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Full Length Research Paper

Characterisation of a chimeric *Phanerochaete chrysosporium* cellobiohydrolase expressed from *Escherichia coli*

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The aim of this study was to purify and analyse a *Phanerochaete chrysosporium cbh*I.1 gene-product expressed as an inducible, secreted, heterologous protein from an *Escerichia coli* pGEX*cbh* I.1 clone. Using glutathione Sepharose 4B affinity chromatography, the expressed protein was purified from the supernatant of an induced *E. coli* transformed with pGEX*cbh*I.1 and ran as a single band on a Sodium dodecyl sulphate-polyacrylamide gel. The glutathione S-transferase (GST) fused CBHI.1 was approximately 80 kDa in size, approximately 2.2 kDa smaller than the theoretically predicted size. The purified protein exhibited time dependent hydrolytic reaction against carboxy-methyl-cellulose (CMC) and Avicel. On CMC the highest hydrolytic reaction occurred at 120 min. whereas for Avicel it was at 150 min. Optimum pH and temperature for activity of the protein against these cellulose substrates were pH 6 and 55^{o}C, respectively, and the protein remained stable under these optimum conditions for 24 h.

Key words: *Phanerochaete chrysosporium*, cellobiohydrolase purification, heterologus expression.

INTRODUCTION

We previously reported in the African Journal of Biotechnology (Howard et al., 2003) on the successful cloning and expression of a functionally active *Phanerochaete chrysosporium* cellobiohydrolase (CBHI.1) protein from *Escherichia coli*. The heterologously expressed glutathione S-transferase (GST) fused CBHI.1 protein exhibited hydrolytic activities against derived carboxy-methyl-cellulose (CMC) and microcrystalline Avicel without requiring *in vitro* chemical refolding. This represents significant improvement on previous cloning and expression attempts (Howard, 1997; van Rensburg et al., 1996). This paper is a continuation of our previous publication (Howard et al., 2003) with the emphasise shifting towards describing the biochemical nature of the fused CBHI.1 protein. Here we report on the purification, characterisation and enzymatic activity of the fused CBHI.1 protein.

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MATERIALS AND METHODS

Clones

E. coli BL21 cells transformed with pGEX*cbh*I.1 and pGEX plasmids used in this study were constructed by Masoko (2001) and have been previously described (Howard et al., 2003).

Purification of fused protein

A single colony of a positive clone (pGEX*cbh*I.1) or transformants harbouring pGEX was inoculated into Luria-Bertani (LB) broth containing 50 µg/ml cabenicillin and grown overnight at 37^{o}C in a shaker incubator. The overnight cultures were diluted 1:40 into fresh LB broth containing the appropriate antibiotic and grown with shaking at 37^{o}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 for 2–3 h. After induction, the cells were spun- down at 2 449 x g for 10 min. at 4^{o}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 all 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 kept at 4^{o}C. The supernatant that did not pass through the membrane was collected, assayed or aliquoted, and stored at 4^{o}C. Affinity chromatography was performed using pre-packaged Glutathionine Sepharose 4B GST Purification Modules (Pharmacia Biotech) according the manufacturer's instructions to purify the fused protein present in the concentrated supernatant fluid.

Protein concentration determination

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

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE)

Denaturing 12% SDS polyacrylamide slab gels were prepared for the Bio- rad mini-gel system: samples were treated, loaded and proteins electrophoresed for 1–2 h at 100–200 constant voltage and the gels stained with Coomassie blue, washed and proteins visualised using standard methods (Sambrook et al., 1982).

Plate enzyme assays

Plate assays using purified protein from induced cells were performed according to the method of Teather and Wood (1982) on both 0.5% w/v CMC or 0.5% w/v Avicel, separately. About 5–10 µg/ml purified protein was loaded in a well in the centre of the plate. The inoculated plates were placed in a plastic box containing soaked tissue paper, the lid of the plastic box was closed and the reaction were incubated at 37^{o}C for 3–4 days. The plastic box with soaked tissue paper provided a humid environment that prevented 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. A clearing zone surrounded by a red background was indicative of the hydrolysis of CMC or Avicel.

Reducing sugar assay

The quantification of hydrolysis of 0.5% w/v CMC and 0.5% w/v Avicel, separately, by the purified protein was determined using the standard method of Bailey and Poutanen, (1989). One international unit (IU) of enzyme activity was defined as the amount of enzyme producing 1 µmol of reducing sugars in glucose equivalents per min under the assay conditions.

pH Effect assay

The following buffers were used at 50 mM concentration: citrate phosphate (pH 3–7), phosphate (pH 8), and carbonate bicarbonate (pH 9–10). In the various buffers, substrate was added and the reducing sugar assay was carried out in 50 mM citrate phosphate buffer pH 6 at 55^{o} C. For pH stability studies, the enzyme was preincubated for 24 h at the different pH in the appropriate buffer; substrate was then added and the reaction performed in 50 mM citrate phosphate buffer pH 6 at 55^{o}C.

Temperature effect assay

The effect of temperature on the enzyme activity was conducted under temperatures ranging from 25–105^{o}C. The reducing sugar assay was conducted at the various temperatures in 50 mM citrate phosphate buffer pH 6 for 55^{o}C. For thermal stability, the protein was pre-incubated for 24 h at the various corresponding temperatures and the residual activity was assayed in 50 mM citrate phosphate buffer pH 6 for 55^{o}C.

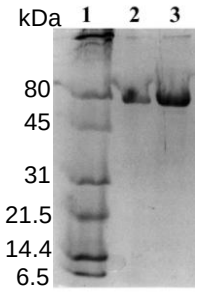

Figure 1. SDS- PAGE of the purified GST-CBHI.1 protein. Lane 1, molecular mass standards. Lanes 1 (5 µg) and 2 (10 µg) of the GST-CBHI.1 protein.

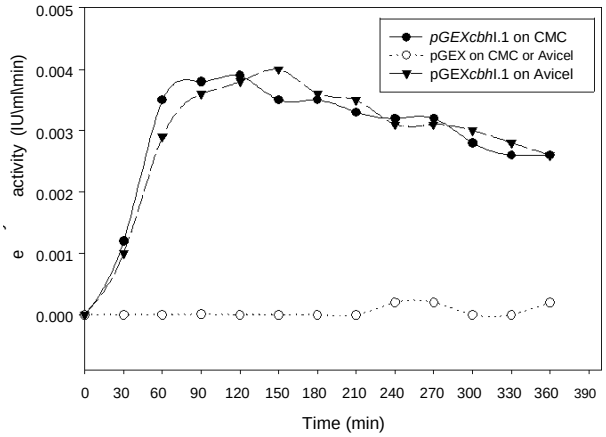

Figure 2. Hydrolysis of CMC and Avicel profiles.

RESULTS

SDS-PAGE and enzymatic profiles

A single dominant protein band of approximately 80 kDa was observed when the purified protein was electrophoresed on SDS- PAGE (Figure 1). This purified protein exhibited a time dependent hydrolytic reaction against CMC and Avicel (Figure 2) whereas 10-fold concentrated supernatant from induced, negative control, host carrying pGEX reaction was almost constant around

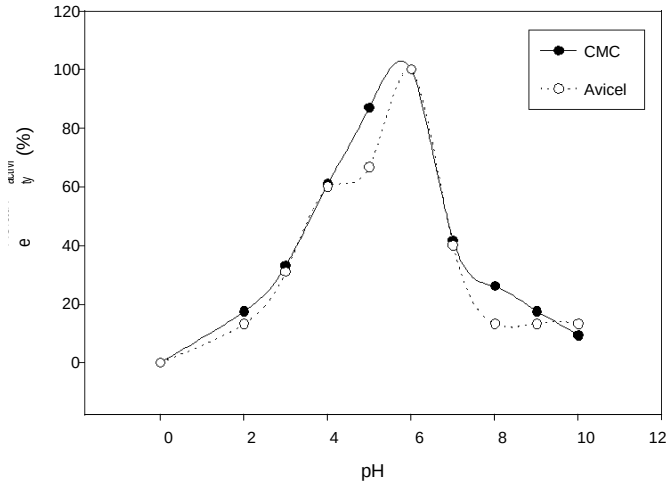

Figure 3. Optimum pH of GST-CBHI.1 on CMC and Avicel.

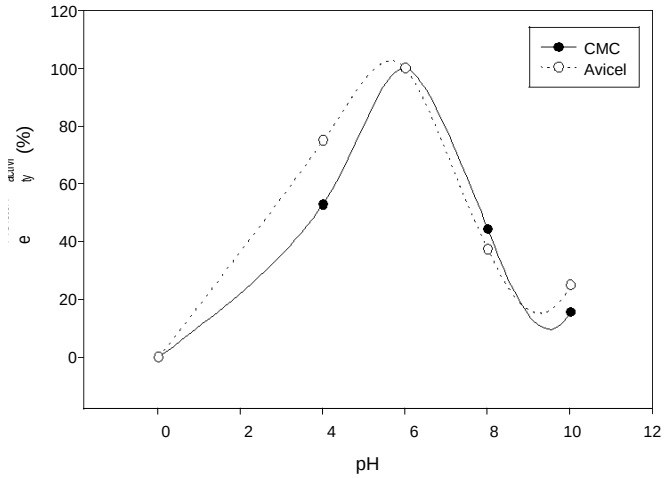

Figure 4. pH stability of GST-CBHI.1 on CMC and Avicel.

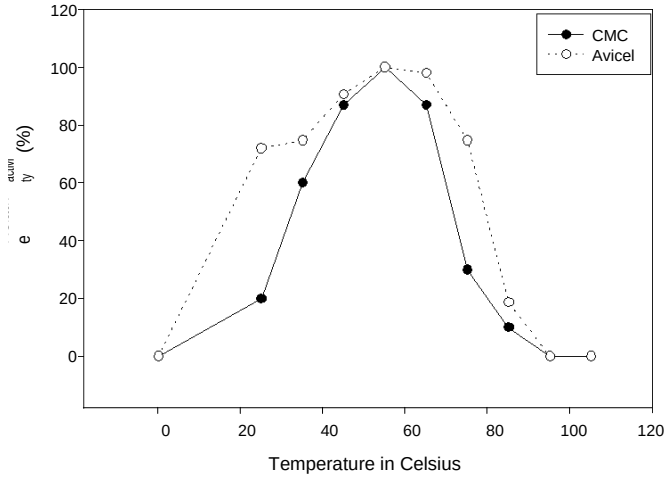

Figure 5. Optimum temperature of GST-CBHI.1 on CMC and Avicel.

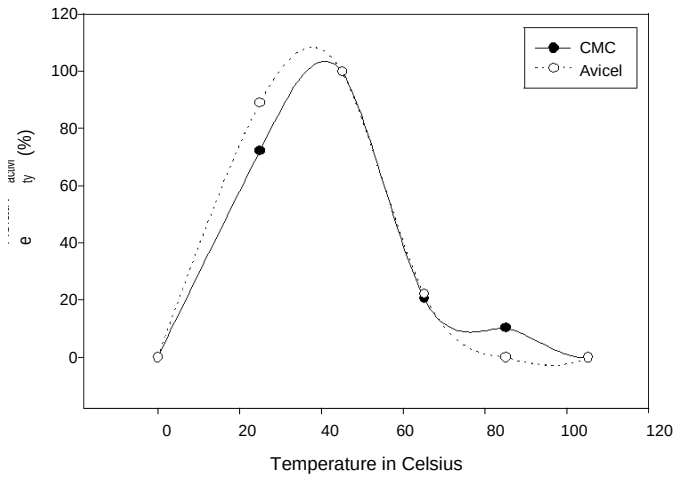

Figure 6. Temperature stability of GST-CBHI.1 on CMC and Avicel

the zero reading. On CMC the highest hydrolytic reaction occurred at 120 min whereas for Avicel it was at 150 min.

Optimum pH and temperature, pH and thermal stability

The optimum pH for the purified protein on both CMC and Avicel was at pH 6 (Figure 3) and at this pH the expressed protein remained stable for 24 h (Figure 4). The optimum temperature for the purified protein on both CMC and Avicel was 55^{O}C (Figure 5) and this protein remained stable at this temperature for 24 h at pH 6 retaining almost 100% of its residual activity (Figure 6).

DISCUSSION

A *cbh*I.1 cDNA (Z22528 in the EMBL/Genbank/DDBJ gene-libraries) fragment of approximately 1.6 Kbp was inserted into the pGEX vector. From the published sequence data of *cbh*I.1 cDNA (Sims et al., 1994) the open reading frame would encode a protein of approximately 516 amino acids. But the engineering of an additional *Hin*d III site up-stream at the 5 -end of *cbh*I.1 when it was cloned into a pET vector to generate clone pET*cbh*I.1 (Howard, 1997) resulted in the removal of the open-reading frame encoding the leader peptide of *cbh*I.1. Therefore, the expected extrapolated gene product would be smaller by 19 amino acids. Essentially the *cbh*I.1 cDNA cloned into the pGEX vector to generate the pGEX*cbh*I.1 clone (Masoko, 2001; Howard et al., 2003) should encode a predicted protein of approximately 497 amino acids (± 55.2 kDa). The gene-sequence encoding the glutathione-S -transferase, the sequence to which *cbh* I.1 was fused in the pGEX vector, encodes a protein of 26 kDa. (Pharmacia Biotech). Therefore, a fused-gene (GST-*cbh*I.1) product of approximately 82.2

kDa was expected, however a product of approximately 80 kDa was obtained which was approximately 2.2 kDa smaller then expected. Re- digestion of the purified pGEX*cbh*I.1 in a double digest with *Bam* HI and *Not* I, the original restriction enzymes used to cut *cbh*I.1 from the pET*cbh*I.1 when it was cloned into the pGEX vector (Masoko, 2001) released a fragment of approximately 1.6 kDa (results not shown) demonstrating that the fragment with the expected size corresponding to *cbh*I.1 was indeed cloned. Moreover, the expected GST-CBHI.1 protein did bind to glutathionine-Sepharaose 4B and was eluted as a single band suggesting that the GST was successfully fused to CBHI.1 since binding to the affinity matrix is specific for GST and the resultant product is certainly larger than the GST protein on its own. The induced gene-product was biologically active suggesting that the *cbh*I.1 gene was successfully fused in-frame to the sequence encoding GST. The slight variation in size of the actual translated product from the theoretical determined size of the expected chemic produced is still unclear and more precise measurements of the protein need to be done.

In conclusion, the pGEX vector system appears to be an ideal system for the expression of fully functional CBHI.1 proteins, which retain their expected hydrolytic activity against derived and native cellulose substrates. We propose that using this system the various *P. chrysosporium cbh*I-like genes can be cloned and expressed, which will permit us to begin to understand the complex nature of the various *cbh*I-like transcripts and their role in cellulose degradation in *P. chrysosporium*.

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