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Temperature effect on expression of recombinant human prethrombin-2 in *Escherichia coli* BL21(DE3) ArcticExpress

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Abstract:

Many proteins produced in *E. coli* accumulate in inclusion bodies. This study aims to detect the role of temperature in reducing the formation of inclusion bodies during recombinant human prethrombin-2expressed in *E. coli* BL21 (DE3) Arctic Express host. In this study, we created a host growth curve to find out the right time to add an inducer. The inducer used in our experiment was IPTG 0.1 mM. The fermentation process use a temperature of 12°C and 22°C. The results showed that recombinant human prethrombin-2 was successfully expressed as protein soluble using an optimum temperature of 12°C in *E. coli* BL21 (DE3) Arctic Express. It was indicated from the 63kDa protein band obtained from the soluble fraction on SDS-PAGE. The higher temperature of fermentation increased the amount of protein in the insoluble fraction due. It concluded that the fermentation temperature affect the rate of prethrombin-2 expression.

Keywords: *E. coli* BL21(DE3) ArcticExpress, prethrombin-2, soluble, temperature

Introduction

The protease α-thrombin is a key enzyme that functions both as a procoagulant and anticoagulant. Thrombin can be potentially applied in replacing suture technic (Spotnitz and Prabhu, 2005). Fibrin glue as a bioadhesive material composed of fibrinogen, thrombin, calcium chloride and factor XIII, is designed to resemble the final stages of coagulation by forming a fibrin clot. When used in wounds, these exogenous components can help endogenous proteins to reach hemostasis (Laurens, 2006). The principle is that fibrinogen is converted into fibrin monomers by thrombin, then fibrin monomers are modified by factor XIII and calcium chloride becomes a fibrin polymer as the final form of fibrin clots through cross-linking (Bonatti et al. 2007).

Prethrombin-2 (a single polypeptide of 308 residues which corresponding to residues from Thr272 to Glu579 in prothrombin), which is a product produced by the removal of the Gla domain and two kringle domains from prothrombin, is known to be the smallest single-chain precursor to α-thrombin (Choi et al. 1989; Yonemura et al. 2004). Prethrombin-2, structurally has one glycosylation site (at Asn373) and four disulphide bonds (Cys293-Cys439, Cys348-Cys364, Cys493-Cys507, and Cys521-Cys551) (Soejima et al., 2001). Pretrombin-2 can be converted to thrombin by FXa enzyme activators (Owen et al. 1974), or with ecarin, namely metalloprotease in the venom of snake *Echis carinatus* species (So et al. 1992; DiBella et al. 1995; Jonebring et al. 2012; Yonemura et al. 2004; Subroto et al. 2016).

In plasma, thrombin is formed from prothrombin which consists of several domains, whereas recombinant thrombin usually originates from the expression of the prethrombin-2 gene.

Production of recombinant thrombin precursors has also been described in mammalian Chinese hamster ovary cells, e.g., meizothrombin (Côté et al. 1994), prethrombin-1 (Bishop et al. 2006), and prethrombin-2 (Russo et al. 1997).There are also attempts to prepare an active recombinant human thrombin with the use of *E. coli* expression. *E. coli* remains the system of first-choice for expressing proteins, as it is cheap, easy to be handled, and has short-life cycle (Cabrita et al. 2006). Additionally, the genome of *E. coli* well studied, so it can be easily to be manipulated. This system remains to be one of the simplest and most preferred bacterial expression systems for heterologous expression of proteins for academic researches as well as of industrial and therapeutic recombinant proteins (Chen, 2012; Osadska et al. 2014). However, inability of *E. coli* to express proteins with high molecular weight, disulphide bond rich, or posttranslational modification become a bottleneck.Prethrombin-2 had previously been expressed in *E. coli* (Choi et al. 1989; So et al. 1992; DiBella et al. 1995; Soejima et al. 2001; Silaban et al. 2015; ; SIlaban et al. 2017).

Soejima et al. (2001), reported a new method of easy expression and efficient refolding of recombinant human prethrombin-2 using an *E. coli* expression system, which could be usable also for the pharmaceutical industry. The final yield of purified prethrombin-2 was 0.5–1%, which means that from 1 liter of *E. coli* bacterial culture only 1 mg of the recombinant protein could be recovered. Prethrombin-2 is expressed in *E. coli*, only a small amount of thrombin is active due to the formation of inclusion bodies (Freydell et al. 2007; Silaban et al. 2016).

Inclusion bodies is a dynamic structure that formed by an imbalance between the aggregates and soluble proteins from *E. coli*. Inclusion bodies formation can be caused by the partially folding of polypeptides. It occurs as are suil of accumulation of partial folding proteins that have been expressed in the aggregates forms through non-covalent hydrophobic interactions, ionic interactions, or both (Rizkia et al. 2015; Silaban et al. 2018).The formation of inclusion bodies in recombinant protein expression in *E. coli* can be minimized by reducing the temperature and expression rate during the production process (Freydell et al. 2007; Hartinger et al. 2010).

Materials and Methods

Strains, chemicals, vector and medium

Transformation and cloning was performed using *E. coli* TOP10F' (Invitrogen, USA). *E. coli* BL21(DE3) ArcticExpress expression host were gifts from Dr. Jiri Damborsky (Masaryk University, Brno, Czech Republic).The hosts were cultivated in Luria Bertani medium, (1% tryptone, 0.5% yeast extract, and 1% sodium chloride) supplemented by appropriate antibiotics (tetracycline 100 µg mL-1 or ampicillin 100 µg mL-1). Luria Bertani medium with addition of 2% agar was used as solid medium. All restriction enzymes, T4 DNA ligase, and pTWIN1 expression vector were purchased from New Englands (New England Biolabs, USA). Human prethrombin-2 (hPT-2) gene was commercially synthesized by GeneArt (Life Technologies, Germany). Isolation kit (Roche Applied Science, USA) and gene extraction kit (Geneaid, Taiwan) were purchased commercially. Isopropyl-β-D-thiogalactoside (IPTG) and β-mercaptoethanol (βME) were from Sigma Aldrich (Sigma Aldrich, USA). Polyacrilamide and Commasie Brilliant Blue were from Biorad (BioRad, Richmond, USA).

Transformation of E. coli BL21(DE3) ArcticExpress using pTWIN1-hPT-2 plasmid

Plasmid pTWIN1-hPT-2 was isolated from *E. coli* TOP10F', then used to transform the competent expression host cells.A total of 5 µL pTWIN1-hPT-2 was added to the microtube containing 100 µL competent cells, and then incubated for 30 min at -4°C. Heat shock was carried out at 42°C for 90 seconds, then was immediately cooled on ice for 10 minutes. This mixture was added to 900 µL of liquid LB medium and was incubated at 37°C for 2h with agitation rate of 150 rpm. Centrifugation was performed at 12,000 g for 1 min seconds. A total of 900 µL supernatant was removed, and the remaining of 100 µL of mixture was grown in LB medium containing 10 µg/mLof

tetracycline, 10 µg/mL of ampicillin and 20 µg/mL of gentamicin, and then incubated at 37°C for 18 hours(Sambrook and Russell, 2001).

Transformation of *E. coli* that grows was *E. coli* which has brought recombinant plasmids. To ensure that *E. coli* transforman had carried the correct recombinant plasmid, then analysis was carried out through recombinant plasmid isolation from *E. coli* transformants, restriction analysis and determination of hPT-2 nucleotide sequences.

hPT-2 Expression with induction temperature variation

Preliminary test of rhPT-2 fragment expression in *E. coli* BL21(DE3) ArcticExpress was carried out by growing *E. coli* BL21(DE3) ArcticExpress [pTWIN1-hPT-2]. A total of 1% (v/v) inoculates culture *E. coli* BL21(DE3) ArcticExpress carried pTWIN1-hPT-2 was grown in a medium containing 10 µg/mL of ampicillin. Induction was done by using concentration of IPTG 0.1 mM, with agitation rate of 100 rpm at a temperature variation of 12°C and 22°C. Cells were harvested after 18 hours of incubation. Cell debris was separated from the supernatant by centrifugation at 12.000*g* speeds. hPT-2 expression results in the supernatant and cell debris were characterized using 12% SDS-PAGE (Bollag et al. 1996).

Results

Isolation of pTWIN1-hPT-2 from E. coli TOP10F'

Plasmid isolation of pTWIN1-hPT-2 from *E. coli* TOP10F cells was carried out using a plasmid isolation kit (Geneaid). To ensure that the isolation process was successfully carried out, then pTWIN1-hPT-2 plasmid was characterized using one enzyme (*Nde*I) and two enzymes (*Nde*I and *Xho*I). Characterization results using this enzyme, then characterized by electrophoresis using 1% agarose gel (Fig 1). Characterization results using one enzyme obtained a linear band measuring 7633 bp in accordance with the size of recombinant plasmid (Fig 1A lane 1). While the characterization using two enzymes obtained two bands measuring 6697 bp as pTWIN1 (Fig 1B lane 2) and 936 bp as hPT-2 fragments (Fig 1B lane 3).

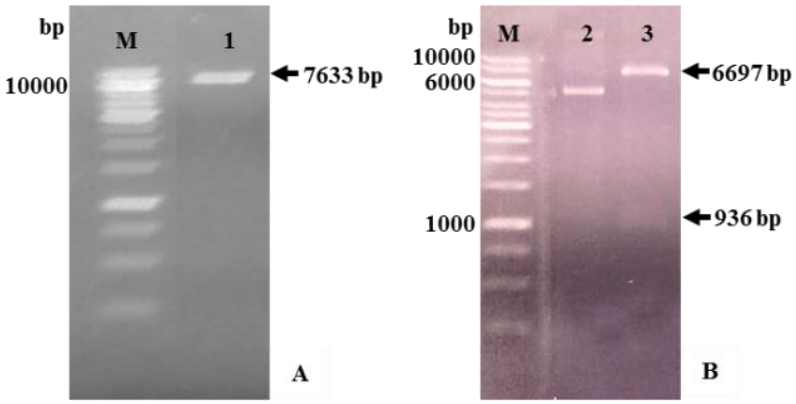

Fig 1. Isolation analysis of pTWIN1-hPT-2 from *E. coli* TOP10F' by using 1% agarose gel electrophoresis. M: 1 kb DNA marker; line 1 figure 1A: pTWIN1-hPT-2 was cut with BamHI; line 2 figure 1B: pTWIN1-hPT-2; line 3 figure 1B: double-digested plasmid resulting pTWIN1 and rhPT-2.

Transformation of E. coli BL21 (DE3)Arctic Express

The *E. coli* strain BL21(DE3) ArcticExpress was transformed by inserting the pTWIN1-hPT2 plasmid, so that for the next phase the cell can produce hPT-2 protein. Selection of transformants in this research was conducted using tetracycline, ampicillin and gentamicin antibiotic resistance. The pTWIN1-hPT-2 plasmid carrying the gene encode enzyme which can degrades ampicillin, so the cells that able to absorb the plasmid will survive, while the others will be dead. The *E. coli* BL21(DE3) ArcticExpress which carry pTWIN1-hPT-2 were grown in LB medium. Colonies which

can grow on selection media (Fig 2A), were grown back in liquid LB medium and isolated as shown in Fig 2B.

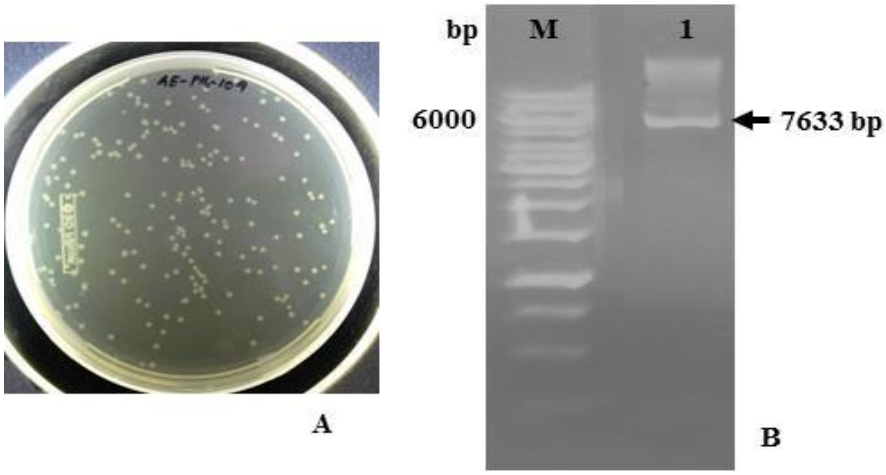

Fig 2. Transformants analysis by using 1% agarose gel electrophoresis. 2A: transformants *E. coli* BL21(DE3) ArcticExpress carrier of pTWIN1-hPT-2 plasmid. 2B: isolation results of pTWIN1-hPT-2 from *E. coli* BL21(DE3) ArcticExpress M: 1 kb DNA marker; line 1 figure 2B: pTWIN1-hPT-2.

hPT-2 expression in E. coli BL21 (DE3)Arctic Express

The effect of induction temperature were investigated.The effect of induction temperature were investigated. The inducer used in our experiment was IPTG. IPTG inducer is a critical factor for gene expression in *E. coli* host (Moradian et al. 203; Rizkia et al. 2015; Wang et al. 2018; Silaban et al. 2019).The cells culture was induced using IPTG with a final concentration of 0.1 mM, and incubated for 16 hours at temperature variations of 12 and 22°C.The rhPT-2 was designed as fusion protein. The total molecular weight of fusion protein was 63 kDa. The protein expression was analyzed by 12% SDS-PAGE.The results showed that induction temperature 12°C for expression of *E. coli* BL21(DE3) ArcticExpress could increase soluble CBD-Intein rhPT-2 fusion protein (Fig 3A line 2). In contrast, induction temperature 22°C unable to increase rhPT-2 fusion protein insoluble form (Fig 3B line 2).

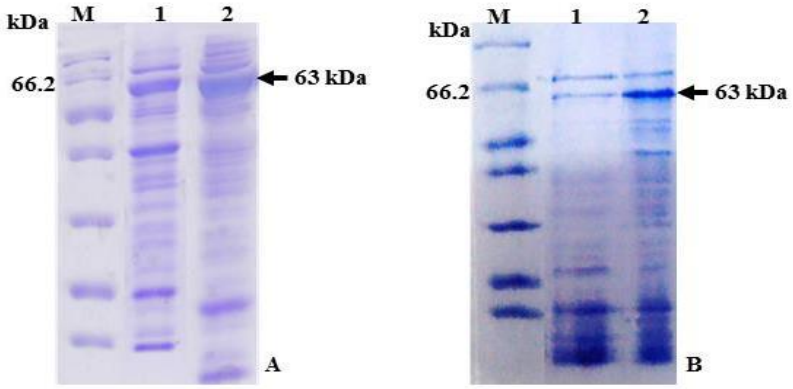

Fig 3. SDS-PAGE analysis of protein expression of CBD-Intein-rhPT-2 with temperature variations. 3A: 12°C induction temperature. 3B: 22°C induction temperature. M: molecular weight marker; line 1 figure 3A & 3B: the insoluble fraction; line 2 figure 3A & 3B:soluble fraction of CBD-Intein-rhPT-2 fusion protein.

Discussion

Formation of inclusion bodies in *E. coli* is a major problem in the expression of recombinant protein (Freydell et al. 2007). Although it may also offer an advantage, that it is easily purified, as long as the protein can latter be refolded (Singh and Panda 2005). However, refolding involves multistep processes and the recovery of correctly folded and active enzyme are still uncertain (Soejima et al. 2001).

Bacterial growth and recombinant protein expression are influenced by several parameters, such as the composition of the medium, oxygen content, pH, fermentation temperature and induction (Freydell et al. 2007; Hartinger et al. 2010). It is well-known that *E. coli* is the most widely used and suitable host of heterologous proteins at 21-49°C, with an optimum at abou 37°C (Ferrer et al. 2003), but when high expression of protein is needed, the *E. coli*'s capability to accurately express recombinant proteins decreased, and inclusion bodies can be formed. Low temperature has been proposed to improve protein solubility, but slower growth and low synthesis rates may downgrade protein yields (Sorensen and Mortensen 2005).

From two temperature variations, only at 12°C, more soluble proteins are produced, while the induction temperature of 22°C produces insoluble proteins.Thus, a high amount of soluble fusion protein rhPT-2 in *E. coli* BL21(DE3) ArcticExpress can be achieved at an optimum temperature of 12°C.This success was allegedly influenced by the presence of a chaperon (CPn60 / Cpn10) carried by *E. coli* BL21(DE3) ArcticExpress host. These chaperones display high refolding activities and govern growth of *E. coli* at low temperatures (Ferrer et al. 2004; Strocchi et al. 2006). As expected, expression analyses demonstrated that *E. coli* BL21(DE3) ArcticExpress could produce rhPT-2 in soluble form.

Conclusion

It can be concluded that the synergy among low induction temperature and *E. coli* BL21(DE3) ArcticExpress as a host, could be used to successfully produce a soluble form of rhPT-2.

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