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Full Length Research Paper

Cloning and expression of *Phanerochaete chrysosporium* cellobiohydrolase (*cbhl.2*)

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The *Phanerochaete chrysosporium* ME446 *cbhl.2* cDNA was cloned and expressed in *Escherichia coli* using a pET system. Pulse-labelling revealed the expression of an induced protein of approximately 50-60 kDa that reacted with anti-*P. chrysosporium* ME446 CBH antibodies. The expressed protein remained undegraded *in vivo* for 135 min but was biologically inactive *in vitro*.

Key words: *Phanerochaete chrysosporium*, *cbhl.2*, cloning and expression, pulse-labelling.

INTRODUCTION

The importance of lignocellulose biotechnology and the many potential applications of lignocellulose enzymes in various industries such as chemicals, fuel, food, brewery and wine, animal-feed, pulp-and-paper, textile and agriculture are well documented (Gavrilescu and Chisti, 2005; Howard et al., 2003; Bhat, 2000; Sun and Cheng, 2002). *Phanerochaete chrysosporium*, being an efficient degrader of lignin, hemicellulose and cellulose, the major constituent components of lignocellulose, is suitable for various industrial applications. Also, it is an appropriate organism for studying the interaction of genes and enzymes involved in the complex lignocellulose degradation process.

Multiple, non-allelic, cellobiohydrolase (*cbh*)-like sequences/genes, which are likely to be involved in cellulose degradation, have been identified (Covert et al., 1992; Sims et al., 1994). Two of these sequences/genes, namely, *cbhl.1* and *cbhl.2* were characterised from genomic and cDNA libraries (Sims et al., 1994). Howard et al. (2004) reported on *cbhl.1*, and hence *cbhl.2* is the focus of this report. The sequence data for *P. chrysosporium* ME446 *cbhl.2* appears in the EMBL/GenBank/DDBJ Sequence Data Library under the accession numbers Z22527 (genomic) and Z29653 (cDNA). *cbhl.2* has two internal introns at the same positions as *cbhl.1* but with different sequences, and a single up-stream intron whereas *cbhl.1* has two up-stream introns. One of *cbhl.2* internal introns near the 3'-end was shown to be excised from only some of the cDNA library clones (Sims et al., 1994). Since this "intron" contains no stop codon, it means translation of

mRNA containing this "intron" will produce a "CBHI.2" with a significantly altered C-terminus (Broda et al., 1994; Birch et al., 1995). Hence two "CBHI.2" proteins could theoretically be formed either through selective alteration of CBHI.2 at protein level or differential splicing at mRNA level. Some cellulases may regulate their substrate specificities through proteolytic removal of their C-terminus (Knowles et al., 1987) while another mechanism maybe differential removal of the 3' intron (Broda et al., 1994; Birch et al., 1995). Birch et al. (1995) demonstrated that two types of mRNA are produced for *P. chrysosporium* ME446 *cbhl.2* and that differential splicing of the 3' intron is substrate dependent. We still do not know whether both mRNAs are translated, the nature of the proteins and the mechanisms involved in cellulose hydrolysis. Cloning and expression of the two forms of cDNA (with and without the "3' intron") will assist in addressing the latter issues.

Previous, unsuccessful, attempts were made to express *cbhl.2* in *Escherichia coli* using pUC18 and pKKScxyl(H) vectors (Howard, 1997). This study investigated cloning and expression of *cbhl.2* in *E. coli* using a pET system.

MATERIALS AND METHODS

Strains and vectors

E. coli BL21 or *E. coli* (DE3)pLysS transformed with pET-22b(+) with or without the gene insert were used as expression hosts. Novagen supplied the host strains and the pET vector.

Antibodies

Anti-rabbit IgG alkaline phosphatase-conjugated (Promega) diluted 1:7500 in Tris Buffered Saline Tween 20 (10mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.05% Tween 20) was used as a secondary antibody. Rabbit anti-*P. chrysosporium* ME446 CBH synthetic peptide, *E. coli* depleted, antibody (CBH-No 3) was supplied by the Lignocellulose Group, University of Manchester Institute of Science and Technology, Manchester, UK. A 2000-fold-diluted antiserum was able to detect 10 pg of the synthetic CBH peptide.

cbh1.2 gene

The sequence data for *P. chrysosporium* ME446 *cbh1.2* appears in the EMBL/GenBank/DDBJ Sequence Data Library under the accession numbers Z22527 (genomic) and Z29653 (cDNA). Clone *E. coli* XL1-Blue-pUC18*cbh1.2*(H) carrying the fully-spliced *cbh1.2* cDNA open reading frame encoding the mature CBH1.2 protein was previously constructed (Howard, 1997). A 1.6 kb *Hind*III fragment containing the *cbh1.2* opening reading was isolated from the latter clone and used in this study.

Media

Clone *E. coli* XL1-Blue-pUC18*cbh1.2*(H) was grown on Luria-Bertani (LB) broth or solid media containing 50 µg/ml cabenicillin at 37°C. *E. coli* BL21(DE3)pLysS was grown on LB media containing 34 µg/ml chloramphenicol at 37°C and when transformed with pET, 50 µg/ml cabenicillin was also added to the same media.

DNA manipulations and colony hybridisation

Mini-prep DNA isolation, restriction endonuclease digestion, ligation and transformation, agarose gel electrophoresis and colony hybridisation were performed according to standard methods described by Sambrook et al. (1989). Both pUC18*cbh1.2*(H) and pET were separately digested with *Hind*III and separated on agarose by electrophoresis. The *Hind* III *cbh1.2* fragment of approximately 1.6 kb and the linearised pET vector were purified from the agarose gel using QIAGEN Agarose Gel DNA Extraction Kit according to the manufacturer's instructions. To prevent self-religation of the vector, purified, linearised vector was dephosphorylated using calf intestinal alkaline phosphatase (CIP) (Promega) and repurified following standard methods (Sambrook et al., 1989). The purified *cbh1.2* was then religated to the pET vector and transformed into competent *E. coli* BL21 cells for vector amplification, repurified from the latter host and transformed into competent *E. coli* (DE3)pLysS for protein expression. Transformants were screened for clones carrying pET*cbh1.2* using colony hybridisation. The purified *Hind*III *cbh1.2* was random primed labelled using either radioactive α -³²PdATP (Sambrook et al., 1989) or Digoxigenin (DIG) Labelling Kit (Boehringer) according to the manufacturer's instructions; and the labelled *cbh1.2* was used as a probe in the colony hybridisation screening.

PCR and DNA sequencing

Plasmids used for sequencing were purified using a QIAprep miniprep kit (QIAGEN). PCR amplification was done using the ABI PRISM™ Dye Terminator Cycle Sequencing Ready Reaction Kit (Perkin Elmer) and cycle-sequenced on an ABI 373 Automatic Fluorescence Sequencer (Perkin Elmer), according to the manufacturer's instructions (Perkin Elmer, protocol P/N 402078, revision A, 1995). The T7 promoter primer 5'-

TAATACGACTCTATAGG (Novagen) was used in DNA sequencing to verify the 5' DNA region spanning: the pET promoter, signal peptide encoding sequence and *cbh1.2* sequence.

Protein blotting and immuno-detection

Dot blots and Western blots were prepared according to standard procedures (Sambrook et al., 1989). Ten-fold concentrated supernatant from *P. chrysosporium* grown on ball-milled straw was used as a positive control. The immuno-detection was done using the ProBlot® Western Blot AP System (Promega) according to the manufacturer's instructions.

Radioactive pulse-labelling and chase

Radioactive labelling, chasing and autoradiographic analyses were essentially done according to the method described by S. Tabor (Department of Biological Chemistry, Harvard Medical School, Boston, MA 02115). A single colony of an overnight culture (*E. coli* BL21 transformed with pET only or pET*cbh1.2*) was inoculated into 5 ml LB media the appropriate antibiotics as mentioned under media section. Cultures were incubated overnight at 37°C with shaking. 100 µl of the overnight culture was transferred to an Eppendorf tube and 500 µl of pre-warmed (37°C) LB broth media containing the appropriate antibiotics was added. The culture was mixed by vortexing briefly, centrifuged at 13,000 rpm for 30 s and the pellet was resuspended in 100 µl of the same media as before. The later culture was further diluted 1:40 in the same LB media. The culture was incubated at 37°C for 3 to 4 h with shaking and until cell density reached an OD_{590nm} ≈ 0.5. One microlitre of the latter culture was centrifuged at 13,000 rpm for 30 s, washed with 5 ml pre-warmed (37°C) 2 x M9/1% glucose media (Sambrook et al., 1989), briefly vortexed and centrifuged as previously. The cell pellet was resuspended in 2 ml pre-warmed 1 x M9 media containing 0.5% glucose, 0.02% mixture of 18 amino acids (except cysteine and methionine) and 0.5% thiamine. Cells were grown with shaking at 37°C for 60 min to deplete methionine reserves, so that radioactive-labelled methionine was readily used in protein synthesis. One millilitre of the culture was induced with 1 mM IPTG for 20 min and the other 1 ml served as the un-induced culture. Rifampicin to a final concentration of 200 µg/ml was added to both the induced and un-induced cultures to inhibit *E. coli* RNA polymerase and cultures were incubated at 37°C with shaking. After 15-20 min 10 µCi ³⁵S-methionine was added to both cultures and the cells were incubated at 37°C with shaking for 10 to 20 min. The cells were harvested at 13,000 rpm for 20 s and analysed for protein content. Supernatant fluid, whole cell and various fractions obtained from the periplasm, cytoplasm, insoluble fraction (membrane and inclusion bodies) were prepared according to procedures described by Sambrook et al. (1989). Supernatant fluid, whole cell and the various fractions were analysed using SDS-PAGE, and stained or unstained cling-wrapped gels were autoradiographed at room temperature for 1-7 days (Sambrook et al., 1989).

The stability of the expressed proteins were monitored following the same procedure as for the labelling experiment, except that cells were pulse-labelled for 15 min and chased using non-radioactive methionine at a final concentration of 0.5%. At intervals of 45, 90 and 135 min after incubation 0.5 ml aliquots were removed centrifuged as before and the whole cell proteins were analysed by SDS-PAGE and autoradiography as indicated above.

Crude protein extract

Single colonies of cells carrying pET and pET*cbh1.2*(H), respectively, were separately inoculated into 5 ml LB broth

containing the appropriate antibiotics 50 µg/ml cabcenicillin and 34 µg/ml chloramphenicol, and grown overnight at 37°C in a shaker incubator. One hundred microlitres of each culture was washed with fresh LB broth containing the appropriate antibiotics, centrifuged at 13,000 rpm for 30 s and resuspended in fresh media. These 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 0.8-1.0. Cells were then induced with 1 mM final concentration of IPTG for 30 min at 30°C, after which rifampicin was added to a final concentration of 200 µg/ml. After 2 h cells were harvested by centrifugation at 4500 rpm for 10 min at room temperature. Cells were resuspended in 4 ml of 50 mM TE buffer pH 6.8 containing 10 mM DTT, 174 µg/ml PMSF and β-mercaptoethanol (1:1000). The cells were lysed by successive cycles of freezing (liquid nitrogen) and thawing by sonication for 20 s at half power (14 mA) until a clear cell lysate was obtained. This mix was used as crude protein extracts or alternatively the sonicated mixture was centrifuged at 13,000 rpm for 2 min to remove cell debris, and this cleared supernatant fluid was used as crude proteins extracts.

The crude protein extracts and cellular fractions regarded as crude enzyme extracts were collected, assayed or aliquoted and stored at 4°C.

Enzyme assays

Plate assays using either cells or crude enzyme extracts were performed according to the method of Teather and Wood, (1982) on 0.5% w/v CMC and 0.5% w/v Avicel, separately. Crude enzyme extracts were also assayed for activity using the method described by Bailey and Poutanen (1989) for the formation of reducing sugars from 0.5% w/v CMC or Avicel in 50 mM citrate buffer pH 5 at 50°C. Alternatively, crude enzymes were tested for activity using two other methods: one as described by Laymon et al. (1996) for hydrolysis of 0.5 mM 4-methylumbelliferyl-β-D-cellobioside/lactopyranoside (MUC or MUL) (Sigma) in 50 mM sodium acetate buffer pH 5 at 50°C. And the other method as described by Cummings and Fowler (1996) for hydrolysis of 2 mM p-Nitrophenyl-cellobioside/lactoside (PNP-C or PNP-L) in 50 mM sodium acetate buffer pH 5 at 30°C.

RESULTS

Colony hybridisation and DNA sequencing

Eight positive clones carrying the *cbhl.2* DNA sequence were identified using plate hybridisation. DNA sequencing revealed that only two of the eight clones carried the *cbhl.2* sequence in the correct orientation and in-frame to the sequence encoding the signal peptide. One of these clones designated pET*cbhl.2*(H) was subsequently used in all further experiments.

Pulse-labelling and chase

The protein bands of expected size (Figure 1) were observed in whole cell extracts (lane 5) and in the insoluble fraction (membrane and inclusion bodies) (lane 8), from induced pET*cbhl.2*(H) cells. Comparison of a Comassie-stained SDS-PAGE loaded with the same samples and with appropriate protein molecular weight

markers, and electrophoresed in parallel with the gel prepared for autoradiography was used to visually estimate the size of the positive bands and these were estimated to be approximately between 50-60 kDa. Interestingly, in each of the positive fractions duplex signals were present. No positive signals corresponding in size to the expected CBH1.2 protein were observed in the soluble periplasmic (lane 6) or soluble cytoplasmic (lane 7) fractions. None of the fractions from induced cells carrying the pET vector only showed any positive signals corresponding in prominence to those found in lanes 5 and 8 of pET*cbhl.2*(H). The signal intensity for the induced protein (whole cell extracts) from pET*cbhl.2*(H) remained almost constant throughout the chase periods of 45, 90 and 135 min (lanes 1-3) (Figure 2).

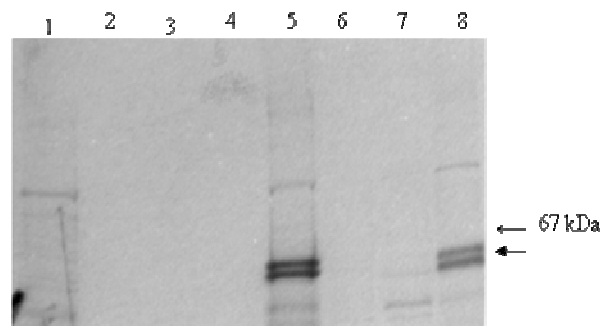


Figure 1. An autoradiograph showing signals from pulsed labelled proteins. Lanes 1-4 contained whole cell extract, soluble periplasmic, soluble cytoplasmic and insoluble fractions, respectively from induced pET only cells. Lanes 5-8 contained the same extract and fractions, respectively, as for the latter cells but these were obtained from induced pET*cbhl.2* cells. The top arrow indicates the estimated size of the nearest molecular mass standard. The bottom arrow points to the duplex bands in lanes 5 and 8.

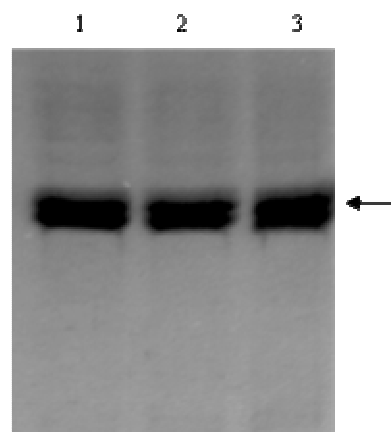


Figure 2. An autoradiograph of pulse-labelled and chased whole cell extract proteins from induced pET*cbhl.2*. Cells were labelled for 15 min and lanes 1-3 represent chased periods of 45, 90 and 135 min, respectively.

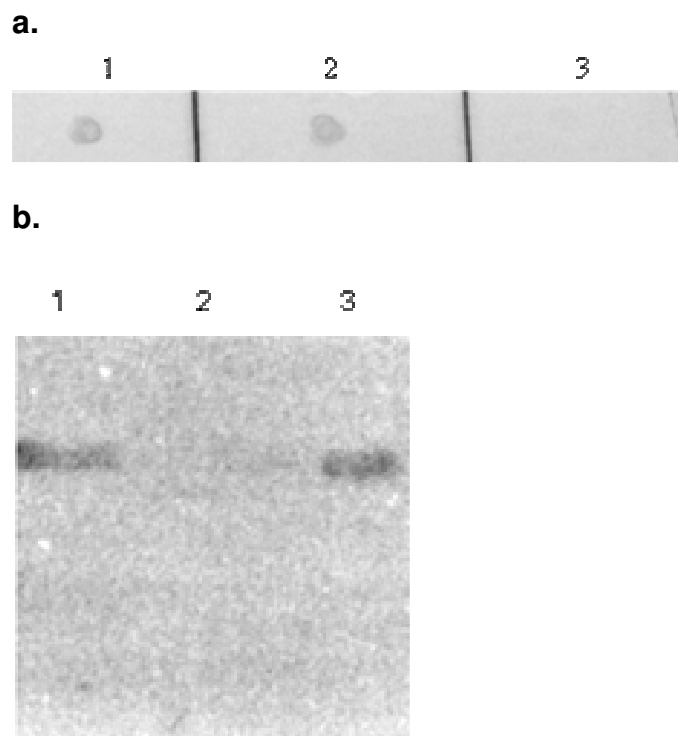


Figure 3. Dot Blot (a) and Western blot (b). a1 and b1, positive controls; a2 and b3, pET $cbhl.2(H)$; and a3 and b2, pET only.

Dot-blot and western blot

Positive reactions with CBH-No 3 antibody were observed in both the dot-blot and Western blot (Figures 3a and 3b, respectively) for insoluble fractions from induced pET $cbhl.2(H)$ and the positive controls but no reaction occurred with crude protein extracts from induced cells carrying the pET vector.

DISCUSSION

Although the cDNA coding for mature CBHI.2 was successfully fused in frame to the *peB* signal peptide sequence of the pET vector various enzyme assays showed no activity and no proteins consistent with the theoretical predicted size of approximately 56.2 kDa (correctly processed CBHI.2 without the *peB* signal peptide) or 58.7 kDa (CBHI.2 still containing *peB* signal peptide) were detected using SDS-PAGE analysis. These observations suggested that either no expression occurred or that a very low undetectable level of an inactive gene-product was made. Results from the pulse-labelling experiments demonstrated that expression did occur. Since the presumptive CBHI.2 protein was not present in the supernatant fluid or soluble fractions but only in the whole cell and insoluble fractions suggested that this protein was insoluble. Most eucaryotic proteins

expressed in *E. coli* often accumulate within the cell as insoluble protein aggregates or inclusion bodies which are biologically inactive (Guise et al., 1996; Cardamone et al., 1995; Doyle and Smith, 1996).

Two closely associated bands consistent with the predicted CBHI.2 size were observed in the insoluble fractions for induced pET $cbhl.2$. The sizes of these bands suggested that these proteins were two forms of presumptive CBHI.2; the top band represented unprocessed CBHI.2, still carrying the signal peptide amino acid sequence, and the lower band the fully spliced protein. It is interesting to note while the signal peptide appeared to have been correctly processed in one variant form of the presumptive CBHI.2, this protein did not appear to have been transported across the cytoplasmic membrane since no signal for this protein was detected in the periplasm. Transport of protein across the cytoplasmic membrane involves various transport factors, including various host cell proteins such as docking proteins, SRP and SecB-dependent complexes, and chaperons (Fekkes and Driessen, 1999). Addition of rifampicin inhibits host RNA polymerase and this might have limited expression of all the necessary host proteins required for efficient transport of presumptive CBHI.2.

The pET 22b is driven by a strong, *lac* inducible, selective T7 RNA bacteriophage promoter and the host BL21 cell carries the genes for a *lac* inducible T7 RNA polymerase that selectively drives transcription of genes cloned in-frame to the T7 RNA promoter. This unique vector-host combination allows for high-level expression of foreign genes, almost 50% of total cell proteins (Novagen), however, in the case of the presumptive CBHI.2 the expression level appears to be much lower since expressed proteins could not be detected by SDS-PAGE analysis but only by radioactive-labelling. Expression of eukaryotic genes in *E. coli* is not always efficient. Makrides (1996) reviews the various factors which might influence expression, some of these include: major differences in codon usage between the foreign gene and native *E. coli* genes, stability and translational efficiency of the foreign-gene's mRNA, unique and subtle gene sequence organisation differences, vector stability and copy number, inadequate protein folding, protein-degradation and toxicity of the foreign protein to *E. coli*. But in this study we eliminated the possibility that the low level accumulation of presumptive CBHI.2 was due to instability, i.e. the protein was significantly degraded by host proteases. Teeri (1987) observed that *T. reesei* CBHI expressed as a non-fused protein in *E. coli* was rapidly degraded by host proteolytic enzymes. Almost 50% of labelled proteins with abnormal structures were degraded within the first hour in *E. coli* (Prouty and Goldberg, 1972). Since the signal intensity remained constant during the chase periods and did not decrease with time suggested that the presumptive CBHI.2 was stable. So it is not clear which of the other factors were

responsible for the low level expression.

The size of the positive signals from the labelling experiment and the fact that these signals were only present in induced pET $cbh1.2(H)$ cells were compelling evidence that the protein might be CBHI.2 but this did not rule out the possibility of some spurious gene-product since none of the enzyme assays showed any cellulolytic activity. But the results from the dot- and Western blots make the latter highly unlikely and hence from the collective evidence it is concluded that a biological inactive CBHI.2 was successfully expressed from *E. coli*. A conclusion that is further supported and reported on in the second part dealing with the chemical refolding and enzymatic activity of CBHI.2.

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