Full Length Research Paper

Enzyme activity of a *Phanerochaete chrysosporium* cellobiohydrolase (CBHI.1) expressed as a heterologous protein from *Escherichia coli*

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The aim of this study was to produce a secreted, heterologously expressed *Phanerochaete chrysosporium* cellobiohydrolase (CBHI.1) protein that required no *in vitro* chemical refolding and to investigate the cellulolytic activity of the clone expressing the glutathione S-transferase (GST) fused CBHI.1 protein. Plate enzyme activity screening of *E. coli* cells transformed with pGEX*cbh*I.1 vector on carboxy-methyl-cellulose (CMC) produced several clones which produced clearing zones on CMC when induced. A randomly selected representative pGEX*cbh*I.1 clone produced hydrolysis on both Avicel and CMC when induced. Crude protein extracts obtained from the induced pGEX*cbh*I.1 clone exhibited time dependent enzymatic activity against both CMC and Avicel.

Key words: *Phanerochaete chrysosporium*, cellobiohydrolase, cellulase activity, heterologous expression.

INTRODUCTION

Lignocellulolytic microorganisms and their lignocellulolytic enzymes have many potential biotechnological applications ranging from the production of bio-fuel, chemicals, proteins, and to improving textiles, wood-pulping and animal feeds for domesticated herbivores (Bhat and Bhat, 1997; Coughlan, 1985; Kotchoni et al., 2003; Ojumu et al., 2003). The white-rot, basidiomycete fungus, *Phanerochaete chrysosporium*, has attracted considerable research interest since it efficiently degrades all three components of lignocellulose (Baldrian, 2003; Tuomela et al., 2002; Yadav 2003). *P. chrysosporium* employs various enzymes in the process of lignocellulose biodegradation but the enzymatic mechanisms and genes involved in this complex process are still poorly understood (Henriksson et al., 2000; Sims et al., 1994; Birch et al., 1995; Broda et al., 1996).

Cellulose degradation by fungi is generally as a result of the induction of a family of cellulolytic enzymes which can be classified into three major classes: endoglucanases, exoglucanases, also commonly referred to as cellobiohydrolases, and glucosidases (Knowles et al., 1987).

In 1988, Sims et al., identified, cloned and characterised the first gene from *P. chrysosporium* ME446 that showed strong homology to the exo-cellobiohydrolase I (*cbh*I) gene from *Trichoderma reesei*. Covert et al. (1992), using a different strain of *P. chrysosporium* (BKM-F-1767), reported five additional *cbh*I-like genes. In 1994, Sims et al. demonstrated the differential expression of multiple *cbh*I-like genes in *P. chrysosporium* ME446, identified and characterised an

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additional *cbh*I gene that was termed *cbh*I.2 to differentiate it from the first gene, which is now called *cbh*I.1. The latter two genes were shown to be non-allelic *cbh*I-like genes with introns which differ in number, position, sequence and length. Northern analysis using sequence-specific olignonucleotides for each of the two genes demonstrated that both *cbh*I.1 and *cbh*I.2 mRNA were expressed when *P. chrysosporium* mycelium was grown on ball-milled straw but neither was expressed when glucose was used as the sole carbon source. Sims et al., (1994) using protein data obtained from three purified cellobiohydrolases (Uzcategui et al., 1991) demonstrated that the protein designated CBHI by the latter researchers is encoded by *cbh*I.2 but also possibly by *cbh*I-3 and *cbh*I-6 genes, which were identified by Covert et al., (1992), and that the CBH62 protein is encoded by *cbh*I.1. CBHI and CBH62 proteins interact synergistically to give enhanced degradation of crystalline cellulose when either is paired with the *P. chrysosporium* CBHII protein (Sims et al., 1994). *P. chrysosporium* is known to produce a number of proteins showing endoglucanse activity (Eriksson and Pettersson, 1975a,b; Uzcategui et al., 1991), however, despite extensive search, no gene or sequence equivalent to those of *Trichoderma* EGI and EGIII has yet been identified (Sims et al., 1994). Because of the latter, it has been speculated that the *cbh*I-like sequences/genes might encode endoglucacanase and exoglucanase-like activities and the specific type of activity displayed by a particular transcript is regulated by substrate-dependent differential splicing of introns in these *cbh*I-like sequences/genes (Birch et al., 1995). In order to investigate the possible differences in substrate affinities encoded by the *cbh*I-like sequences/genes and to begin to understand the differences in intron splicing (Birch et al., 1995) attempts have been made to clone and identify *cbh*I-like genes/sequences. Howard (1997) cloned and expressed *P. chrysosporium cbh*I.1 cDNA in *E. coli* using a pET cloning and expression system. A small amount of an inactive, internalised product was produced that had to be refolded using tedious *in vitro* chemical methods to restore enzymatic activity. The refolded CBHI.1 protein exhibited activity only against artificial substrates such as 4-methylumbelliferyl-ß-D-cellobioside/lactopyranoside (MUC or MUL) but not against p-nitrophenyl-ß-D-cellobioside/lactopyranoside (PNP-C or PNP-L) nor against cellulose substrates such as carboxy-methyl-cellulose (CMC) and Avicel. It was speculated that the loss of activity against derived cellulose (CMC) and native cellulose (Avicel), substrates which cellobiohydrolases are expected to hydrolyse, was probably associated with "partial or incorrect" refolding of the protein. Interestingly Laymon et al., (1996) made a similar observation with a heterologously expressed, refolded *T. reesei* CBHI, but they could not offer any plausible explanation. To eliminate the speculative

possibility of "refolding problems" we attempted to produce a heterologously expressed CBHI.1 protein that was secreted and that required no *in vitro* refolding; and here we report on the cellulolytic activities observed for a secreted, glutathione S-transferase (GST) fused CBHI.1 protein expressed from *E. coli*.

MATERIALS AND METHODS

Strains and vectors

E. coli BL21(DE3)p*Lys*pET*cbh*I.1, *E. coli* BL21(DE3)p*Lys*pET strains were previously constructed by Howard, (1997). The *E. coli* BL21 strain and pGEX-4T-3 vector were supplied by Pharmacia Biotech.

General DNA manipulations

Mini-scale preparation of *E. coli* plasmids harbouring pET*cbh*I.1 cDNA or pET or pGEX-4T-3 was done using standard methods as described by Sambrook et al., (1982). Both pET*cbh*I.1 and pGEX-4T-3 were separately double digested with *Bam* HI and *Not* I using standard methods (Sambrook et al., 1982). After polyacrylamide gel electrophoresis (PAGE), the *Bam* HI - *Not* I flanked *cbh*I.1 fragment and the linearised pGEX was purified from the gel using a QIAGEN agarose gel DNA Extraction Kit according to the manufacturer's instructions. To prevent recircularisation and religation of the vector, purified, linearised vector was dephosphorylated using calf intestinal alkaline phosphatase (CIP) (Promega) and repurified following standard methods (Sambrook et al., 1982). The purified *cbh*I.1 fragment was then ligated to the pGEX vector and transformed into competent *E. coli* XL BL21 cells using standard methods (Sambrook et al., 1982). Transformed cells were spread-plated onto LB agar plates containing 50 µg/ml cabenicillin and incubated overnight at 37°C.

Plate enzyme assay screening

After incubation, hundred randomly selected colonies were replica plated onto duplicate solid media consisting of Luria-Bertani (LB) agar containing 0.5% w/v CMC, 50 µg/ml cabenicillin and 1 mM IPTG. Inoculated plates were placed in a plastic box containing soaked tissue paper, the lid of the plastic box was closed and the cultures were incubated at 37°C for 3-4 days. The plastic box with soaked tissue paper provided a humid environment that prevents the media from drying out. After incubation, the plates were flooded with Congo red (1 mg/ml) solution, incubated at room temperature for 15 min and washed several times with sterile 1 M NaCl solution to remove unbounded excess dye. Transformants which produced clearing zones, indicative of hydrolysis of CMC, were selected as positive clones.

Crude protein extract

A single colony of a positive pGEX*cbh*I.1 clone or a transformant harbouring pGEX was separately inoculated into 10 ml LB broth containing 50 µg/ml cabenicillin and grown overnight at 37°C in a shaker incubator. The overnight cultures were diluted 1:40 into fresh LB broth containing the appropriate antibiotics and grown with shaking at 37°C until the cell density reached an OD_{590nm} of

approximately 0.5-0.6. Cells were then induced with 1 mM final concentration of IPTG and incubated as before for 2-3 h. After induction, cells were spun-down at 2449 x g for 10 min at 4°C and the supernatant was collect and transferred into a clean, sterile flask and filtered at 100 psi through a sterile Millipore vacuum filtration unit fitted with a 0.22 μm pore size TF–Millipore membrane to remove any residual cells. The proteins in the cell-free supernatant were concentrated 10-fold using an Amicon ultra-filtration stirrer cell fitted with a PM10 Amicon membrane with a 10 000 molecular cut-off, at 75 psi pressure with the unit being kept at 4°C. The supernatant that did not pass through the membrane was collected, assayed or aliquoted and stored at 4°C.

Protein concentration determination

Protein concentration was determined using a Bio-rad protein assay kit according to the manufacturer's instructions.

Plate enzyme assays

Plate assays using either cells or concentrated supernatant from induced cells were performed according to the method of Teather and Wood, (1982) on 0.5% w/v CMC and 0.5% w/v Avicel, separately, following the method described under plate enzyme assay screening, except that when the supernatant was used no antibiotic and IPTG were added.

Reducing sugar assay

The quantification of hydrolysis of 0.5% w/v, CMC or Avicel, by the crude concentrated supernatant proteins was determined using a standard method (Bailey and Poutanen, 1989) in 50 mM citrate phosphate buffer pH 6 at 55° C. One international unit (IU) of enzyme activity was defined as the amount of enzyme producing 1 μmol of reducing sugars in glucose equivalents per minute under the above assay conditions.

RESULTS

Initial screening of cells transformed with pGEXcbhI.1 on plates containing the appropriate antibiotics on CMC produced almost 90% of individual transformants which produced clearing zones that where not present in the negative control, cell carrying pGEX only. Three of these positive clones were randomly selected and further assessed for their hydrolysis of CMC and Avicel against the appropriate controls. Figure 1 shows the results for one representative pGEXcbhI.1 clone. Only the positive clone exhibited a clearing zone on both CMC (Figure 1a) and Avicel (Figure 1b) but no clearing zones were observed for the negative control pGEX and for pETcbhI.1.

The production of a clearing zone on CMC and Avicel plates only when IPTG was present by pGEXcbhI.1 suggested that the translated product came from an inducible gene and was secreted. Therefore, the supernatant from this inducible clone was collected,
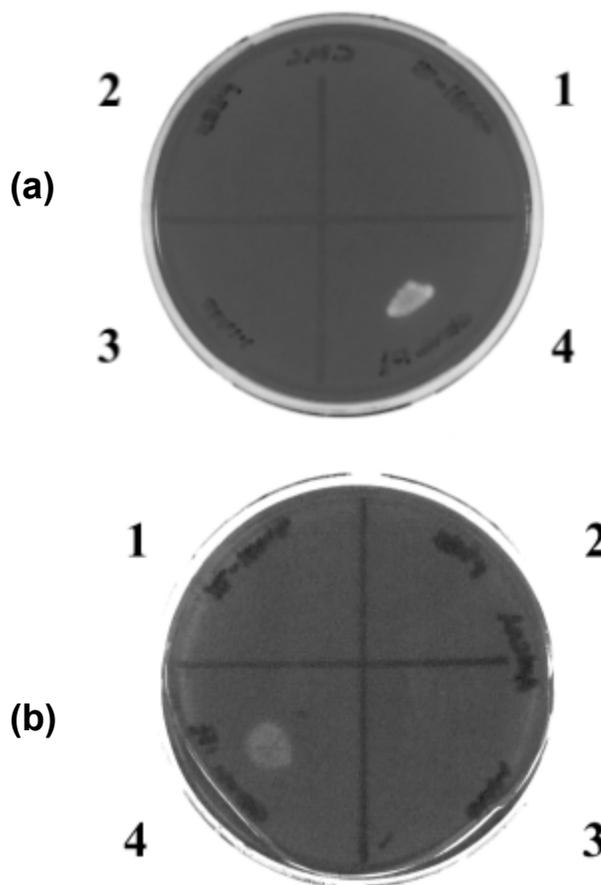

Figure 1. A Congo red stained LB agar plate containing 5% v/w CMC (a) or 5% v/w Avicel (b), IPTG and cabenicillin, after the plate was incubated for 3-4 days at 37°C. The different numbered sectors on the plate were inoculated with the following cells: 1, a clone harbouring pETcbhI.1; 2, BL12 cell only (control); 3, a transformant harbouring pGEX (control) and 4, a clone harbouring pGEXcbhI.1. The clearing in sector 4 is indicative of CMC (a) and Avicel (b) hydrolyses.

concentrated, and loaded in the middle of a well on plates containing CMC and Avicel, and the results are shown in Figure 2a and 2b. Once again we observed that the secreted protein produced larger clearing zones larger than when intact pGEXcbhI.1 cells were used (Figures 1a and b) suggesting that the protein responsible for the hydrolysis of cellulose was indeed present in the supernatant fluid. Boiling an aliquot of this crude protein extract at 100°C for 5 min and performing the same plate assays did not produce any clearing zones suggesting that the inducible, secreted gene product was indeed due to the action of a protein enzyme.

The crude, concentrated protein exhibited a time dependent hydrolytic reaction against CMC and Avicel (Figure 3) whereas concentrated supernatant from

induced, negative control pGEX did not produce any reaction. On CMC the highest hydrolytic reaction occurred at 120 min whereas for Avicel it was at 150 min. Comparing the reducing sugar profiles (Figure 1) it appears that the hydrolysis of both substrates were similar.
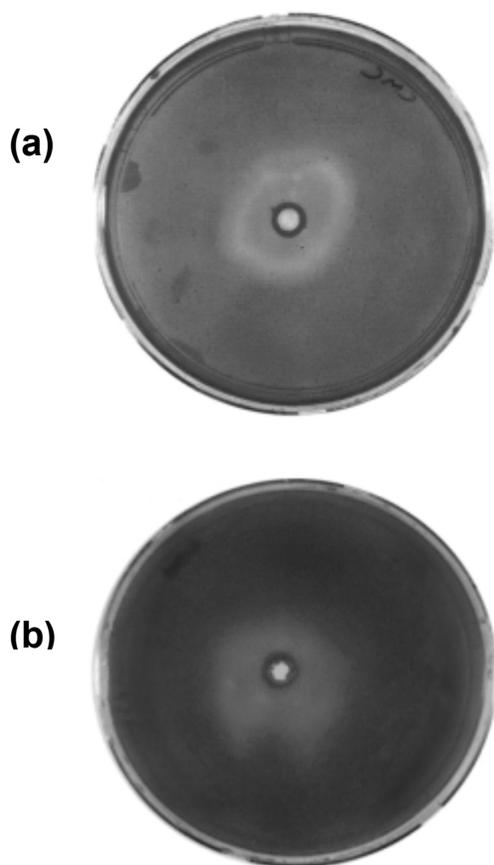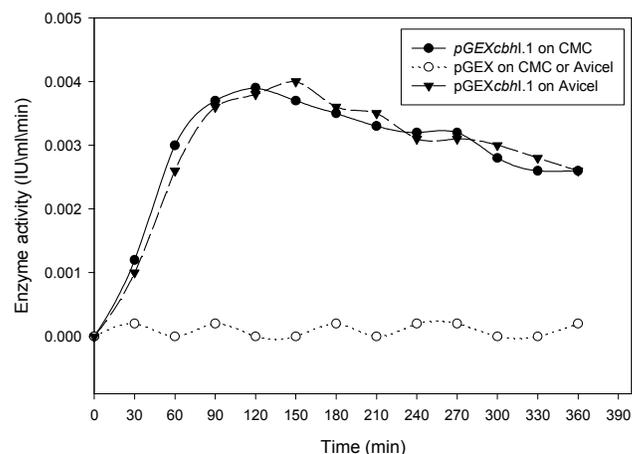

Figure 3. The enzymatic profiles showing the time dependent production of reducing sugars from CMC and Avicel by concentrated supernatant-fluid harvested from the induced pGEXcbhI.1 clone.

Figure 2. A Congo red stained LB agar plate containing 5% v/w CMC (a) or Avicel (b), after the plate was incubated for 3-4 days at 37ᵒC. The centre well was inoculated with 50 μl concentrated supernatant-fluid harvested from the induced pGEXcbhI.1 clone. The clearing is indicative of CMC (a) and Avicel (b) hydrolyses by crude proteins secreted by the clone.

DISCUSSION

Because clearing zones, indicative of hydrolysis of cellulose, were present in only the clone and not in the negative controls suggested that this clone produced an inducible product that was secreted. The only difference between the pGEX transformant and pGEXcbhI.1 was that the latter carried the cbhI.1 insert whereas the former did not. These observations, therefore, suggest that the

protein that hydrolysed cellulose must be a result of the induction of cbhI.1.

In our introduction, we indicated that the clone pETcbhI.1 produced an internalised, inactive gene product that did not hydrolyse derived or native cellulose. In this study we have confirmed the previous observation (Howard, 1977) that the clone carrying pETcbhI.1 did not hydrolyse CMC or Avicel whereas the protein produced by pGEXcbhI.1 did indeed hydrolyse these cellulose substrates without the need for *in vitro* refolding. Therefore, the pGEX system appears to be appropriate for expressing a fully, functional CBHI.1 protein. From these preliminary enzymatic results it appears that the protein, likely to be GST-CBHI.1, hydrolyses CMC and Avicel equally well. CBHI.1 is postulated to be an exo-cellobiohydrolase (Sims et al., 1994) therefore our expectation was that it should not exhibit significant activity against CMC because the latter activity is more typical of endoglucanase rather than exo-cellobiohydrolase activity.

Studies are currently underway to purify the expected chimeric CBHI.1 protein and to conduct more extensive characterisation and enzymatic studies on the purified gene-product. Such studies will indeed enable us to understand the complex nature of cellulose degradation by *P. chrysosporium*.

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