Reconstruction and Optimized Expression of a Synthetic Secretory Leukocyte Protease Inhibitor (SLPI) Gene in *Escherichia coli* BL21

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Abstract

An active substance that has the greatest effect on wound healing is Secretory Leukocyte Protease Inhibitor (SLPI). It is known that the SLPI encoding genes can be isolated and expressed on amnion membrane. Previous studies, we isolated and optimized the SLPI gene through Echerrchia coli BL21 (DE3) mediated pET101/DTOPO, which expressed active recombinant human SLPI (rhSLPI) stored in pET-ESLPI. However, the expression of the rhSLPI products has not yet been accomplished.

In this study, we optimized SLPI expression by developing a synthetic SLPI gene based on amino acid sequences with codons and expressed in E. coli BL21 to give the maximum expression. We used pUC57 and pET-32a plasmids to promote the cloning of synthetic SLPI genes. A codon-optimized SLPI gene was successfully synthesized with codon adaptation index value showing the distribution of codon usage frequency along the length of the gene sequence.

In addition, the pET-SLPIopt fusion protein was successfully optimized with band sizes of 5900bp (pET-32a) and 413bp (SLPI) by double-digestion of NcoI and EcoI restriction enzymes. After the pET-SLPIopt was induced with various IPTG concentrations (50, 100, and 500 uM) at 30 °C, both soluble and insoluble fractions were analyzed as a result of SDS-PAGE which showed that the fusion protein, expressed predominantly in the supernatant, was 29.18 kDa. Our reported findings the recombinant protein of SLPI through pET-32a plasmid could be expressed in dissolved form.

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Introduction

Secretory Leukocyte Protease Inhibitor (SLPI) is a protease inhibitor generated by different cells, including mast cells, macrophages and neutrophils[1,2,3], which expressed particularly in amnion membrane, cervical, seminal plasma, mucosal secretion, bronchial mucus, and nasal.[4] As a protease inhibitor, SLPI can protect epithelial tissue during inflammation. Therefore, this protein is used for the treatment of cystic firbrosis, emphysema, chronic obstructive pulmonary disease (COPD) and bronchitis.[5] A number of studies on SLPI showed various functions of SLPI besides protease inhibitor, such as antimicrobial activity against numerous microorganisms[6], to inhibit the transmission of HIV-1, to reduce ROS (reactive oxygen species) production from hydrogen peroxide-induced cardiac fibroblast[6], and wound healing activities.[5,8]

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Based on the crystallography, structure of SLPI consists of two homologous domains (N-terminal and C-terminal domains). Each domain of SLPI has eight cysteine residues that

establish four intramolecular disulfide bonds that cause SLPI has a compact protein structure.[6,9] The N-terminal part of SLPI is related to stabilize the protease inhibitor complex, and the C-terminal part has a protease binding side that functions as an inhibitory region.[6]

In our previous study, we cloned the SLPI gene from amniotic membranes and expressed it in *E.coli* BL21 by using pET101/DTOPO, which expressed active recombinant human SLPI (rhSLPI) stored in pET-ESLPI. This rhSLPI was deposited in the GenBank databases of National Center for Biotechnology Information (NCBI) under accession number of ABV21606.[9,10] However, the expression of rhSLPI products have not maximum yet. Thus, in this study, we have set up a synthetic SLPI gene based on amino acid sequences with codons, and expressed in *E. coli* BL21 to yield the optimum expression. In order to facilitate the cloning of the synthetic SLPI gene we used pUC57 plasmid to improve the number of synthetic SLPI gene copies, and pET-32a, a new expression vector for creating and increasing SLPI expression. Those vectors have a multiple cloning site (MCS) which contains typically unique restriction sites. Moreover, the pET-32a vector also contains polyhistidine tag (His-tag) in both sites of N- and C-terminals, while the amniotic membrane of SLPI gene merely carries his-tag at the C-terminal[9] that can help for simplifying purification of the recombinant protein.

Materials and methods

Research Samples

Reagents, Strain, and Vectors

As a cloning host E. coli strain TOP10 (Invitrogen, Carlsbad, CA, USA). The E. coli BL21 (Novagen, Darmstadt, Germany) stored in pET-ESLPI derived from previous work was used for an expression host. pET32a (Novagen) plasmids was used for the construction of the expression system. GeneJET plasmid miniprep kit (Thermo Fisher Scientific, USA), the cloning process used restriction enzymes of NcoI, XhoI, EcoRI, and DNA ligase (Thermo Fisher Scientific, USA). Tag DNA polymerase (Thermo Fisher Scientific, USA). The primers were synthesized by Institute of Tropical Disease (ITD). DNA was extracted and purified using a QIAamp Kit (Qiagen, Carlsbad, CA, USA). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) buffer, buffer A (50 mM Phospate buffer; 100 mM NaCl; dan 5 mM Imidazol), buffer B (50 mM Phospate buffer; 100 mM NaCl; dan 500 mM imidazol), elution buffer (200 mmol/l imidazole in PBS), Luria-Bertani (LB) medium supplemented with kanamycin (50 µg ml-1) and Isopropyl β-D-1-thiogalactopyranosides (IPTGs) were used for the growth of recombinant bacteria and protein expression. Porcine Pancreatic Elastase (PPE) and N-succinyil-alanyl-Lalanyl-L-prolyl-L-phenylalanyl-4-nitroanalide (NPN) (Sigma, St. Louis, MO, USA) were used as enzyme and substrate, respectively, to examine inhibitor activity.

Research Methods

Construction of synthetic SLPI encoding genes

1. ***Cloning and expression of synthetic SLPI genes***

A full-length rhSLPI coding sequence stored in pET-ESLPI was isolated using GeneJET plasmid miniprep kit (Thermo Fisher Scientific, USA). Thus the sequence was used as template for polymerase chain reaction (PCR) amplification. Designed gene was synthesized by Genscript and optimally analyzed by OptimumGeneTM based on the preferred codons in E. coli. The DNA fragment of synthetic SLPI gene was amplified by using designed primer pairs' sequences with forward primer, 5'-GATTAGAATTCATGAAGTCCAGCGGCCTCTT CCC -3' and reverse primer, 5'-CGACCTCGAGTCAATGGTGATGGTGATGAT GAC -3'. PCR was performed in a final volume of 10 µl using Tag DNA polymerase under the following conditions: a pre-denaturation and denaturation step of 94 ºC for 5 minutes and 55 seconds, respectively. Followed by 30 cycles of 72 ºC for 60 sec, annealing at 60 ºC for 30 sec, and extension at 72 ºC for 30 sec. The post-extension was completed at 72 ºC for 5 min. The PCR product was analyzed in 1% agarose gel electrophoresis.

For cloning, we used a high-copy pUC57 vector to generate pUC-SLPIopt. The PCR product was ligated into the pUC57 vector between the NcoI and XhoI sites in-frame and a pUC-SLPIopt was amplified by using designed primer pairs' sequences with addition of NcoI restriction enzyme (underlined) to the forward primer, 5'-CGCCATGGAGTGGTAAAGCC-3' and XhoI enzyme (underlined) to the reverse

primer, 5'-CGACCTCGAGTCAGGCTTTCACC-3', then the pUC-SLPIopt recombinant plasmid was transformed into E. coli strain TOP10.

For protein expression, a pET-32a plasmid used with NcoI/EcoI restriction enzymes. The SLPI gene was inserted into pET-32a plasmid and transformed E. coli strain BL21 were cultured and the recombinant plasmid was identified by PCR. The recombinant plasmid was named as pET-SLPIopt, and kept at -20 °C until use.

2. *Transformation of synthetic SLPI gene*

The SLPI gene transformation performed using a heat shock method at 42 °C for 45-90 seconds, then a single colony anchoring pUC-SLPIopt and pET-SLPIopt plasmids were cultivated into Luria-Bertani (LB) liquid medium supplemented with 50µg/ml kanamycin for 16 – 18 hours in a shaker incubator with 150 rpm speed at 37 ºC and inducted by 0.5 *mM* isopropyl β-D-1-thiogalactopyranosides (IPTG), respectively. The cell was then harvested 3 hours after IPTG induction by centrifugation. The cell pellet was subsequently resuspended in lysis-equilibration wash (LEW) buffer. The cell was messed by sonication at 4 MHz frequency, and then the cell debris separated by centrifugation for three minutes, the supernatant was collected.

The protein expression was analyzed by sodium dodecyl sulfate polyacrylamide gel elelctrophoresis (SDS-PAGE). All the constructs were verified by DNA sequencing. Genomic DNA was extracted from transformants to confirm that SLPI gene was integrated into the *E. coli* genome, and analyzed through PCR by using the cloning primers.

3. Fourier Transform Infrared (FTIR) Characterization

The fusion gene pE-SLPIopt was expressed following the conventional protocol to obtain the fusion protein pE-SLPIopt. Briefly, an overnight culture of BL21 containing the recombinant plasmid pE-SLPIopt was refreshed with LB broth containing 50 mg/L ampicillin, when the culture grew to an optical density (OD) of 0.6 at 600 nm, the cells were induced with 50, 100 and 500 uM of IPTG for 3 hours.

Then, the cells were harvested by centrifugation at 13,000 rpm for 1 min at 4 °C. The cell sediment was collected and resuspended with sterilized H_2O and equal volume of 2×SDS-PAGE buffer, and SDS-PAGE was performed to detect the fusion protein. Different induction IPTG concentration was compared to study the optimal expressing conditions. To detect the solubility of the fusion protein pE-SLPIopt, BL21 containing the recombinant plasmid pE-SLPIopt was induced by IPTG following the optimal expressing conditions as optimized above. Cells were harvested by centrifugation at 13,000 rpm for 1 min at 4 °C, resuspended in 20 mmol/l Tris-HCl (pH 8.0). The mixture was broken by sonication process on ice for 15 min. The total lysate was divided into soluble (supernatant), and insoluble (pellet) fractions by centrifugation at 13,000 rpm for 5 min at 4 °C. The soluble was filtered by Sartorius™ Minisart™ NML Syringe Filters.

The soluble and insoluble fractions were analyzed by 12% SDS-PAGE, and visualized using Coomassie Brilliant Blue. Ni-NTA of BioLogic DuoFlow™ Medium-Pressure chromatography was used to purify protein from the expressing cells broken by sonication process with the conventional protocol mentioned above. The supernatant was passed through the His-Trap 1 mL (GE Healthcare) column, followed by washing the column with 8 ml buffer A (pH 7.2) and 8 ml wash buffer (20 mmol/l imidazole in PBS), respectively. The target protein was then eluted with 2 ml of elution buffer. The eluted fractions were detected by 12% SDS-PAGE.

Results

Optimized synthetic SLPI gene design

A codon-optimized SLPI gene was successfully synthesized by the Genscript software (www.genscript.com) to obtain SLPI gene with enhanced translation efficiency. The Codon Adaptation Index (CAI) value was increased from 0.60 (before optimization) to 0.90 (after optimization) shown the distribution of codon usage frequency along the length of the gene sequence. A CAI of 1.0 is considered perfect in the desired expression organism in terms of high gene expression level. The GC content of the sequence was 52.45% (before optimization) increased to be 54.57% (after optimization), and the value of 100 was set for the codon with the highest usage frequency for a given amino acid in the organisms with the desired expression (Figure 1).

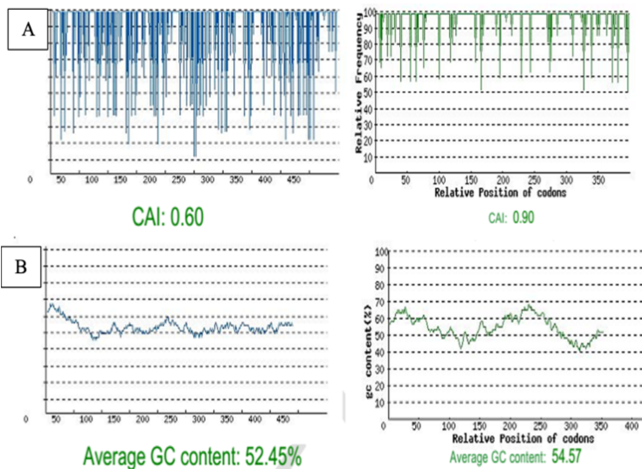

Figure 1. A bioinformatics analysis of synthetic SLPI gene using Genescript codon software with before optimization (left) and after optimization (right). **(A)** Codon adaptation index of SLPI gene; **(B)** GC content of SLPI gene.

Construction of synthetic SLPI encoding genes

In this research, an analysis of the surface structure and morphology of the material was carried out using SEM. SEM images on BAM at 50x and 500x magnification (figures 2a, 2b, 3) show a flat surface. Meanwhile, the modified BAM showed macro and micro pore structures with magnifications of 50x and 500x (Figures 4a, 4b, 5).

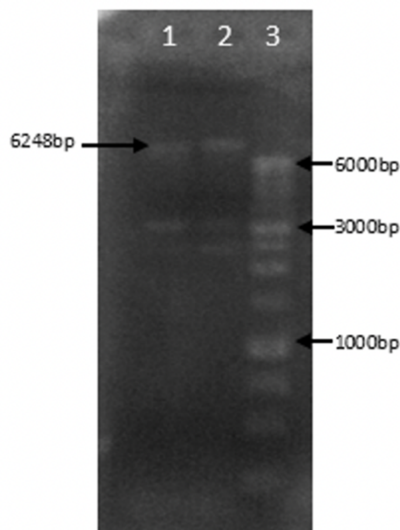

Figure 2a. PCR amplification of pET-ESLPI recombinant plasmid. Lane 1-2: Amplified product of pET- ESLPI recombinant plasmid (aprroximately 6248 bp); Lane 3: molecular weight standard.

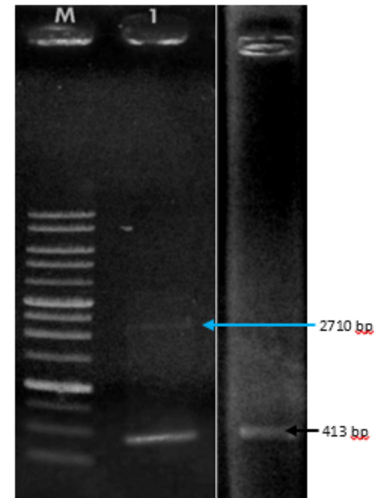

Figure 2b. PCR amplification of pUC-SLPIopt in *E. coli*. TOP10 **A**. Restriction profile of pUC-SLPIopt in *E. coli*. Lane M: molecular weight standard, Lane 1: pUC-SLPIopt with digested by *XhoI* and *NcoI*. **B**. PCR amplification of positive clone.

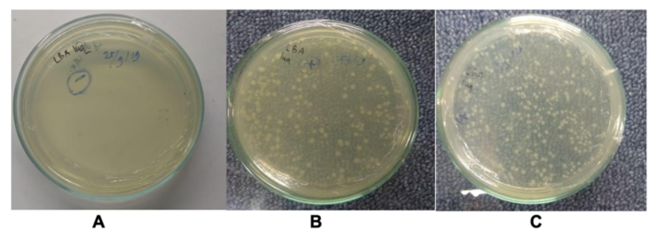

Figure 3. Transformant in *E. coli* BL21. **A**. Negative control; **B**. Positive control (pET-32a); **C**. Sample pET-SLPIopt.

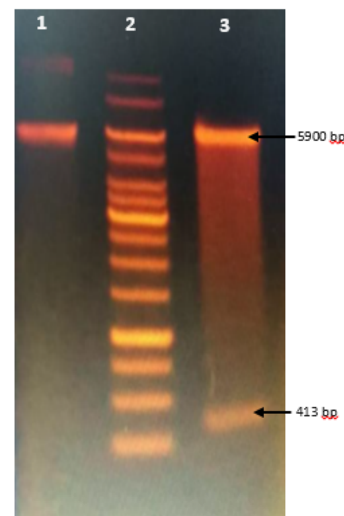

Figure 4a. Restriction profile of pET-SLPIopt in *E. coli* B21. Lane 1: pET-SLPIopt without digestion; Lane 2: molecular weight standard; Lane 3: pET-SLPIopt with digested by *NcoI* and *EcoI.*

Expression of the pET-SLPIopt recombinant plasmid

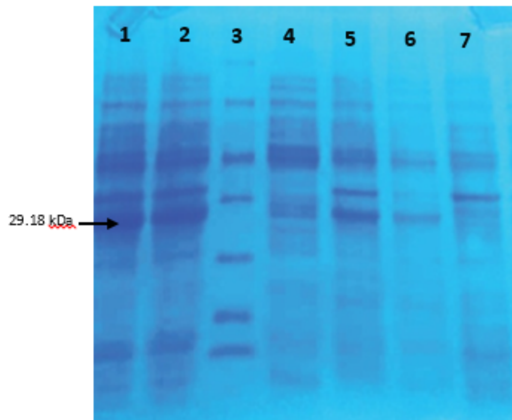

Figure 4b. SDS-PAGE of the pET-SLPIopt fusion protein. Lane 1: Insoluble fraction with 500 uM IPTG induction; Lane 2: Insoluble fraction with 100 uM IPTG induction; Lane 3: Molecular weight standard; Lanes 4: Insoluble fraction with 50 uM IPTG induction; Lane 5: Soluble fraction with 500 uM IPTG induction; Lane 6: Soluble fraction with 100 uM; Lane 7: Soluble fraction with 50 uM. The main fusion protein band was denoted with an arrow.

Analysis of fusion protein pE-SLPIopt

The SLPI enzyme expressed in the pET-32a plasmid carrying the His-tag marker can be purified by Ni-NTA chromatography. The soluble was filtered by Sartorius™ Minisart™ NML Syringe Filters. The soluble and insoluble fractions were analyzed by 12% SDS-PAGE, and visualized using Coomassie Brilliant Blue (CBB), and fusion protein was mainly expressed in the supernatant, although not pure yet (Figure 7).

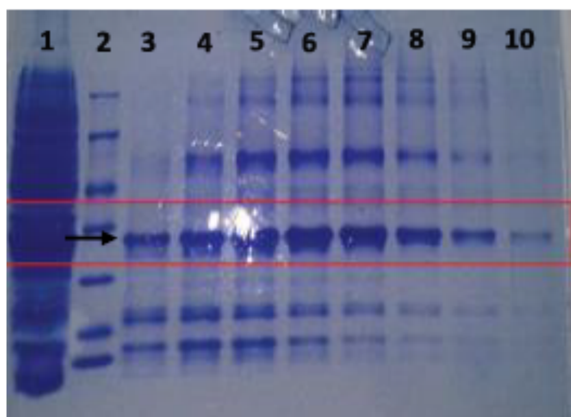

Figure 5. SDS-PAGE of the pET-SLPIopt fusion protein before and after running by Ni-NTA chromatography. Lane 1: Soluble fraction before Ni-NTA; Lane 2: Molecular weight standard; Lane 3-10: Soluble fraction after Ni-NTA. The main fusion protein band was denoted with an arrow.

Discussion

This study indicated that *E. coli* strain B21 can serve as an expression host for efficient and optimizing production of functionally active human SLPI. The efficiency of gene expression in organisms is influenced by several factors such as codon usage bias, codon frequency distribution, and GC content.[11] The CAI values clearly parallel levels of gene expression, and have generally high CAI values. Regarding to Gouy and Gautier (1982) that in *E. coli* the relationship between codon bias and gene expression is illustrated by considering operon, seems firmly established[12], and that certain codons affect expression level, this suggests that for a heterologous gene to have a maximal level of expression its codon usage need to correspond to that of the host.[13,14]

The pUC-SLPIopt recombinant plasmid resulted from the genscript synthesis process was transformed into *E. coli* TOP10, and yield more than 10 colonies of transformants. The transformed colonies then isolated and analyzed to confirm the plasmid and DNA insertion. PCR product of pUC-SLPIopt recombinant plasmid has detected by PCR with F/R primers (mentioned above) with double-digestion of *XhoI* and *NcoI* restriction enzymes at 2710 bp and 413 bp band sizes (Figure 3) The band size of 413 base-pairs is the optimized SLPI suitable to previous study.[15,16]

The transformation was done using the transformation kit, and transformants obtained on LB medium (Figure 4) Double digested synthetic SLIPI gene by *XhoI* and *NcoI* restriction enzymes from pUC-SLPIopt was inserted into the expressing plasmid pET-32a. The pET-SLPIopt plasmids was cultivated into Luria-Bertani (LB) liquid medium supplemented with 50µg/ml kanamycin for 16 – 18 hours in a shaker incubator with 150 rpm speed at 37ºC and inducted by 0.5 *mM* isopropyl β-D-1-thiogalactopyranosides (IPTG). Transformation of the pET-SLPIopt recombinant plasmid was isolated and analyzed by PCR with a pairs of primers (F/R) and *NcoI* and *EcoI* restriction

enzymes showed the successful construction of pET-SLPIopt with two bands consist of pET-32a vector 5.9 kb and insert 413 bp (Figure 5)

The pET-SLPIopt recombinant plasmid was expressed in host bacteria *E. coli* BL21. Several expression conditions were experimented, when the cell density for pET-SLPIopt induction was at OD600 of 0.6, and the optimal protein expression occurred at 3 hours post-induction with various IPTG concentrations (50, 100 and 500 uM) at 30 °C, both soluble and insoluble fractions were analyzed by 12% SDS-PAGE, and visualized using Coomassie Brilliant Blue. SDS-PAGE result showed that the molecular weight of 29.18 kDa, and the fusion protein appeared mainly in the pellet of the induced pET-SLPIopt transformed bacteria (Figure 6). In contrast to the expression of pET-SLPIopt after lysis by sonication, fusion protein was mainly expressed in the supernatant (Figure 7).

Conclusions

The synthetic SLPI gene whose codons were optimized according to the codon usage frequencies of the host E. coli strain B21 can serve as an expression host for efficient and optimizing production of SLPI, pET-SLPIopt. The product of the recombinant protein of pET-SLPIopt was analyzed by SDS-PAGE result showed that the fusion protein mainly expressed in the supernatant. That means the recombinant protein could be expressed in dissolved form.

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Declaration Of Interest

The authors report no conflict of interest.

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