial concentration of cellulose substrate in the reaction mixture, the final level of solubilization effected by the cellulosome alone (without added β-glucosidase) was surprisingly constant (about 15 mg/mL). Similar results were obtained employing crude β-glucosidase (Novozym) to give equivalent levels of activity as described above for the combined enzyme system containing the purified β-glucosidase preparation.

DISCUSSION

Recent advances in the understanding of the cellulolytic system of *C. thermocellum* have led to the renewed interest in its cellulases, and in particular to the cellulosome, as a practical means for the conversion of cellulose to soluble sugars. The theoretical cellulolytic potential of this bacterial system has been estimated to be about 50-fold better than that of funal systems (2), although their utilization for industrial processes has yet to be realized.

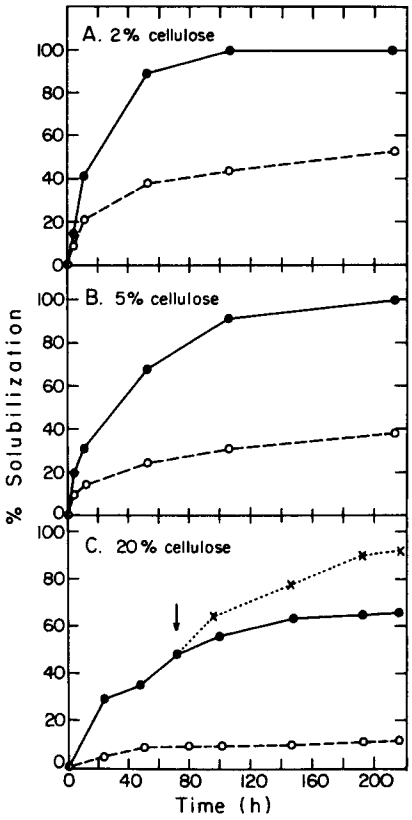

Fig. 4. Solubilization of various concentrations of cellulose by the combined cellulosome-β-glucosidase system vs that of the cellulosome alone. The figures show the time course of solubilization of the indicated concentrations of cellulose either by a combination (●) of the cellulosome (8 μg/mg cellulose) and the β-glucosidase (0.04 cellobiase U/mg substrate), or by an equivalent amount of the cellulosome alone (○). At the highest level of cellulose concentration (Panel C), the extent of solubilization reached a plateau at about 60%. The reaction could be increased to near completion (X) by the introduction (arrow) of a second sample of the combined enzyme system.

In order to achieve optimal levels of product formation, it is imperative to understand the key requirements for this system. In this regard, it has previously been demonstrated that the solubilization of cellulose, either by the crude cellulase system of *C. thermocellum* (*2,8*) or by the purified cellulosome (*6*), is highly dependent upon the conditions of its medium. The presence of Ca^{2+} and reducing conditions (e.g., thiols) markedly enhances the degradation of the insoluble substrate. Even more critical is the status of the major enzymatic end product, cellobiose, the accumulation of which results in the potent inhibition of the solubilizing action. Despite such modulating effects on the degradation of microcrystalline cellulose, the latter substances do not affect the enzymatic activity on a

variety of modified substances (e.g., CMC, dyed cellulosics or amorphous cellulose).

In designing a process for efficient enzymatic degradation of cellulose, the major consideration is to rid the system of the resultant cellobiose so as to prevent feedback inhibition of the cellulase apparatus. The cell apparently does so in nature by a combination of mechanisms (*1*). Firstly, the substrate is degraded extracellularly by means of polycellulosomal protuberances; part of the product is converted to glucose by the resident membrane-associated β-glucosidase, the glucose and the residual cellobiose are then incorporated intracellularly via the appropriate transport system. Moreover, additional amounts of cellobiose may be utilized in nature by other saccharolytic (but non-depolymerizing) satellite bacterial strains that occupy the same ecosystem. In this way, cellobiose is effectively removed from the environment and the cellulase system (specifically the cellulosome) continues to degrade the substrate efficiently. In the cell-free state, the cellulosome produces saturating levels of cellobiose, and cellulolysis is thus inhibited.

Conceptually, the combination of a cellulase system with β-glucosidase is not new. This approach has been tried in the past using fungal systems with varying degrees of success (*15–18*). Different strategies have been examined, including the batchwise mixture of the enzymes, the recycling of cellulase reaction products through immobilized β-glucosidase, and co-immobilization of both cellulase and β-glucosidase components. Nevertheless, these processes were economically prohibitive, since catalytic efficiencies were extremely low; very high enzyme-to-substrate ratios were required to effect high levels of solubilization. In another recent example where a bacterial system was employed, Kadam and Demain (*7*) combined a cloned β-glucosidase from *C. thermocellum* ATCC 27405 with the crude cellulase system from the same strain and demonstrated the enhanced degradation of relatively low concentrations of cellulose.

The major contribution of the present study is that a purified cellulolytic entity, the cellulosome of *C. thermocellum,* can be combined with a readily available β-glucosidase (Novozym) to effect efficient solubilization of very high substrate concentrations. In doing so, we employed relatively low concentrations of the respective enzymes compared with the fungal system. In applying the cellulosome system for naturally occurring cellulosics, it is noteworthy that the cellulosome already comprises numerous xylanases among its components that complement the inherent cellulolytic activity thus effecting concomitant solubilization of hemicellulose (*19,20*). Indeed, preliminary experiments led to significant solubilization of dried alfalfa using the combined cellulosome-β-glucosidase system. In future applications, we plan to extend these studies by combining other enzymes with the cellulosome system for the improved solubilization of dense concentrations of natural cellulosic substrates.

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