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

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Abstract. Prothrombin-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 n 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', *Bam*HI and *Xho*I respectively then were cloned by using the pMAT vector in *E. coli*. Moreover, hPT2 fragment ligated into an expression vector, pTWIN1, for *E. coli*. The result from the characterization hPT2 gene in pTWIN1 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 time 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 which 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 refraction [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 its 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 (100 ug / mL) and ampicillin (100 ug / 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-intein Ssp DnaB-hPT2) synthesized by GeneArt AG (Germany).

2.2. Design and codon optimization of hPT2

The hPT2 synthetic gene was designed based on the amino acid sequence in GenBank (Accession number: NM_000506.3). Codon preference 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.urv.es/OPTIMIZER) and Graphical Codon Usage Analyzer (http://gcua.schoedl.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 *Bam*HI and *Xho*I restriction site at the intern. To combine the hPT2 synthetic and the pTWIN1 expression vector, pMAT-hPT2 cut using restriction enzymes *Bam*HI and *Xho*I. 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 Qiagen'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 designing 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 unmatch 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) S: art, tcg, tcc, tca, (2) E: gag, (3) T: act, aca, (4) G: gga, ggg, (5) R: agg, aga, cga, cgg, (6) P: cct, ccc, (7) K: aag, (8) L: ctc, ctt, ttg, (9) V: g[illegible], (10) Q: caa. While hPT2 codon that have the relatively more than 50% to nearly 100% consists of (1) A: gcc, gca, gct, (2) Y: tac, (3) F: ttc, (4) N: aat, (5) D: gac, (6) C: tgt, (7) I: atc, ata, (8) H: cac, (9) V: gtt, (10) T: acg, 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

pTWIN1 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 *Bam*HI (**ggatcc**) at the 5' end and *Xho*I (**ctcgag**) at the 3' end that the hPT2 gene combined with pTWIN1. The synthetic design of hPT2 gene for CBD-intein-hPT2 as follows:

ctcgagaccgcgaccagcgaatatcagacctttttaacccgcgcacctttggcagcggcgaagcggattgcggcctgcgcccgctgtttgaaaaa
aaaagcctggaagataaaaccgaacgcgaactgctggaaagctatattgatggccgcattgtggaaggcagcgatgcggaaattggcatgagccc
gtggcaggtgatgctgtttcgcaaaagcccgcaggaactgctgtgcggcgcgagcctgattagcgatcgctgggtgctgaccgcggcgcattgcc
tgctgtatccgccgtgggataaaaactttaccgaaaacgatctgctggtgcgcattggcaaacatagccgcacccgctatgaacgcaacattgaaaa
aattagcatgctggaaaaaatttatattcatccgcgctataactggcgcgaaaacctggatcgcgatattgcgctgatgaaactgaaaaaaccggtgg
cgtttagcgattatattcatccggtgtgcctgccggatcgcgaaaccgcggcgagcctgctgcaggcgggctataaaggccgcgtgaccggctgg
ggcaacctgaaagaaacctggaccgcgaacgtgggcaaaggccagccgagcgtgctgcaggtggtgaacctgccgattgtggaacgcccggtg
tgcaaagatagcacccgcattcgcattaccgataacatgttttgcgcgggctataaaccggatgaaggcaaacgcggcgatgcgtgcgaaggcgat
agcggcggcccgtttgtgatgaaaagcccgtttaacaaccgctggtatcagatgggcattgtgagctggggcgaaggctgcgatcgcgatggcaa
atatggcttttatacccatgtgtttcgcctgaaaaaatggattcagaaagtgattgatcagtttggcgaataa**ggatcc**

Inserted of hPT2 synthetic gene in pTWIN1 was done on the intein Ssp DnaB. To enable the process of protein expression, at the 3'end hPT2 added a stop codon (taa) before the *Bam*HI fusion restriction so that Mxe GyrA did not express because it would increase the size of the resulting protein. The start codon added because it included in the CBD-intein SspDnaB in pTWIN1.

3.3. Cloning of hPT2 genes

To obtained hPT2 fragment, the pMAT-hPT2 plasmid cut by using BamHI and XhoI based on the previous synthetic genes design. Cutting result of pMAT-hPT2 characterized using 1% agarose gel electrophoresis. The analysis showed that the hPT2 fragment successfully cut from the pMAT vector. Two bands with molecular weight approximately 2374 bp and 939 bp indicated as pMA-T and hPT2, respectively (Figure 1). Using the same enzyme, pTWIN1 was cut and then isolated for hPT2 ligation process. Figure 2 shown that pTWIN1 expression vector successfully cut. There were two bands with a molecular weight of 6536 bp as pTWIN1 vector, and bands with the molecular weight are 839 bp as Multi Cloning Site (MCS) fragment, which released from pTWIN1 vector (Figure 2).

hPT2 fragments ligated to the pTWIN1 expression vector using T4 DNA ligase (Fermentas), Next, the ligation results of isolated and purified by DNA isolation kit (Roche). Refining result characterized by 1% agarose gel electrophoresis. As seen in Figure 3, agarose gel electrophoresis results showed that hPT2 fragments (939 bp) and pTWIN1 vector (6536 bp) was successfully isolated and purified from the agarose gel.

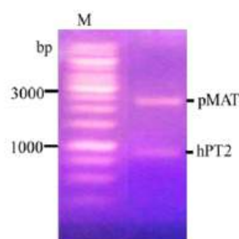

Figure 1. Electrophoresis analyses of results of pMAT-hPT2 (3313 bp) plasmid cut with *Bam*HI and *Xho*I enzymes. M: 1 kb DNA Marker, pMAT with a molecular weight of 2374 bp, hPT2 with a molecular weight of 939 bp.

3.4. Gene cloning characterization

Confirming the successful ligation result, and to ensure similarity the nucleotide sequence of hPT2 gene cloned results with the designed results, as many as 10 μL hPT2-pTWIN1 plasmid with a

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.

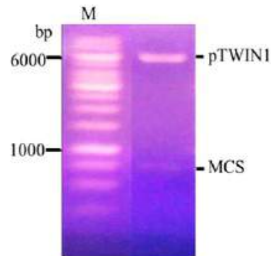

Figure 2. Electrophoresis analyses of results of pTWIN1 (7375 bp) plasmid cut with BamHI and XhoI enzymes. M: 1 kb DNA Marker, pTWIN1 with a molecular weight of 6536 bp, MCS with a molecular weight of 839 bp.

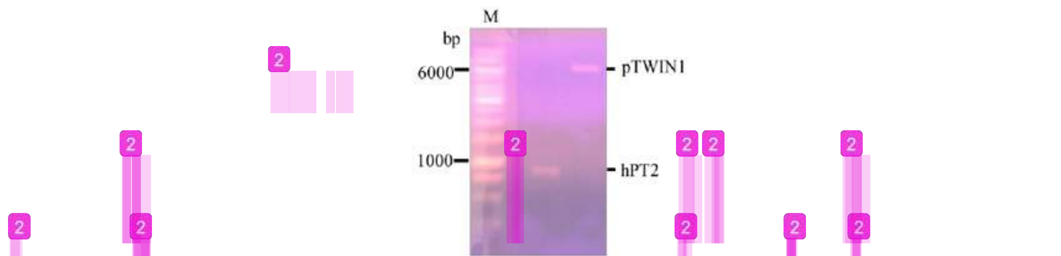

Figure 3. Extraction analyses of pTWIN1 and hPT2 by using agarose gel. M: 1 kb DNA Marker, pTWIN1 with a molecular weight is 6536 bp, hPT2 with a molecular weight is 939 bp.

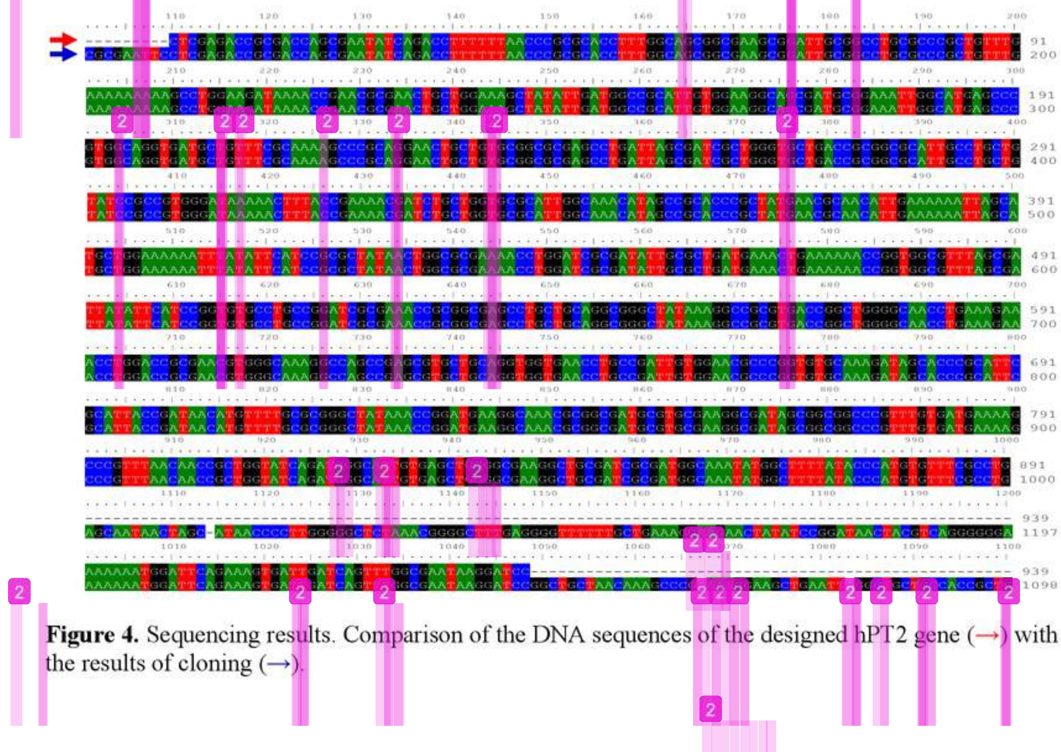

Figure 4. Sequencing results. Comparison of the DNA sequences of the designed hPT2 gene (→) with the results of cloning (→).

4. Conclusions

hPT2 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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