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Effect of IPTG Concentration on Recombinant Human Prethrombin-2 Expression in *Escherichia coli* BL21(DE3) ArcticExpress

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Abstract. Production of recombinant proteins in *E. coli* still faces a bottle neck due to formation of inclusion bodies. The level of gene transcription can be regulated by using appropriate concentration of isopropyl- β -D-thiogalactoside (IPTG) inducer. The purpose of this study is to determine the effect of IPTG concentration on human prethrombin-2 (hPT-2) expression. The hPT-2 expression was induced by various concentrations of IPTG (0.01 mM, 0.025 mM, 0.05 mM, 0.075 mM, 0.1 mM, 0.2 mM and 0.3 mM) at 12°C for 18 hours hosted by *E. coli* BL21(DE3) ArcticExpress. The result shows that the suitable IPTG concentration for induction of hPT-2 in *E. coli* BL21(DE3) ArcticExpress was 0.1 mM. It was indicated from the 62-kDa protein band obtained from the soluble fraction on SDS-PAGE. It concluded that the IPTG concentration affects the rate of rhPT-2 expression.

Keywords: *E. coli* BL21(DE3)ArcticExpress; expression; IPTG concentration; prethrombin-2

1. Introduction

Prethrombin-2, the smallest single-chain immediate precursor of α -thrombin that structurally has one glycosylation site and four disulphide bonds [1; 2]. Human prethrombin-2 is a single polypeptide of 308 amino acid residues which corresponds to the residues from 272 to 579, the carboxy terminal portion of prothrombin [3]. Prethrombin-2 has been identified as one of intermediates, which is generated during activation of prothrombin to thrombin [4]. Prethrombin-2 can be converted to thrombin by activation with Factor Xa [5; 6].

To date, there is an effort to express a recombinant human prethrombin-2 hosted by microorganisms for commercial purposes [7]. *E. coli* remains the system of first-choice for expressing proteins, as it is cheap and easy to handle, however many mammalian proteins cannot be successfully expressed in *E. coli* [8; 9]. Prethrombin-2 had previously been expressed in *E. coli*, however as is often the case, this normally soluble eukaryotic protein was found to be insoluble in *E. coli*, yielding inclusion bodies from which the protein had to be solubilized and refolded [1,5]. Inclusion bodies formation can be caused by the partially folding of polypeptides. Inclusion bodies is a dynamic structure that formed by an imbalance between the aggregates and soluble proteins from *E. coli*. It occurs as a result of accumulation of partial folding proteins that have been expressed in the aggregates forms through non-covalent hydrophobic interactions, ionic interactions, or both [10; 11]. Soejima et al [1], 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 [10; 12; 13]. Because the level of gene transcription is affected by IPTG, using the suitable concentration IPTG is one of approaches to avoid or reduce inclusion bodies.

IPTG is commonly used for inducing expression from the lac promoter and offers a number of distinct advantages, especially in small scale experiments. IPTG functions by binding to the lacI



repressor and altering its conformation, which prevents the repression of the β -galactosidase coding gene *lacZ*. Unlike lactose and other galactosides, IPTG is a metabolic-free, or gratuitous inducer, because it is not metabolized by the cell. It can ensure that the level of induction remains constant following the addition of IPTG to the growth medium [14]. This research aims was to determine the effect of IPTG concentration on prethrombin-2 expression in *E. coli* BL21(DE3) ArcticExpress. The *E. coli* BL21(DE3) ArcticExpress used as expression host strain because it is a components cell that chemically can be used for the expression of target genes in inserted to a vector IMPACT [15]. *E. coli* BL21(DE3) ArcticExpress carrying the T7RNA polymerase gene inserted in the *lacZ* gene under the control of the T7 promoter.

2. Experimental Method

2.1. Strains, chemicals, vector, and medium

Transformation and cloning was performed using *E. coli* TOP10F' (Invitrogen, USA). *E. coli* BL21(DE3) ArcticExpress expression host were kind 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 μ g mL⁻¹ or ampicillin 100 μ g 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 England (New England Biolabs, USA). Prethrombin-2 gene was commercially synthesized by GeneArt (Life Technologies, Jerman). Isolation kit (Roche Applied Science, USA) and gene extraction kit (Geneaid, Taiwan) were purchased commercially. Isopropyl- β -D-thiogalactoside (IPTG) and β -mercaptoethanol (β ME) were from Sigma Aldrich (Sigma Aldrich, USA). Polyacrilamide and Commasie Brilliant Blue were from Biorad (BioRad, Richmond, USA).

2.2. Construction and codon optimization of hPT-2

The hPT-2 synthetic gene was designed to be cloned into pTWIN1 expression vector, using two restriction sites at 5' and 3' ends, consecutive XhoI and BamHI. The amino acid sequence of hPT-2 1-308 obtained from database GenBank (GenBank accession number AID16055.1). The amino acid sequence of hPT-2 1-308 was then converted to a nucleotide and was optimized using OPTIMIZER (<http://genomes.urv.es/OPTIMIZER>) [16]. An *E. coli* codon usage table was adapted from a codon usage database (www.kazusa.or.jp/codon). Subsequently, optimized codon was analyzed by Graphical Codon Usage Analyzer (<http://gcua.schoedl.de/>) [17].

2.3. Cloning

All stages of cloning was performed follow the method Sambrook and Russel [18]. Plasmid pMA-T-hPT-2 was cut by XhoI and BamHI. In parallel, pTWIN1 was also cut by same restriction enzymes. The restriction fragments were characterized using 1% agarose gel electrophoresis. Then, hPT-2 and pTWIN1 fragments were extracted from the agarose. The extracted hPT-2 was ligated into pTWIN1 by T4 DNA ligase resulting pTWIN1-hPT-2. Furthermore pTWIN1-hPT-2 is cloned to *E. coli* TOP10F'. Screening of the TOP10F' *E. coli* transformants, isolation and characterization of pTWIN1-hPT-2 plasmid from *E. coli* TOP10F' were also performed. The cloned gene was verified by DNA sequencing (MacroGene, Korea).

2.4. Transformation of *E. coli* BL21(DE3) ArcticExpress

A total of 5 μ L pTWIN1-hPT-2 was added to the microtube containing 100 μ L 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 μ L 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 μ L supernatant was removed, and the remaining of 100 μ L of mixture was grown in LB medium containing 10 μ g/mL of tetracycline, 10 μ g/mL of ampicillin and 20 μ g/mL of gentamicin, and then incubated at 37°C for 18 hours.

2.5. rhPT-2 expression with IPTG induction

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 various concentrations of IPTG (0.01 mM, 0.025 mM, 0.05 mM, 0.075 mM, 0.1 mM, 0.2 mM and 0.3 mM) with agitation rate of 100 rpm at a temperature of 12°C. Cells were harvested after 18 hours of incubation. Cell debris was separated from the supernatant by centrifugation at 12,000 g speeds. Prethrombin-2 expression results in the supernatant and cell debris were characterized using 12% SDS-PAGE [19].

3. Results and Discussion

3.1. Construction and codon optimization of hPT-2

Nucleotide sequence of hPT-2 was analysed using OPTIMIZER. The result showed that the nucleotide sequence of hPT-2 measuring 924 bp, GC percentage was 52.56% with CAI 0.89. Analysis of the codons by Graphical Codon Usage Analyzer (GCUA) using *E. coli* codon preference indicated that 9 amino acids (RDVHISFGY) were encoded by non-preference codon. After codon optimization, GC percentage of hPT-2 was 54.71% with CAI of 1.00.

The first step in this study was codon optimization of hPT-2 gene according to the codon preference of *E. coli* [2]. Rarely used codons were replaced with high frequency codons [20; 21]. Every gene in the genome has numeric number called codon adaptation index (CAI). The CAI can be used predict the expression of heterologous protein [22].

3.2. Cloning

The plasmid map of synthetic hPT-2 is shown in Figure 1. The hPT-2 gene was cut from plasmid pMA-T-hPT-2 using XhoI and BamHI. In parallel, vector pTWIN1 was cut with the same restriction enzymes. The agarose gel electrophoresis showed that hPT-2 fragment was successfully cut from pMA-T. Two bands with molecular weight approximately 939 bp and 2.374 bp were indicated as hPT-2 and pMA-T. pTWIN1 digest also produced two bands with molecular weight 6.536 bp (pTWIN1) and 839 bp (MCS/Multi Cloning Site). Then, the hPT-2 fragment was ligated in to pTWIN1 vector using T4 DNA ligase. To confirm the ligation process, the sequence pTWIN1-hPT-2 was verified by DNA sequencing. The nucleotide alignment revealed that hPT-2 was successfully inserted into pTWIN1 [2].

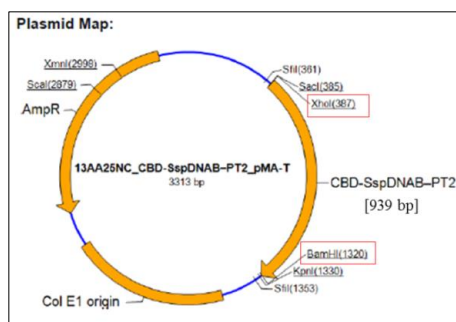


Figure 1. The plasmid map of synthetic hPT-2.

3.3. Transformation of *E. coli* BL21(DE3) ArcticExpress

The competent *E. coli* BL21 (DE3) ArcticExpress cell was transformed using pTWIN1-hPT-2 by the heat shock method [18], so that for the next phase the host 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 BL21(DE3) ArcticExpress which carry pTWIN1-hPT-2 were grown in LB medium. Colonies which can grow on selection media (Figure 2a), were grown back in liquid LB medium and isolated as shown in Figure 2b.

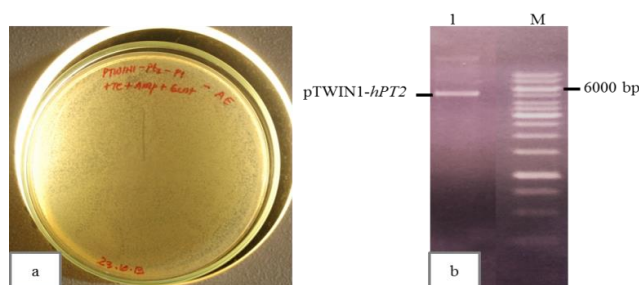


Figure 2. Characterization of transformants. 2a: transformant colonies; 2b: Plasmid pTWIN1-hPT-2 recombinant insulation from *E. coli* BL21(DE3) ArcticExpress. M: 1 kb DNA marker.

3.4. rhPT-2 expression with IPTG induction

The effect of final IPTG concentration were investigated. The inducer used in our experiment was IPTG. Inducer concentration used for inducing gene expression is a critical factor because it can influence overall costs, total biomass productivity, and protein yield per cell and it also has a potential inhibitory effect on cell growth [23; 24]. Thus, a suitable IPTG concentration is of prime importance. The concentration of IPTG has been reported to affect protein folding [25]. The IPTG inducer it also is reported could to prevent lac repressor bound to the lac operator thus inducing expression to form active proteins [26]. When *E. coli* BL21(DE3) ArcticExpress carrying plasmid pTWIN1-hPT-2 was grown for 18 h at different concentrations of IPTG, the results showed that the optimal value for IPTG concentration was 0.1 mM (Figure 3). As displayed in Figure 3, *E. coli* were successfully expressed rhPT-2 using various concentrations of IPTG, this was indicated by the 62-kDa protein band that obtained.

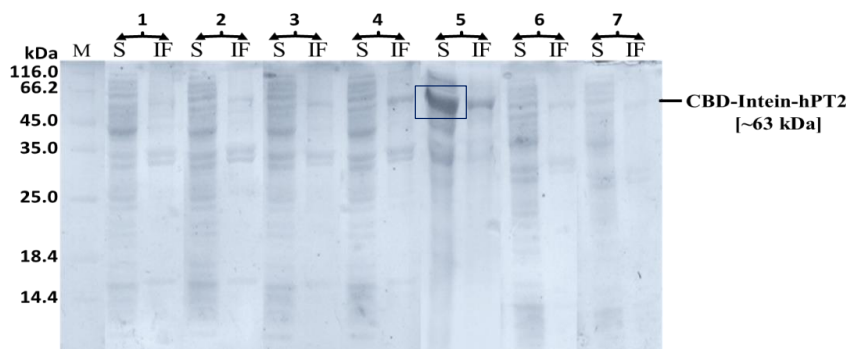


Figure 3. Characterization of expression with various concentrations of IPTG using SDS-PAGE. IPTG concentration of 0.01 mM; 2: 0.025 mM; 3: 0.05 mM; 4: 0.075 mM; 5: 0.1 mM; 6: 0.2 mM; 7: 0.3 mM. M: Marker; S: soluble fraction; IF: insoluble fraction

4. Conclusions

Differences of IPTG concentration affect the rhPT-2 expression rate and formation of inclusion bodies. Best IPTG concentration for induction of rhPT-2 gene expression in *E. coli* BL21(DE3) ArcticExpress was 0.1 mM. These results provide a basis for further study of the the effect of pre-induction inoculum OD_{600} , inducing time, and inducing temperature on the expression of rhPT-2.

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