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Cellobiose oxidase of *Phanerochaete chrysosporium* enhances crystalline cellulose degradation by cellulases

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The effect of *Phanerochaete chrysosporium* cellobiose oxidase (CBO) on microcrystalline cellulose hydrolysis by *Trichoderma* cellulases was determined. Addition of 10 $\mu g \cdot ml^{-1}$ CBO to a reaction mixture containing *T. viride* cellulase increased glucose and cellobiose production by 10% and 48%, respectively. Cellulose weight loss was also enhanced by 19%. At higher concentrations (20–80 $\mu g \cdot ml^{-1}$), CBO decreased glucose and cellobiose production. Cellulose weight loss at 60 $\mu g \cdot ml^{-1}$ CBO was 76% compared to control cellulase reactions. This decrease appears to be due to inactivation of cellulase by H_2O_2 produced via CBO reaction, because addition of catalase enhances sugar production and cellulose weight loss. These findings suggest that at low, perhaps physiologically relevant concentrations, CBO enhances crystalline cellulose degradation by cellulases.

Cellobiose oxidase; Crystalline cellulose; Cellulose degradation; Cellulase; *Phanerochaete chrysosporium*; *Trichoderma viride*

1. INTRODUCTION

Cellulose constitutes 40–60% of woody plant cell walls and its bioconversion to fuels and chemicals is of great interest [1]. The cellulase systems of several fungi have been studied intensively [2]. Some cellulolytic fungi produce extracellular cellobiose-oxidizing enzymes in addition to cellulases [3–8]. However, the role of these enzymes in cellulose degradation is not understood. *Phanerochaete chrysosporium* produces two cellobiose-oxidizing enzymes, cellobiose oxidase (CBO) and cellobiose:quinone oxidoreductase (CBQase) [3,4]. CBO is a hemoflavoprotein and appears to require molecular O_2 for activity [3], whereas CBQase is a flavoprotein and requires a quinone for activity [4]. Both enzymes oxidize cellobiose to cellobionolactone. Recently, cellobiose dehydrogenase from *Sporotrichum (chrysosporium) thermophile* has also been demonstrated to be a hemoflavo enzyme [5]. We demonstrated that CBO binds to microcrystalline cellulose and suggested that it may be organized into two domains, a cellulose-binding domain and a catalytic domain [9]. Previously only cellulases were known to have this structural organization [10,11]. In this study, the effect of CBO addition on *Trichoderma viride* and *Trichoderma reesei* cellulase-catalyzed hydrolysis of microcrystalline cellulose is investigated. Our findings suggest that CBO enhances crystalline cellulose hydrolysis by these cellulases.

Abbreviations: CBO, cellobiose oxidase; FPLC, fast protein liquid chromatography.

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2. MATERIALS AND METHODS

2.1. *Cellobiose oxidase*

P. chrysosporium strain OGC 101 [12] was grown with cotton linters (10 $g \cdot l^{-1}$) in agitated cultures as described previously [9]. CBO was purified from the extracellular medium by a procedure involving ammonium sulfate precipitation and DEAE-Sephadex, Phenyl Sepharose, Sephacryl S-200 and Mono-Q (FPLC) chromatography (Bao, Usha and Renganathan, unpublished results). The purified CBO (M_r ~90,000) was homogeneous as judged by SDS-polyacrylamide gel electrophoresis and did not contain any contaminant proteins. The specific activity of the purified enzyme, as determined with ferricytochrome *c* as the electron acceptor was 9.7 $U \cdot mg^{-1}$.

2.2. *Estimation of glucose, cellobiose and cellobionolactone*

Glucose was estimated by the glucose-6-phosphate dehydrogenase assay [13]. The sample containing glucose (10 μl) was incubated with ATP (1 μmol), $MgCl_2$ (1 μmol), hexokinase (0.3 U) and Tris–HCl (25 μmol) in a total volume of 0.3 ml at 37°C for 15 min. The reaction was arrested by incubating the sample at 100°C for 2 min. The heat-treated sample was then incubated with NADP (0.5 μmol), $MgCl_2$ (20 μmol), Tris–HCl (100 μmol) and glucose-6-phosphate dehydrogenase (0.5 U) in a total volume of 2 ml, at 37°C for 30 min. The amount of NADPH formed was determined spectrophotometrically at 340 nm. Concentration of glucose was determined from a standard curve.

Cellobiose concentration was estimated using a CBO assay. A sample (5 μl) containing cellobiose was incubated with CBO (1 μg, 0.01 U) and cytochrome *c* (37.5 μM) in 20 mM succinate, pH 4.5, at room temperature for 30 min. The amount of reduced cytochrome *c* formed was determined at 550 nm. Cellobiose concentration was determined from a standard curve.

Cellobionolactone was estimated by modification of a previously reported method [14]. The lactone containing solution (50 μl) was reacted with 25 mM periodate in 0.35% H_2SO_4 solution (0.1 ml) at room temperature for 30 min. Sodium metabisulfite (1 M, 50 μl) was then added to decompose excess periodate. After 5 min, 0.1 ml of

2,3,4-trihydroxybenzoic acid (10 mg·ml^{-1}) was added, followed by 2.7 ml of concentrated H_2SO_4. Finally, 20 μl each of ammonium sulfate (0.5 g·ml^{-1}) and ferric nitrate (10 mg·ml^{-1}) was added and the solution was heated at 40°C for 30 min. The blue color which developed was measured at 590 nm.

2.3. *Microcrystalline cellulose hydrolysis by* Trichoderma *cellulases—Effect of CBO*

Microcrystalline cellulose (20 mg, Sigmacell type 50) was incubated with crude, dialyzed cellulase from *T. viride* or *T. reesei* (0.5 mg) in 2.5 ml of 50 mM acetate buffer (pH 5) at 28°C with shaking (150 rpm) for 18 h. Tetracycline (10 μg·ml^{-1}) was included in these incubations to inhibit bacterial growth. At the end of the incubation, the reaction was stopped by heating the reaction mixture at 100°C for 2 min, and the reaction mixture was then centrifuged at 12,000 × *g*. The amounts of glucose, cellobiose and cellobionolactone in the supernatant were determined. The conditions for determining the effect of CBO on cellulose hydrolysis were identical except that 5–80 μg of CBO were added.

For determining the cellulose weight loss, cellulose (600 mg) was incubated with crude *T. viride* cellulase (6 mg) and 0, 0.3 mg or 1.8 mg CBO in 30 ml 50 mM acetate, pH 5, as described above. The concentration of catalase, when added to these incubations, was 0.15 mg. At the end of the incubation, the reaction mixture was filtered through a pre-weighed Buchner funnel with medium porosity fritted disk to remove the unhydrolyzed cellulose. The funnel containing the cellulose was dried in an oven at 100°C, cooled, and weighed to determine the cellulose weight loss. Quadruplicate samples were used in each experiment.

2.4. *Chemicals*

All chemicals were reagent grade. Cytochrome *c* microcrystalline cellulose (Sigmacell type 50), and cellulases were purchased from Sigma Chemical Company, 2,3,4-Trihydroxybenzoic acid was obtained from Aldrich Chemical Company.

3. RESULTS

Low concentration of CBO (5–10 μg·ml^{-1}) enhanced glucose and cellobiose generation in *Trichoderma* cellulase catalyzed hydrolysis of microcrystalline cellulose (Fig. 1). When CBO (10 μg·ml^{-1}) was added to *T. viride* cellulase, glucose production was increased by approximately 10%, whereas cellobiose yield was increased by 48% (Fig. 1A). Similarly, glucose and cellobiose production by *T. reesei* cellulase increased by approximately 10% and 20%, respectively, in the presence of low concentrations of CBO (Fig. 1B). However, at higher concentrations of CBO, reducing sugar production by *T. viride* and *T. reesei* cellulases decreased (Fig. 1). This decrease was due to the CBO-catalyzed oxidation of cellobiose to cellobionolactone.

Cellobionolactone was estimated using an extensive modification of a previously reported colorimetric method [14]. This method is not specific for cellobionolactone in that other sugar lactones, such as gluconolactone, also give a positive response. The crude cellulase preparations used in these experiments possibly contained glucose oxidase as a contaminant resulting in the generation of gluconolactone as one of the cellulose degradation products. Therefore, to determine the amount of cellobionolactone formed with CBO, lactone produced in the absence of CBO was subtracted from the total lactone formed in the presence of CBO. In the *T. viride* cellulase reaction, cellobionolactone generation increased slowly between 10–40 μg of added CBO, and rapidly above the 40 μg level (Fig. 1A). In the case of *T. reesei*, cellobionolactone yield increased linearly as the CBO concentration in the reaction was increased (Fig. 1B).

The effect of CBO (10 μg or 60 μg ml^{-1}) addition on cellulose weight loss was also determined (Table I). In

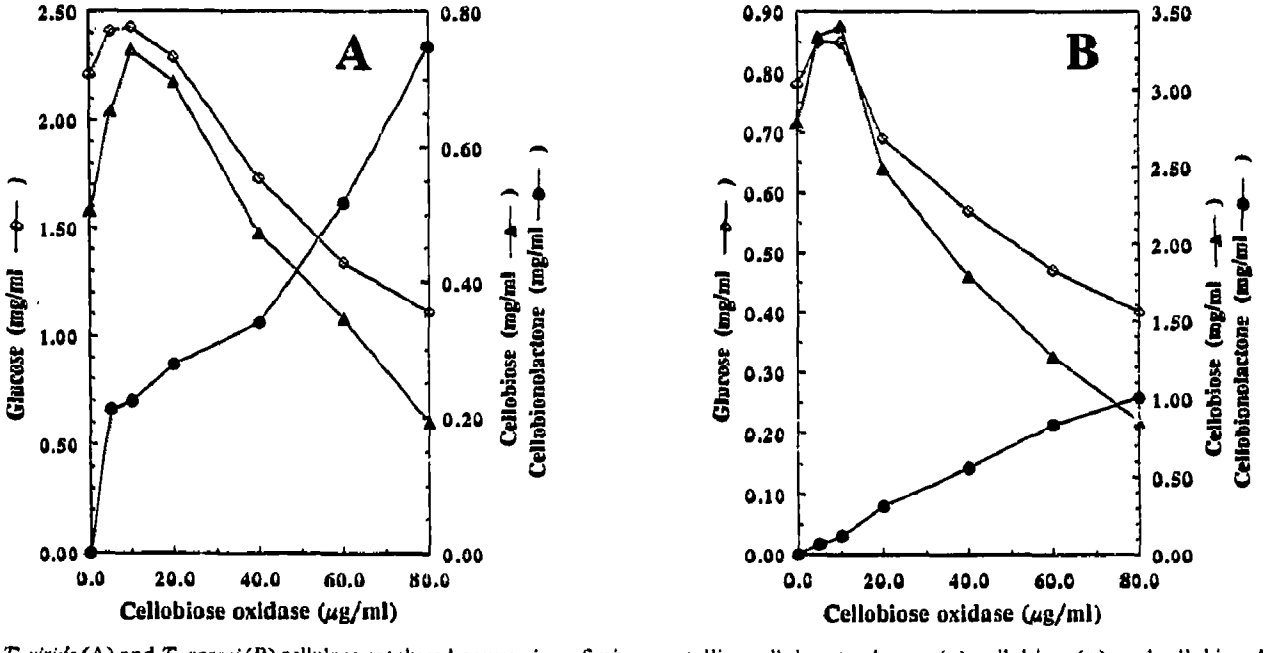

Fig. 1. *T. viride* (A) and *T. reesei* (B) cellulase catalyzed conversion of microcrystalline cellulose to glucose (◇), cellobiose (▲), and cellobionolactone (●), in the presence of varying concentrations of CBO.

the presence of 10 $\mu g \cdot ml^{-1}$ of CBO, cellulose weight loss was increased by approximately 19%. Inclusion of 5 $\mu g \cdot ml^{-1}$ catalase in this reaction did not increase the weight loss further. In the presence of 60 $\mu g \cdot ml^{-1}$ CBO, the weight loss decreased to 76% compared to control cellulase incubations. However, catalase addition further increased this weight loss to 94.8%. Catalase alone, in the absence of CBO, did not increase the weight loss.

4. DISCUSSION

Although Ayers et al. discovered CBO in the cellulose-degrading cultures of *P. chrysosporium* in 1978 [3], the role of CBO in cellulose degradation is still not well understood. One approach to determining the contribution of CBO to cellulose degradation is to study the effect of CBO addition on cellulose hydrolysis by cellulolytic systems such as those from *T. viride* and *T. reesei* which apparently do not contain CBO. Following such an approach, we have established that CBO enhances crystalline cellulose degradation by *Trichoderma* cellulases (Fig. 1, Table I). At low concentrations (5–10 $\mu g \cdot ml^{-1}$), CBO increases both glucose and cellobiose production (Fig. 1). However, at higher levels, reducing sugar generation decreases and cellobionolactone yield increases. In enzymatic cellulose hydrolysis, the synergistic actions of endocellulase and exocellobiohydrolase hydrolyze cellulose to cellobiose [1,2]. β-Glucosidase then hydrolyzes cellobiose to glucose. When CBO is included in a cellulase reaction, it oxidizes cellobiose to cellobionolactone. Therefore, as the concentration of CBO in a cellulase reaction is increased, generation of cellobiose and glucose, which is formed from cellobiose, will decrease and cellobionolactone production will increase (Fig. 1).

Table I

Effect of CBO and/or catalase addition on cellulose weight loss[a]

Enzymes	Weight loss (mg)	Percentage weight loss relative to control[c]
1. Cellulase[b] (0.2 $mg \cdot ml^{-1}$) (control)	91.5 ± 1.0	100 ± 1.1
2. Cellulase (0.2 $mg \cdot ml^{-1}$) + CBO (10 $\mu g \cdot ml^{-1}$)	108.5 ± 0.6	118.6 ± 0.6
3. Cellulase (0.2 $mg \cdot ml^{-1}$) + CBO (60 $\mu g \cdot ml^{-1}$)	69.5 ± 0.1	76.0 ± 0.1
4. Cellulase (0.2 $mg \cdot ml^{-1}$) + CBO (10 $\mu g \cdot ml^{-1}$) + catalase (5 $\mu g \cdot ml^{-1}$)[d]	109.2 ± 2.3	119.3 ± 2.1
5. Cellulase (0.2 $mg \cdot ml^{-1}$) + CBO (60 $\mu g \cdot ml^{-1}$) + catalase (5 $\mu g \cdot ml^{-1}$)[d]	86.7 ± 0.2	94.8 ± 0.2

[a] Enzymes were incubated with 600 mg microcrystalline cellulose in 50 mM acetate, pH 5, in a total volume of 30 ml. Weight loss was determined as described in section 2.

[b] Cellulase is from *T. viride*.

[c] Cellulose weight loss observed in the absence of CBO and catalase was considered to be 100%.

[d] In a separate experiment, cellulose weight loss for the control in the presence of 5 $\mu g \cdot ml^{-1}$ catalase was determined to be 97.8 ± 0.9%.

Cellulose weight loss experiments suggest that, in the presence of 10 $\mu g \cdot ml^{-1}$ CBO, cellulose weight loss is increased by 19% compared to the control cellulase reactions (Table I). However at 60 $\mu g \cdot ml^{-1}$ of CBO, the cellulose weight loss is only 76% compared to that of control. CBO produces H_2O_2 during its catalytic cycle (Bao, Usha, and Renganathan, unpublished results) and the oxy-radicals resulting from the H_2O_2 may inactivate the cellulases. Therefore, the effect of catalase addition on cellulose weight loss was examined. Catalase increases the weight loss in 60 $\mu g \cdot ml^{-1}$ CBO incubations by 19% (Table I). However, catalase addition does not increase the weight loss in the control and 10 $\mu g \cdot ml^{-1}$ CBO incubations, possibly because the level of H_2O_2 generated in these incubations is not deleterious to the enzymes. Although catalase did not have any effect on glucose and cellobiose production at 10 μg CBO, it enhances glucose and cellobiose yield at 60 μg CBO (data not shown). The lignin-degrading system of *P. chrysosporium* contains two important peroxidases, which require H_2O_2 as a substrate [15,16]. During lignocellulose degradation, H_2O_2 produced via CBO may be consumed by lignin-degradation reactions, thus avoiding H_2O_2-dependent cellulase inactivation.

Hydrolysis of the crystalline structure of cellulose has been recognized to be rate-limiting in the saccharification of cellulose to glucose [17]. Findings presented in this report suggest that at low, perhaps physiologically relevant concentrations, CBO enhances cellulose degradation by cellulases and this may be the physiological function of CBO. Further experiments, aimed at understanding the interaction of CBO and *P. chrysosporium* cellulase in cellulose degradation and the mechanism of CBO-dependent enhancement of cellulose hydrolysis, are planned.

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