Construction and Optimization of Prethrombin-2 Human Genes in E. coli for the Production of Active Thrombin

by Saronom Silaban
Construction and Optimization of Prethrombin-2 Human Genes in E. coli for the Production of Active Thrombin

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Abstract. Prethrombin-2 is a thrombin precursor, which plays an essential role in the conversion of fibrinogen to fibrin during the blood clotting process. In this study, we designed and constructed the human prethrombin-2 (hPT2) gene. hPT2 gene fused with a tag at the C terminal position, which contains the sequence intein followed by chitin binding domain (CBD) that useful for the purification process. The digestion process of hPT2 tag induced by changing its pH/temperature. The hPT2 codon that used in this study designed according to the preference codon of E. coli. hPT2 gene was created using the OPTIMIZER software with the addition of two side restriction at the 5' and 3', BamHI and XhoI respectively then were cloned by using the pMAT vector in E. coli. Moreover, hPT2 fragment ligated into an expression vector, pTW1N1, for E. coli. The result from the characterization hPT2 gene in pTW1N1 that using DNA sequencing method shown that the designed hPT2 gene cloned. Thus, the cloned hPT2 gene used as a precursor for PT2 expression in the E. coli host.

Keywords. prethrombin-2, thrombin, codon preferences, E. coli

1. Introduction
Covering the wound surgery method used a stitches technique, the gold standard applied. Although it is the gold standard, this technique caused several problems, such as prolonged time wound healing and surgery process time, additional trauma (during sew and retraction threads), increasing the inflammatory, and the possibility of the complication relating to stitches of infection [1].

Fibrin glue (LF) replacement stitching techniques can glue and close the wound. LF as bioadhesive material, composed of thrombin, fibrinogen, calcium, and XIII factors. The content is designed to resemble the final stages of coagulation to form a fibrin clot. LF used as a hemostatic material that stops bleeding from the incision gap, matrix for wound healing and tissue adhesive [2]. Currently, thrombin in the commercial LF usually made from frozen and fresh beef plasm [3], commercial LF instead of stitching gives a lot of advantages, such as the surgery is more convenient, faster and can avoid losses due to suture [4].

The extensive use of E. coli as a host in the production of recombinant proteins is due to its nature that can overgrow with a short life cycle, information and genomes' characters completely so that easily manipulated, production costs are relatively cheap, high-level expression of the target protein, fast, and the technology is established [5]. However, in addition to the advantages mentioned above, the host also has a weakness, such as the phenomenon of codon refract [6], and the potential to produce complex and inactive aggregates protein that it knew as inclusion bodies [7].

The first strategy needs to be done to solve this weakness that mentioned above is by optimizing codon of the target gene to codon preference of the host [8]. This strategy aims to address the lack of protein expression of the target gene. This optimization process is done by changing the codon encoding a particular amino acid derived from another source into codons with high frequency in the expression host. The expression level of genes composed codon optimization results more elevated than the genes without optimization [9]. The results of gene expression increased at least threefold with codon optimization [10]. The second strategy was used to the synthetic gene technology based on the ability to change codon refraction from the target gene into the host's match with codon preference recombinant [11]. Another advantage of this technology is the effectiveness and efficiency than the isolation process, and to avoid the diseases transmission and allergic reactions [12].
2. Materials and Methods

2.1. Strains, vectors, media, chemicals

_E. coli_ TOP10F' is a host strain for plasmid cloning and rejuvenation. pMAT is a commercial cloning vector. Strains were grown in Luria Bertani medium (LB) with composition (tryptone 1%, yeast extract 0.5%, and 1% sodium chloride) supplemented with tetracycline antibiotics (10 μg / mL) and ampicillin (100 μg / mL). For solid media, LB media components added with 2% agar. All restriction enzymes and T4 DNA ligase obtained commercially from Fermentas (Canada). PTWIN1 expression vector obtained commercially from New England Biolabs, NEB. hPT2 synthetic gene (CBD-interm Ssp DNA H-hPT2) synthesized by GenArt AG (Germany).

2.2. Design and codon optimization of hPT2

The hPT2 synthetic gene was designed based on the amino acid sequence from GenBank (Accession number: NM_000506.3). Codon preferences of _E. coli_ that contained in the Codon Usage Database (http://www.kazusa.or.jp/codon/). Codon optimization is performed using software Optimizer (http://gnomes.tur.es/OPTIMIZER) and Graphical Codon Usage Analyzer (http://gena.schoell.de/).

2.3. Fusion Construction of hPT2 and vector of pTWIN1

Fusion expression protein of hPT2 in _E. coli_ and purification using IMPACT-TWIN system, the synthetic design of hPT2 genes equipped with BamHI and XhoI restriction site at the intern. To combine the hPT2 synthetic and the pTWIN1 expression vector, pMAT-hPT2 cut using restriction enzymes BamHI and XhoI. In parallel, cutting pTWIN1 with the same enzymes. Then, combined pTWIN1 by using T4 DNA ligase to produce pTWIN1-hPT2 plasmid.

2.4. Transformation of _E. coli_ TOP10F'

Transformation of pTWIN1-hPT2 used competent cells of _E. coli_ TOP10F' procured by heat shock [13]. _E. coli_ transformed colonies selected through media contained the tetracycline and ampicillin antibiotic. pTWIN1-hPT2 recombinant plasmid isolated from _E. coli_ TOP10F' transformed colonies using the QIAgen Spin Plasmid Miniprep Test Kit according to Qagen’s protocol. The recombinant plasmid purification results, analyzed by 1% agarose gel electrophoresis. Then, plasmids can be used for restriction analysis and determined the nucleotide sequence using DNA sequencing methods. Sequencing results aligned using Seqman on the Bioedit program.

3. Results and Discussion

3.1. Codon optimization of hPT2

Synthetic design of hPT2 gene made through software. hPT2 synthetic designed based on the amino acid sequence in GenBank (Accession number: NM_000506.3) and used codon preferences of _E. coli_ that contained in the Codon Usage Database. Based on data in GenBank, hPT2 gene consists of 308 amino acids.

The sequence analysis results of hPT2 codons in _E. coli_ signed there was an unmatched codon preference of _E. coli_. Some human hPT2 codons encoding amino acids which have less than 50% similarity to the codon preference of _E. coli_, among others (1) &gt; art, tgg, tca, tca, (2) &gt; gac, (3) T &gt; act, acc, (4) &gt; gga, ggg, (5) &gt; agg, gga, cga, cgg, (6) &gt; ccc, ecc, (7) &gt; arg, (8) L, ctc, ctg, (9) V &gt; gtv, (10) Q &gt; cca. While hPT2 codon that have the relatively more than 50% to nearly 100% consists of (1) A: gcc, gca, got, (2) Y: ttc, (3) F: ttc, (4) N: nat, (5) D: gac, (6) C: tgg, (7) I: atc, atu, (8) H: cac, (9) V &gt; gtt, (10) T: agc, dan (11) G: ggt. hPT2 codons which have not yet reached 100% relative similarity to _E. coli_ optimized up to 100%.

Synthetic gen utilizing can simplify and accelerate desired genes acquisition because unlimited at the natural biological source [8]. Also, Gen Bank’s data can be accessed easily as the basis of determining the sequence of genes to be synthesized. Codon optimization is done because the target genes have potential differences of preference codon from the host genome. Although, some of the target genes have sufficient similarity with the host so the codon optimization is not necessary. In this study, hPT2 gene that expressed in the _E. coli_ host, hPT2 codon choice has low preference codon than _E. coli_ [14]. The previous study reported that the hPT2 gene expressed in _E. coli_ would produce an
inclusion body [15-18]. Therefore, codon optimization of the original organism against preference codon of the host is needed [19-20].

3.2. Recombinant plasmid construction

pTW11 has a gene encoding the chitin binding domain, which can bind chitin to the purification matrix [21]. In this research, hPT2 constructed into fusion form in the N-terminal position (CBD-intein Ssp DnaB-hPT2). At the end both inserted cutting sides of BamHI (ggattc) at the 5' end and XhoI (cctgag) at the 3' end that the hPT2 gene combined with pTW11. The synthetic design of hPT2 gene for CBD-intein-hPT2 as follows:

cctgagacctggaagctagaagttatttcctttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttt
concentration is 100 ng/mL of the nucleotide sequences determined by DNA sequencer by MacroGene (Korea). Comparison of the nucleotide sequence pt2 hPT2 optimization results with cloned presented in Figure 4.

**Figure 2.** Electrophoresis analyses of results of pTWIN1 (7375 bp) plasmid cut with BamHI and XhoI enzymes. M: 1 kb DNA Marker, pTWIN1 with a molecular weight of 6536 bp, MCS with a molecular weight of 839 bp.

**Figure 3.** Extraction analyses of pTWIN1 and hPT2 by using agarose gel. M: 1 kb DNA Marker, pTWIN1 with a molecular weight is 6536 bp, hPT2 with a molecular weight is 939 bp.

**Figure 4.** Sequencing results. Comparison of the DNA sequences of the designed hPT2 gene (→) with the results of cloning (↔).
4. Conclusions
iPT2 gene codon optimization according to codon preference of E. coli can minimize the effects of codon refraction, which the impact on the expression of recombinant proteins. Use of the synthetic gene is more efficient and effective than isolation process from a natural source.

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