International Scholars Journals

African Journal of Virology Research ISSN 2756-3413 Vol. 14 (4), pp. 001-004, April, 2020. Available online at www.internationalscholarsjournals.org © International Scholars Journals

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Full Length Research Paper

Refolding and characterisation of a heterologous expressed *Phanerochaete chrysosporium* cellobiohydrolase (CBHI.2)

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Accepted 17 September, 2019

Cloned *Phanerochaete chrysosporium* ME446 *cbh*I.2 cDNA was successfully expressed in *Escherichia coli* as an insoluble, internal, biologically inactive protein. *In vitro* chemical refolding restored the activity of the crude CBHI.2. However, this enzyme was active against 4-methylumbelliferyl-ß-D-cellobioside (MUC) and 4-methyllumbelliferyl-ß -D- lactopyranoside (MUL) substrates only. The crude enzyme lost almost 50% of its activity at 5 min at 100^{o}C heat treatment whereas total inactivation was achieved at 30 min.

Key words: *Phanerochaete chrysosporium*, CBHI.2, chemical refolding, heat-inactivation.

INTRODUCTION

Most eucaryotic proteins expressed in *Escherichia coli* often accumulate within the cell as insoluble aggregates or inclusion bodies which are biologically inactive (Guise et al., 1996; Cardamone et al., 1995; Doyle and Smith, 1996). The reasons cited for formation of insoluble proteins in *E. coli* include: secretary sequence may obstruct folding; the cytoplasm is a reducing environment that may decrease the stabilising effect of disulfide bonds; and *E. coli*'s inability to glycosylate proteins. Insoluble protein aggregates arise from partially folded intermediates whereas excessive associations of various intermediate forms of the protein arising during the refolding process result in inclusion body formation. Inclusion bodies are not exclusively composed of the expressed protein; it was found that contaminating polypeptides constituted 5-50% and phospholipids 0.5-13% of inclusion bodies depending on the growth conditions of the organism (Middelberg, 1996).

The information in the primary amino acid sequence of a particular protein allows the protein to attain a conformation in an autonomous way under the correct conditions (Anfinsen, 1973). However, the role of other factors such as chaperones, cofactors, pH and ions within the cell have been recognised as other important facilitators of "correct folding" (Mitraki and King, 1989). *In vitro* chemical refolding using various chemicals, and other methods (varying of induction time, temperature and amount of induction agent, and the type of media) have been successfully used to obtain active soluble proteins (Mitraki and King, 1989; Forciniti, 1994; Guise et al., 1996; Kopetzki, et al., 1989) . The amount of active, fully refolded proteins recovered using chemical methods can vary between 1 to 90% depending on the initial concentration of insoluble protein as well as the size and type of protein. Chemical refolding of proteins is rather fortuitous and appropriate conditions have to be empirically determined for each protein.

CBHI.2 was successfully expressed in *E. coli* but accumulated inside the cell and the recovered protein was biologically inactive *in vitro* (Howard, 2005). Teeri (1987) and Laymon et al. (1996) made similar observations for heterologously expressed *T. reesei* CBHI. They found that *T. reesi* CBHI expressed from *E. coli* was localised in insoluble bodies and these had to be purified, and the protein chemically refolded *in vitro* before biological activity was restored. This paper reports on the investigation made to restore the biological activity of CBHI.2 using a chemical refolding method.

MATERIALS AND METHODS

Strains

Clone *E. coli* pET*cbh*I.2(H) and *E. coli* BL21(DE3)p*Lys* S cells carrying vector only were grown on Luria-Bertani (LB) broth or solid media containing 50 g/ml cabenicillin and 34 g/ml chloramphenicol at 37^{o}C.

Refolding of crude protein extracts

Crude protein extracts were obtained as previously described (Howard, 2005). Crude proteins were refolded using the procedure described by Laymon et al. (1996) and essentially involved the following: urea was added to a final concentration of 8 M to the clear cell lysate (crude protein extract obtained after sonication) and denaturation of the proteins was allowed to proceed at 4^{o}C overnight with gentle shaking. 10 mM DTT, 174 g/ml PMSF and ß-mercaptoethanol (1:1000) were added and the lysate was transferred to dialysis tubes (Spectra Por® cellulose ester membrane with a 5000 molecular weight cut- off) and dialysed against 1 l of 50 mM Tris buffer pH 8 containing 5 mM $CaCl_2$ for 2 days using 3 buffer changes (after 2 h the first buffer change was made and dialysis proceeded overnight, the next morning the buffer was again changed and dialysis proceeded for another day) at 4^{o}C with gentle stirring. After dialysis the protein was concentrated with an Amicon ultra- filtration unit fitted with a PM10 Amicon membrane (cut-off, 10,000) at 4^{o}C. Insoluble proteins were removed from the crude protein extract by centrifugation at 13,000 rpm for 2 min, and the supernatant -fluid was assayed immediately. For long-term storage 10 mM DTT and 0.002% (finale concentration) sodium azide were added and the aliquotes were kept at -20^{o}C.

Enzyme assays

Plate assays using crude enzyme extracts 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. Crude enzyme extracts were assayed for activity using methods described by Bailey and Poutanen (1989) for the formation reducing sugars from 0.5% w/v, CMC or Avicel in 50 mM citrate buffer pH 6 at 55^{o}C. Alternatively, 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 -ß-D-cellobioside/lactopyranoside (MUC or MUL) (Sigma) in 50 mM sodium acetate buffer pH 5 at 50^{o}C. The second method described by Cummings and Fowler (1996) involves the hydrolysis of 2 mM p-Nitrophenyl-cellobioside/lactoside (PNP-C or PNP-L) in 50 mM sodium acetate buffer pH 5 at 30^{o}C. For the MUC and MUL assays, specific enzyme activity was defined as mol of MU formed per minute per mg of crude enzyme under the assay conditions.

Protein concentration

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

Protein heat inactivation

Crude enzyme was incubated at 100^{o}C in a water-bath and at various time-intervals (0 to 30 min) aliquots were removed, cooled on ice and stored at room temperature for 5 to 10 min. These aliquots were assayed for residual activity on MUC using the method described by Laymon et al. (1996) for hydrolysis of 2 mM MUC in 50 mM sodium acetate buffer pH 5 at 50^{o}C.

RESULTS

Enzyme assays

Activity was detected on MUC and MUL substrates only (Figure 1). The unfolded crude proteins from pET*cbh*I.2(H) were negative. Similarly crude folded and unfolded proteins from cells carrying the vector only were also negative. The specific enzyme activities of the crude enzymes are shown in Table 1. The activities on the two substrates are comparable with only a 1.35 mol min^{-1} mg^{-1} increase in activity against MUC.

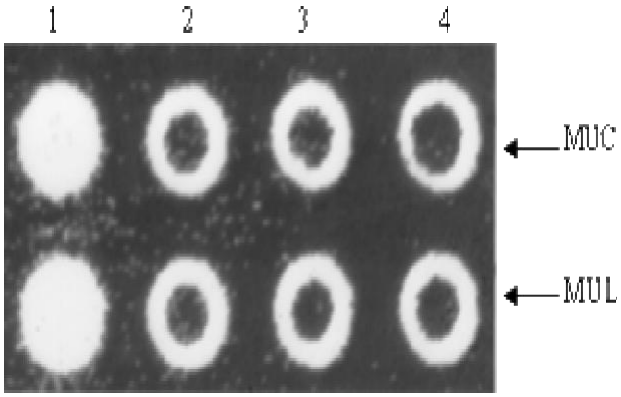

Figure 1. A photo of a micro-titre exposed on a uv-transilluminator. The filled white spots indicate release of fluorescent MU (positive reaction) while the black spots surrounded by white rings are wells where no reaction occurred. Lanes 1 and 2 contained folded and unfolded crude enzyme extracts from pET*cbh*I.2, respectively. Lanes 3 and 4 contained folded and unfolded crude enzyme extracts from pET, respectively.

Table 1. Specific enzyme activity of crude refolded CBHI.2

Substrates	Specific enzyme activity (mol min^{-1} mg^{-1})
MUC	2.15
MUL	1.6

Protein heat inactivation

Almost 50% of the activity was lost at 5 min of heat treatment at 100^{o}C whereas total inactivation was achieved at 30 min (Figure 2).

DISCUSSION

The refolded crude enzyme exhibited activity against fluorgenic-cellobioside and lactoside only. Similar

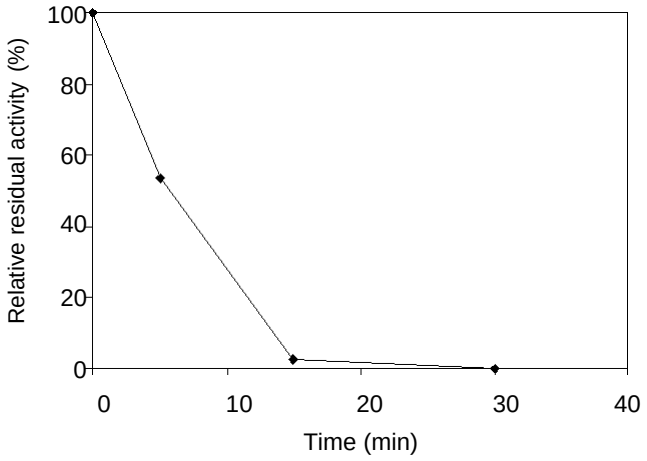

Figure 2. Relative residual enzyme activity on 4-methylumbelliferyl-ß-D-cellobioside (MUC).

findings were made for a heterologously expressed and chemically refolded *T. reesei* CBHI (Laymon et al., 1996). Cellobiohydrolases hydrolyse β-1,4-glycosidic bonds of cellulose removing cellobioside units from the reducing-end and is also able to split cellobiose and lactose from fluorogenic-linked substrates (Rabinovich et al., 2002). But the crude enzyme used in this study showed substrate "preference". Similar substrate "selectivity" was observed for another heterologously expressed *P. chrysosporium* cellobiohydrolase (CBHI.1) that was chemically refolded (Howard, 1997). But a secreted, heterologously expressed CBHI.1 was active against microcrystalline and derived cellulose substrates (Howard et al., 2003, 2004). Similarly other heterologously expressed cellobiohydrolases which did not require refolding exhibited activity against the other cellulose substrates (Takashima et al., 1996; Haakana et al., 2004; Ye et al., 2001). So it appears that substrate "selectivity" of CBHI.2 is due to some protein structural conformational changes that might have been induced during the chemical folding process. Conducting similar enzymatic studies on a natural folded CBHI.2 will assist in clarifying these initial findings.

Although enzyme activities against cellobioside and lactoside substrates were rather low, they are comparable to values obtained for *T. reesei* CBHI (Laymon et al., 1996) on the same fluorgenic substrates. Other reported cellobiohydrolases show greater activity against lactoside substrates (Boer and Koivula, 2003) whereas this crude CBHI.2 seem to prefer cellobioside as a substrate. The findings of this study seem to suggest that CBHI.2 might be more heat resistant then CBHI.1 which lost almost 100% activity at 5 min at 100^{o}C (Howard, 1997). Caution should be exercised in making any substantive deductions with respect to the enzyme's substrate selectivity and heat resistance since these findings are based on studies conducted on the crude enzyme. Also, more extensive studies (Daniel et al., 1996) are required on the heat resistance/tolerance.

Studies conducted on a purified secreted CBHI.2 will certainly provide more informative data.

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