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On-Column Refolding of Recombinant Fungal Endoglucanase

Mohd Jamil Aizat Jamaluddin and Hamzah Mohd. Salleh

Bioprocess and Biomolecular Research Unit (BPMERU), Department of Biotechnology Engineering, International Islamic University Malaysia, Jalan Gombak, P. O. Box 10, 50728 Kuala Lumpur, Malaysia.

Abstract: Endoglucanase is an industrially important enzyme involved synergistically in degradation of cellulosic materials into constitutive monomers with other cellulase enzymes. In this study, overexpression of $(His)_6$-tagged endoglucanase from *Fusarium oxysporum* sp. in *E. coli* BL21(DE3) resulted to high tendency of endoglucanase in the form of inactive inclusion bodies when the host cells were cultured between at 37°C and induced by 0.20 mM (final concentration) IPTG. A simple and time-efficient on-column refolding scheme using immobilized metal-chelate affinity chromatography (IMAC) aided by AKTA purifier, an automated fast protein liquid chromatography (FPLC) system afforded highly purified refolded recombinant endoglucanase that was active in carboxymethyl cellulose (CMC) assay. The on-column refolding scheme is reliable and can be applied for efficient and robust method for lab-scale production of recombinant endoglucanase .

Key words: On-column refolding; recombinant fungal endoglucanase; inclusion bodies; FPLC.

INTRODUCTION

It has been known that expressing eukaryotic proteins in prokaryotic expression system like *Escherichia coli* has been an ideal productive source for straightforward industrial applicability. This, as a result, has raised many studies and developments involving recombinant enzyme production, in particular endoglucanase, in *E. coli* due to its proven feasibility and effectiveness in terms of inexpensive carbon source requirements for growth, rapid biomass accumulation and relatively simple scale-up process (de Marco, 2009; Sahdev *et al.*, 2008; Neubauer, 2006; Weickert *et al.*, 1996; Sorensen *et al.*, 2003; Sorensen and Mortensen, 2005).

However, it has also gradually become a frustration since generally while a considerable amount of recombinant fungal endoglucanase in *E. coli* have indeed been produced, most of it is not soluble. Instead it is persistently found as an inactive insoluble protein aggregates known as inclusion bodies. This formation of insoluble aggregates owing to the high, mostly non-natural expression of proteins has been considered to be an unspecific process driven by contacts between partially folded or unfolded peptides, which are believed to be sourced from *E. coli*'s lack of post-translational modifications required by eukaryotic proteins (de Marco, 2009; Eser *et al.*, 2009; Xu *et al.*, 2008; Xu *et al.*, 2008; Chen *et al.*, 2008; Berndt *et al.*, 2008).

Methods to recover soluble and functional protein from inclusion bodies have been specifically involved unfolding insoluble aggregates and refolding back into its native form (Veldkamp *et al.*, 2007; Gao *et al.*, 2003; Fahey *et al.*, 2000; Jin *et al.*, 2004; Hahm and Chung, 2001; Altamirano *et al.*, 1997; Li *et al.*, 2003 Yoshii *et al.*, 2000; Mayer and Buchner, 2004; Fahey and Chaudhury, 2000; Cho *et al.*, 2001; Shimizu *et al.*, 1999; De Bernardez Clark, 1998; Misawa and Kumagai, 1999; Werner *et al.*, 1994; Tresaugues *et al.*, 2004). The unfolding and refolding processes required for recovering proteins in native form are complex and not always convenient from an industrial point of view. That is probably the reasons why mostly they are considered undesirable. Nonetheless, still they are sometimes become the method of choice if the problem persisted, especially in preparative-scale production. The major obstacles is that both processes often result in poor recovery yields, and often the requirement for optimal protein biological activity and resolubilization protocols affect at some degree their native structure (Mayer and Buchner, 2004; Tresaugues *et al.*, 2004; Miot and Betton, 2004; De Bernardez Clark, 2001; Smith and Walker, 2003; Jurgen *et al.*, 2010). Hence, in light of this, the aim of the present study was to produce soluble and functional recombinant fungal endoglucanase using a "two in one" strategy: affinity chromatography purification combined with an on-column refolding approach using a fast protein liquid chromatography AKTA purifier system.

MATERIALS AND METHODS

Bacterial Strain and Plasmid:

Recombinant *E. coli* strain BL21(DE3) harboring pET28a-wtEGI plasmid construct was kindly provided by Professor S.G. Withers and Professor A.J. Warren from the University of British Columbia, Vancouver, Canada.

Corresponding Author: Hamzah Mohd. Salleh, Department of Biotechnology Engineering, International Islamic University Malaysia, JalanGombak, P.O. Box 10, 50728 Kuala Lumpur, Malaysia.
E-mail: hamzah@iium.edu.my

The pET28a-wtEGI plasmid construct contained a gene that codes for an endoglucanase from *Fusarium oxysporum* sp.

Shake Flask Culture:

A starter culture of *E. coli* BL21(DE3) harboring pET28a-wtEGI plasmid construct was prepared first by transferring 10 ml of LB/kanamycin (50 µg/ml, final concentration) stock solution into a sterile 50-ml tube and was grown overnight for approximately 16 hours at 37°C and 250 rpm. A 2.5 ml (1:100) of the overnight culture was inoculated into a 1-L Erlenmeyer flask containing 250 ml LB/kanamycin medium (50 µg/ml, final concentration) which was prepared in quadruplet. The culture was incubated at 37°C and 250 rpm, and induced at A_{600}~0.6 with 0.2 mM IPTG (final concentration) and incubated again at 37°C and 250 rpm for approximately six hours before cell harvest.

Preparation of Unfolded Endoglucanase Sample:

E. coli cells were harvested by centrifugation at 7000 x *g* for 10 minutes at 4°C and the supernatant was discarded. To prepare sample of unfolded endoglucanase, the cell pellet was resuspended in 15 ml Bugbuster™ protein extraction reagent (Novagen, Darmstadt, Germany) containing 15 µl Benzonase (25 U/µl) and lysozyme (200 µg/ml, final concentration). The suspension was incubated on ice with occasional stirring for 20 minutes before centrifugation at 18,000 x *g* for 30 minutes. The supernatant was discarded and the cell pellet was resuspended with 1:10 diluted Bugbuster™ protein extraction reagent and centrifuged at 18,000 x *g* for 15 minutes. The rinsing step was repeated three times. Finally, the endoglucanase aggregates were solubilized (unfolded) by thoroughly mixing with denaturing lysis/binding buffer (final concentrations: 40 mM NaH_2PO_4 pH 7.8, 6 M urea, 0.5 M NaCl).

On-Column Refolding with IMAC Aided by FPLC:

On-column refolding performed throughout this study was done using a AKTA purifier FPLC system (GE Healthcare Bio-Sciences, USA). The setups of the FPLC system as well as the theoretical curves for on-column refolding as illustrated in figure 1 were followed as recommended by the manufacturer.

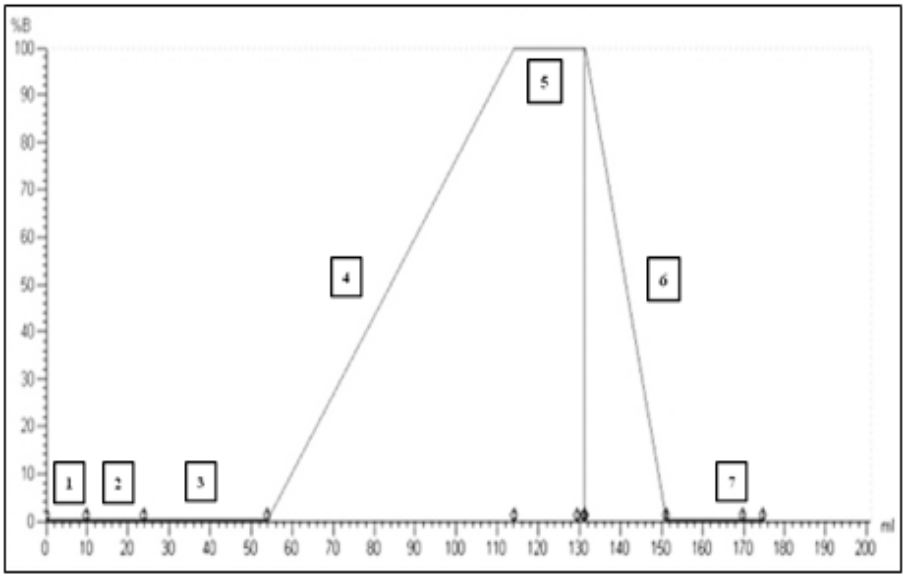

Fig. 1: On-column refolding's theoretical curves. Numbered from left to right: (1) Column equilibration - 10 minutes; (2) Sample injection; (3) Wash of unbound proteins - 20 minutes; (4) Gradient refolding - 60 minutes; (5) Delayed gradient refolding - 20 minutes; (6) Elution - 20 minutes; (7) Re-equilibration - 20 minutes.

A glass chromatography column (0.7 x 10 cm, Bio-Rad, USA) was used and filled with 4 ml bed volume of Ni-NTA His•bind resin (Novagen, Germany) pre-equilibrated with equilibration buffer (40 mM NaH_2PO_4 pH 7.8, 6 M urea, 0.5 M NaCl). The system was set to run at 1 ml/min, and started with equilibration of the column with washing buffer from port A (40 mM NaH_2PO_4 pH 7.8, 6 M urea, 0.5 M NaCl, 20 mM imidazole). After a 5 ml protein sample (fixed) was loaded, the unbound proteins were washed away by addition of the previous washing buffer from port A. At this point, the unfolded endoglucanase bound to the Ni-NTA His-•bind resin

was able to refold by washing the column, gradient-wise, with the refolding buffer from port B1 (40 mM NaH_2PO_4 pH 7.8, 0.15 M NaCl). Finally, as the system reached the elution phase, the system was immediately and manually paused and the pump tubings from port B1 and A were switched to elution buffer in port B2 (40 mM NaH_2PO_4 pH 7.8, 0.15 mM NaCl, 50 mM EDTA) and refolding buffer respectively to elute the Ni-NTA His•bind bound $(His)_6$-tagged endoglucanase.

Elution fractions of 1 ml under the elution peak were identified, and analyzed by SDS-PAGE for verification of the presence of recombinant endoglucanase. From the SDS-PAGE verification step, the fractions were pooled accordingly, and dialyzed three times against 40 mM NaH_2PO_4 pH 7.8 to remove EDTA, otherwise, the recombinant endoglucanase activity would be greatly diminished. The dialyzed recombinant endoglucanase was then concentrated using Amicon ultra (3,000 MWCO) and analyzed by enzyme assay.

Carboxymethyl Cellulose (CMC) Assay:

The activity of recombinant endoglucanase was assayed in triplicate using CMC as the substrate. In each assay contains 50 µl of 2% CMC (prepared in ultra-pure water) and 10 µl citrate buffer (0.5 M, pH 4.8). The hydrolysis of CMC was initiated by addition of 40 µl of purified enzyme. After 30 minutes of incubation at 37°C, the reaction was terminated with 100 µl DNS reagent and then heated at 95°C for ten minutes. Following the color development, 200 µl of each sample was transferred to a flat-bottomed 96-well microplate and the absorbance at 550 nm was measured using an Infinite® M200 plate reader (Tecan Group Ltd., Switzerland).

RESULTS AND DISCUSSION

In this study, a recombinant endoglucanase was expressed under conditions that promote production in the form of inclusion bodies as they are also the source of relatively pure polypeptides due to the fact that parts of them consist mostly the overexpressed recombinant proteins and with little contaminating molecules [32-35]. Hence, they could be used as a source of relatively pure misfolded proteins when refolding yields the functional and soluble proteins, which are easily separable from cellular debris or can be isolated with a reasonably purity by simple detergent-washing procedures (Mayer and Buchner, 2004; De Bernardez Clark, 1998; Werner *et al.*, 1994; Tresaugues *et al.*, 2004; Miot and Betton, 2004; De Bernardez Clark, 2001; Smith and Walker, 2003; Jurgen *et al.*, 2010; Rufdolph and Lilie, 1996; Fahnert *et al.*, 2004; Gergiou and Valax, 1999; Patra *et al.*, 2000; Villaverde and Mar Carrio, 2003; Batas and Chaudhuri, 1996; Zardeneta and Horowitz, 1994; Middelberg, 2002; Lin and S.Y., 1991; Shi *et al.*, 1997; Datar *et al.*, 1993).

Charged Ni-NTA His•bind resin was selected due to its specificity for chelating $(His)_6$-tagged recombinant protein. Refolding of the bound $(His)_6$-tagged endoglucanase was performed using of a 60 minutes long linear 8 to 0 M urea gradient at a flow rate of 1 ml/min. Elution of the refolded protein was performed using a 20 to 500 mM imidazole gradient.

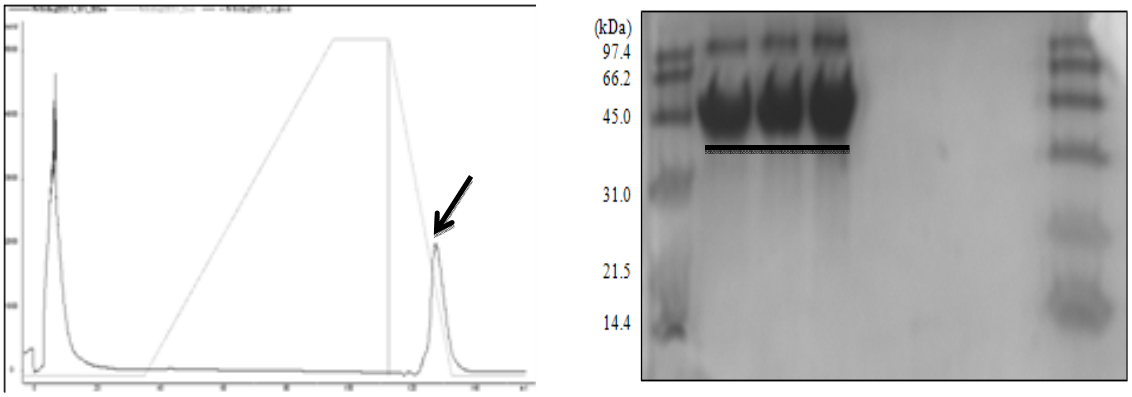

Fig. 1: Chromatogram results (left) from the first attempt of on-column refolding and its SDS-PAGE analysis (right) where each underlined lane represents the purified recombinant endoglucanase (underlined). The arrow points at the elution peak.

Figure 2 and 3 illustrate the results from the on-column refolding procedure. The chromatogram curve for each run shows a consistent and sharp resolved elution peak (indicated by an arrow). This single peak indicates that the presence of 0.5 M NaCl in the buffer was sufficiently able to suppress non-specific interactions between the charged Ni-NTA His•bind resin and the recombinant enzyme. In comparison to approach used by Mitsumori and Minato (2000), gradual dialysis against decreasing concentrations of urea (8 M - 4 M - 2 M) was applied for

renaturation of the recombinant protein after it had been purified using a commercially pre-packed column, HisTrap™ (GE Healthcare Bio-Sciences, USA). This approach, however, only afforded a partially purified recombinant *Fibrobacter succinogenes* endoglucanase F (EGF) (Lin and S.Y., 1991). As for other instances, Sun *et al.,* (2011) applied size exclusion chromatography (SEC) and tested both urea and pH gradients for the on-column refolding of DT389-hIL13 on Superdex-75. The recombinant protein highest purity was reported around 95% and also with high level of biological activity, however the methods applied were quite extensive and time-consuming considering the steps that had been used throughout the course of purification (Shi *et al.,* 1997).

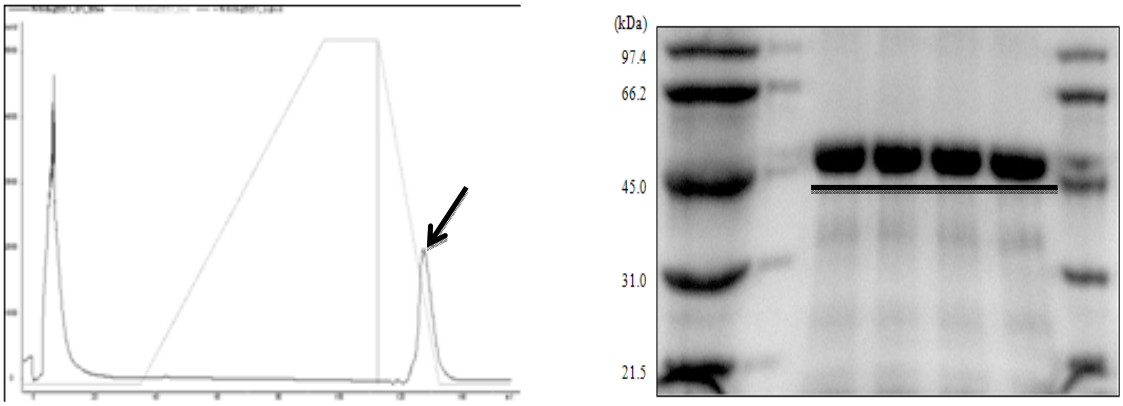

Fig. 2: Chromatogram results (left) from the second attempt of on-column refolding and its SDS-PAGE analysis (right) where each underlined lane represents replicates of the purified recombinant endoglucanase (underlined). The arrow points at the elution peak.

In the present work, SDS-PAGE analyses (figure 2 and 3) indicate high degree of enzyme purity with a consistent single thick protein band at the expected size of ~45kDa. These results show the on-column refolding protocol used throughout this study is reproducible in term of recovery and purity of the recombinant protein. Also, we can say that under these chromatographic conditions, the recombinant endoglucanase was able to unfold and refold quite well as the properly refolded enzyme was indeed active as indicated by the CMC assay results. As enzyme incubation time is prolonged the net absorbance readings increases as a result of more reducing sugar ends - present in smaller CMC oligomers - react with DNS reagent.

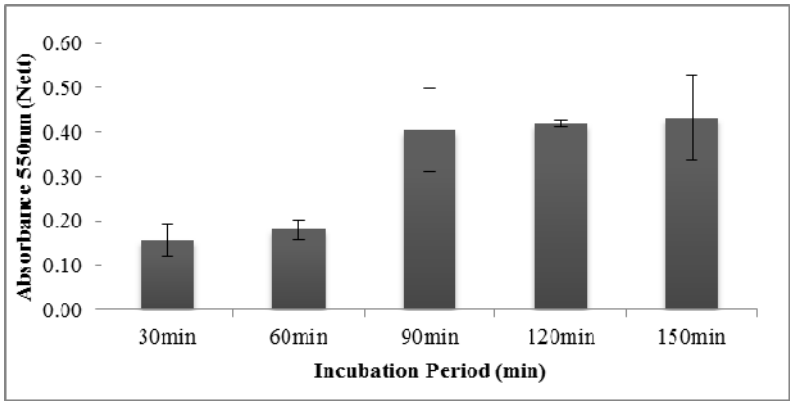

Fig. 4: Net absorbance (at 550nm) in CMC assay at different incubation period indicating functionality of the refolded recombinant endoglucanase.

Conclusion:

The approach used to unfold and refold the recombinant endoglucanase on-column using automated fast protein liquid chromatography system was found to be; (1) reliable - the recombinant enzyme was able to hydrolyze CMC substrate at significant level indicating its functionality, (2) robust - as there is no indication of substantial non-specific interactions between the recombinant enzyme and the charged Ni-NTA His•bind resin, and (3) time-efficient - the procedure only took two and a half hours to complete and required a single-step purification to achieve a high enzyme purity.

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