Volume 327, number 2, 161–164 FEBS 12744 July 1993

Release of the FAD domain from cellobiose oxidase by proteases from cellulolytic cultures of *Phanerochaete chrysosporium*

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Received 18 May 1993; revised version received 7 June 1993

Evidence has previously suggested that cellobiose:quinone oxidoreductase (CBQ) in cellulolytic cultures of *Phanerochaete chrysosporium* might be produced from cellobiose oxidase (CBO) by proteolytic cleavage. This study demonstrates that the ratio of CBO activity to (CBO + CBQ) activity declines with decreasing culture pH, while protease activity increases. Furthermore, we demonstrate that endogenous *P. chrysosporium* proteases can only cleave CBO when the enzyme is bound to cellulose. This is the first demonstration that the proteases produced in cellulolytic cultures of *P. chrysosporium* can release the FAD domain from CBO.

Cellobiose dehydrogenase; Cellobiose-quinone oxidoreductase; Proteolysis; Cellulose degradation; Antibody

1. INTRODUCTION

Phanerochaete chrysosporium is a white rot fungus whose enzyme system for the degradation of lignocellulose has been investigated intensively. This fungus when grown on cellulose as a carbon source produces two extracellular enzymes which, in the presence of a suitable electron acceptor, oxidize the reducing ends of cellobiose, cellodextrins, and cellulose [1,2,3,4,5]. One enzyme is named cellobiose oxidase (CBO) and the other is cellobiose:quinone oxidoreductase (CBQ). Both enzymes carry FAD as a prosthetic group which is responsible for oxidizing the sugar at the reducing end of the substrate. In addition to FAD, CBO harbors a heme prosthetic group. Differences in the catalytic properties of CBO and CBQ have been discussed in detail in a previous report [6]. Both enzymes can utilize dichlorophenol-indophenol (DCPIP) as an electron acceptor; however, only CBO can reduce cytochrome *c*. This difference in cytochrome *c* reducing ability is useful for assessing CBO activity in enzyme mixtures containing both CBO and CBQ.

Henriksson et al. [7] demonstrated that the proteolysis of CBO by papain yielded two major protein fragments, a 35 kDa fragment carrying the heme group and a 55 kDa fragment containing FAD. The 55 kDa fragment harboring FAD retained the ability to reduce 3,5-di-*t*-butyl-*O*-benzoquinone in the presence of cellobiose. This observation suggested a similarity between CBQ and the FAD-containing domain of CBO. Wood and Wood [8] reported that the partial digestion of CBO and CBQ with staphylococcal V8 protease or cyanogen bromide yielded numerous bands having identical mobilities on SDS-polyacrylamide gels. These researchers also showed that polyclonal antibodies to either purified protein cross-reacted with both enzymes, thus indicating a high degree of homology between CBO and CBQ. Furthermore, a heme-containing fragment obtained from culture supernatants cross-reacted with anti-CBO antibodies, but not with anti-CBQ antibodies. Based on these observations, CBQ was proposed to be formed from CBO by proteolytic cleavage.

However, no cleavage of CBO has yet been demonstrated using endogenous proteases produced by *Phanerochaete chrysosporium*. In this paper, we demonstrate release of the FAD domain from CBO using a protease recovered in supernatants from cellulolytic cultures of *P. chrysosporium*. Evidence is presented to show that proteolytic cleavage of CBO is pH dependent and occurs most readily when the enzyme is bound to cellulose.

2. MATERIALS AND METHODS

2.1. *Cultivation*

Phanerochaete chrysosporium, strain K-3, was grown in 1 liter flasks on a medium described by Wood and Wood [8], containing 2% cellulose (Whatman CF-11) as a carbon source. The inoculated flasks were kept at 37°C and shaken at 150 rpm. In experiments to examine the effect of culture pH on CBO/CBQ production, the pH of the culture solution was adjusted to 3.8, 4.3 or 4.8 after three days of cultivation.

2.2 *Preparation of enzymes*

CBO was purified as described in our previous paper [9], with the exception that a final anion exchange chromatography step (Mono-Q FPLC, Pharmacia) was included. CBO was eluted from this column

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using a linear gradient of 20–1000 mM sodium acetate, pH 6.0, in a total volume of 150 ml.

CBQ was purified to homogeneity as described previously [10].

Protease was eluted from the DEAE-Toyopearl 650M (Tosoh Co., Tokyo, Japan) column used for CBO purification after the elution of CBO. The partially purified protease so obtained was confirmed to be free from CBO and CBQ activities, and was subsequently used for the proteolysis of CBO.

2.3. *Enzyme assays*

DCPIP-reducing activity of CBO and CBQ was assayed at 24°C in 100 mM sodium acetate, pH 4.2. Activity was measured as the decrease in absorbance at 600 nm in a solution of 500 μM cellobiose and 200 μM DCPIP. The DCPIP absorption coefficient (ε_{600}) used for calculating enzyme activity was 2.7 $mM^{-1} \cdot cm^{-1}$ One unit of DCPIP-reducing activity was defined as the amount of enzyme necessary to reduce 1 μmol of substrate per min.

Cytochrome *c*-reducing activity of CBO was assayed at 24°C in 100 mM sodium acetate, pH 4.2, as previously described [6]. One unit of cytochrome *c*-reducing activity was defined as the amount of enzyme necessary to reduce 1 μmol of substrate per min

Azocoll hydrolysis was used to measure protease activity. Azocoll (10 mg) was incubated with 1 ml of enzyme solution at 30°C with shaking every 15 min. After 1 h of incubation, 700 μl of the colored supernatant was removed and the pH was adjusted to 5.0 by addition of 300 μl of 500 mM sodium acetate buffer, pH 5.0. The final absorbance of the solution was measured at 550 nm.

2.4. *Proteolysis of CBO*

Partially purified protease(s) were incubated for 36 h at 24°C with 450 mU of pure CBO in 100 mM sodium acetate buffer, pH 4.2, in either the presence or absence of cellulose. Cellulose (Whatman CF-11) was added to a final concentration of 3% (w/v) in the appropriate reaction mixtures.

2.5. *Production of anti-CBO polyclonal antibodies*

Balb/c mice were injected with purified CBO (0.5 mg/mouse) in Freund's complete adjuvant and boosted six weeks later with CBO (0.1 mg/mouse) in Freund's incomplete adjuvant. Polyclonal antibodies were recovered and partially purified from mouse serum by three sequential precipitation/resuspension cycles with ammonium sulfate to 50% saturation.

2.6. *SDS-PAGE and Western blotting*

SDS-PAGE was performed in 10% (T) gels using the Tris-tricine buffer system of Schägger and von Jagow [11]. Resolved proteins were subsequently transferred to polyvinylidene difluoride (PVDF, Immobilon-P, Millipore Corp.) membranes using a semi-dry electroblotter. Blots were developed with anti-CBO polyclonal antibody and alkaline phosphatase-conjugated goat anti-mouse IgG antibodies as previously described [12].

3. RESULTS

3.1. *DCPIP reducing activity of CBO and CBQ*

A time course for the production of CBO and CBQ, as determined by DCPIP-reducing activity, in cultures of *P. chrysosporium* cultivated on cellulose is shown in Fig. 1. DCPIP-reducing activity (CBO + CBQ) was first detected on day 3. At that time, culture pH was adjusted manually to 3.8, 4.3 and 4.8. DCPIP-reducing activity continued to increase through day 7 in the culture at pH 3.8, but it leveled off in the culture at pH 4.8 after day 5. No significant differences in DCPIP-reducing activity were observed among the three pH values before day 5.

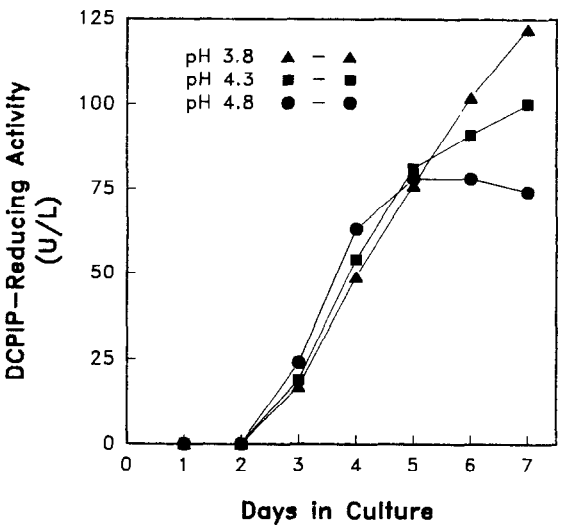

Fig. 1. Effect of culture pH on the time course for production of DCPIP-reducing activity (CBO+CBQ) in cellulolytic cultures of *P. chrysosporium*.

3.2. *Cytochrome c-reducing activity of CBO*

In contrast to DCPIP reducing activity (CBO + CBQ), significant differences in cytochrome c-reducing activity (CBO) were found in the pH-adjusted culture solutions. As can be seen in Fig. 2, the increase in cytochrome *c* activity levels halted immediately after the pH was adjusted to 3.8 on day 3. In cultures which had been adjusted to pH 4.8, cytochrome *c*-reducing activity continued to increase for another one or two days. As both CBO and CBQ can utilize DCPIP as an electron acceptor, while only CBO can effectively reduce cytochrome *c*, the lower cytochrome *c*-reducing activity at lower pHs indicates a higher content of CBQ in acidified culture medium.

3.3. *Protease activity*

A time course for the production of protease activity, as determined using azocoll as substrate, is shown in Fig. 3. As was the case for CBO and CBQ, protease activity levels were found to be dependent upon the pH

Table I

Changes in the DCPIP- and cytochrome *c*-reducing activities of CBO caused by proteolysis in the presence and absence of cellulose

Incubation time (h)	% Relative reducing activity			
	− Cellulose		+ Cellulose	
	DCPIP	Cyt-*c*	DCPIP	Cyt-*c*
0	100	100	100	100
12	ND	ND	95	59
36	94	93	90	14

ND = not determined.

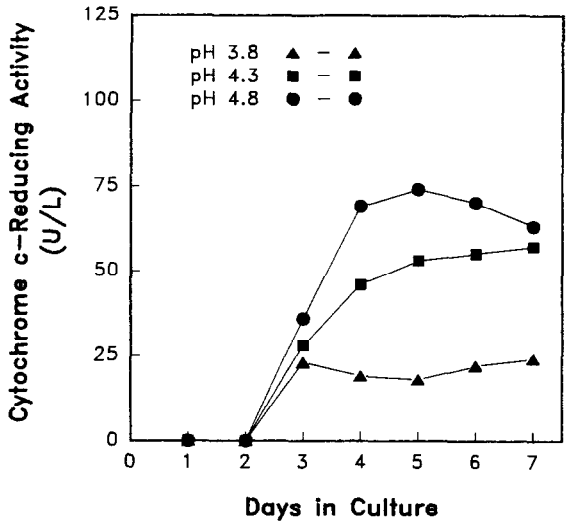

Fig. 2. Effect of culture pH on the time course for production of cytochrome *c*-reducing activity (CBO) in cellulolytic cultures of *P. chrysosporium*.

of the culture solution. Protease activity in cultures at pH 3.8 was found to be more than double the activity in cultures at pH 4.8. The correlation of higher CBQ content with elevated protease activity suggested that the protease activity detected with the azocoll assay might be responsible for the proteolytic conversion of CBO into CBQ.

3.4. *Proteolysis of CBO*

Purified CBO was incubated with the partially purified *P. chrysosporium* protease in either the presence or absence of cellulose. DCPIP and cytochrome *c*-reducing activities, measured before and after 36 h of proteolysis, are shown in Table I. Activities are expressed relative to the initial activity prior to proteolysis. Protease treatment of CBO in the absence of cellulose resulted in no significant decrease in either DCPIP or cytochrome *c*-reducing activities. However, protease treatment in the presence of cellulose led to a marked decrease in cytochrome *c*-reducing activity, while DCPIP-reducing activity remained nearly constant.

Proteins from the protease-treated samples were resolved by SDS-PAGE and transferred to PVDF membranes. For samples containing cellulose, proteins were recovered from the cellulose pellet by extraction directly into SDS-PAGE sample buffer, as little or no DCPIP- or cytochrome *c*-reducing activity could be detected in the supernatants. The blotted proteins were probed with murine polyclonal antibodies raised against CBO, and protein bands which bound antibody were detected using an alkaline phosphatase-conjugated secondary antibody as shown in Fig. 4. The antibodies labeled both purified CBQ and CBO (lanes 1 and 2, respectively). Protease treatment of CBO in the absence of cellulose resulted in a very faint band which had the same mobility as CBQ (lane 3). On the other hand, protease treatment of CBO in the presence of cellulose produced substantially more of the immunodetectable band with mobility equivalent to CBQ (lane 4). Incubation of CBO with cellulose in the absence of protease yielded no bands with mobilities corresponding to CBQ (data not shown).

4. DISCUSSION

Two acidic proteases isolated from cellulolytic cultures of *P. chrysosporium* (*Sporotrichum pulverulentum*) were previously demonstrated to regulate the activity of endo-1,4-β-glucanases in these cultures [13]. Similarly, *P. chrysosporium* grown under lignolytic conditions has been shown to secrete at least two proteases, and increases in the proteolytic activity of these cultures appeared to correlate with a decline in lignin peroxidase activity [14]. Work from other laboratories [7,8] has suggested that CBQ might be formed in cellulolytic cultures of *P. chrysosporium* by the proteolytic cleavage of CBO. However, no proteases produced by *P. chrysosporium* has previously been shown to cleave CBO.

We found that acidification of cellulolytic cultures of *P. chrysosporium* led to an increased ratio of DCPIP- to cytochrome *c*-reducing activity, suggesting that more CBQ was formed under these conditions (Figs. 1, 2). Protease activity, as assessed by azocoll hydrolysis, was also found to increase in response to decreased culture pH (Fig. 3). These observations suggested that the protease activity detected in culture supernatants should catalyze the cleavage of CBO to form CBQ, if this were indeed the mechanism for CBQ formation. However, direct incubation of CBO with the *P. chrysosporium* protease preparation did not lead to a reduction in cytochrome *c*-reducing activity (Table I), nor did it result in

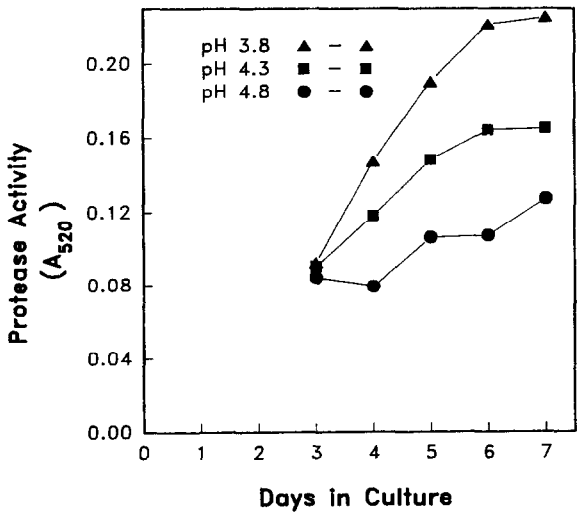

Fig. 3. Effect of culture pH on the time course for production of protease activity in cellulolytic cultures of *P. chrysosporium*.

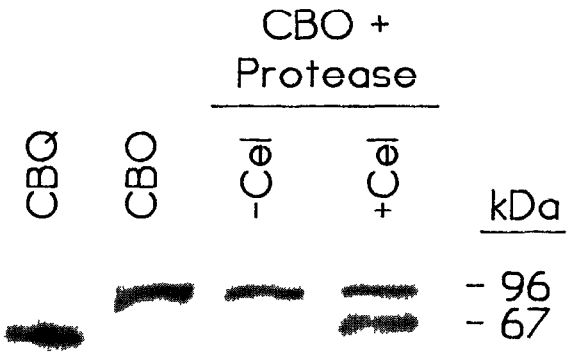

Fig. 4. Immunoblot of purified CBO treated with *P chrysosporium* proteases in the absence or presence of cellulose (Cel). Purified CBO and CBQ are shown for comparison.

any changes to the CBO polypeptide detected in immunoblots (Fig. 4). On the other hand, when CBO was incubated with the same protease preparation in the presence of cellulose, not only did the ratio of cytochrome *c*-reducing activity to DCPIP-reducing activity decline, but a strongly cross-reactive polypeptide with a mobility comparable to that of CBQ was detected on the immunoblot.

CBO has been reported to bind to cellulose [7,15]. In our experiments, little or no DCPIP-reducing activity was detectable in supernatants from samples containing cellulose. As the addition of cellulose did not appear to enhance proteolysis of azocoll by the *P. chrysosporium* protease preparation, our observations indicate that CBO is cleaved only when the enzyme is bound to cellulose. This suggests that the conformation of CBO changes upon binding to cellulose, which in turn facilitates access of the endogenous proteases to the hinge region connecting the FAD- and heme-containing domains of the enzyme. The physiological significance of the proteolytic conversion of CBO to CBQ, as well as the fate of the released heme domain, is as yet unknown. However, the fact that CBO is most easily cleaved when bound to an insoluble substrate suggests that there may be a need to reexamine the effects of endogenous proteases on cellulases and lignin peroxidase from *P. chrysosporium* as these enzymes also interact with similar insoluble substrates.

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