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Research Article

Design and Cloning of Gene Encoding SLPI C-Terminal Domain in *Escherichia coli* TOP10

Evi Umayah Ulfa[1*], Ni Nyoman Tri Puspaningsih[2]

[1] Fakultas Farmasi, Universitas Jember, Indonesia

[2] Fakultas Sains dan Teknologi, Universitas Airlangga, Indonesia

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*Corresponding author: evi.farmasi@unej.ac.id

Abstract

Elevated levels of neutrophil elastase in CPOD (Chronic Obstructive Pulmonary Disease) airways are regarded as the main trigger of lung destruction and inflammation. SLPI (Secretory leukocyte protease inhibitor), an inhibitor protease, represents an attractive candidate for treatment in chronic lung diseases due to proteases excess. The antiprotease active site of SLPI has been located on the C-terminal domain. This study aimed to design and clone the gene encoding the C-terminal domain of SLPI (SLPIc). The gene encoding SLPIc was optimized and predicted solubility using OptimumGene™ and SoDoPe software. The nucleotide sequence of the optimized SLPIc was synthesized, inserted into the pGEX 4T-2 vector commercially by Genscript, and transformed into the *Escherichia coli* TOP10. The pGEX 4T-2 vector contains a glutathione S transferase (GST) gene located before the MCS to generate a recombinant protein for fusion with GST. For purification purposes, the His-tag synthesized together with SLPIc. The optimized SLPIc nucleotide sequence gave a CAI value of 0.81, GC content 52.31, and a CFD of 2%. The solubility probability of SLPI fused with GST increased from 0.124 to 0.4656. Confirmation of the transformant using restriction and sequencing analysis showed that the gene encoding of SLPI domain C-terminal optimized in the pGEX 4T-2 plasmid was successfully transformed into *E. coli* TOP10 as novelty of this study. The optimized SLPIc gene in pGEX 4T-2 has a high probability of being expressed in E. coli based on in-silico analysis.

Keywords: Cloning, codon optimized, SLPI domain C, *Escherichia coli* TOP10.

Desain dan Kloning Gen Penyandi SLPI Domain C-Terminal pada *Escherichia coli* TOP10

Abstrak

Peningkatan kadar neutrofil elastase di saluran udara CPOD (penyakit paru obstruktif kronis) dianggap sebagai pemicu utama kerusakan dan peradangan paru-paru. SLPI (*Secretory Leukocyte Protease Inhibitor*), sebuah protease inhibitor, berpotensi sebagai kandidat untuk pengobatan penyakit paru-paru kronis karena kelebihan protease. Sisi aktif antiprotease SLPI terletak pada domain C terminal. Tujuan penelitian ini adalah melakukan desain dan kloning gen penyandi SLPI domain C (SLPIc). Gen penyandi SLPI domain C dioptimasi kodon menggunakan perangkat lunak OptimumGene™ dan diprediksi solubilisasinya menggunakan perangkat lunak SoDoPe. Urutan nukleotida gen penyandi SLPIc hasil optimasi disintesis, disisipkan pada vektor pGEX 4T-2 secara komersial oleh Genscript dan ditransformasikan ke inang *Escherichia coli* TOP10. Vektor pGEX 4T-2 yang digunakan mengandung glutathione S transferase (GST) sebelum MCS untuk menghasilkan protein rekombinan fusi dengan GST. Pada ujung N terminal dari SLPIc ditambahkan His6 tag untuk memudahkan proses pemurnian. Urutan nukleotida SLPI domain C hasil optimasi memberikan nilai CAI 0,81, GC content 52,31 dan CFD 2%. Probabilitas kelarutan SLPI yang difusikan dengan GST meningkat dari 0,124 menjadi 0,4656. Hasil konfirmasi transforman dengan menggunakan analisis restriksi dan sekuensing menunjukkan

gen penyandi SLPI domain C hasil optimasi dalam plasmid pGEX 4T-2 berhasil ditransformasikan ke dalam *E. coli* TOP10 sebagai kebaruan dari penelitian ini. Berdasarkan hasil ananlisis *in silico* dapat disimpulkan gen SLPIc yang telah dioptimasi kodonnya memiliki kemungkinan tunggi untuk diekspresikan dalam *E. coli*.

Kata Kunci: Kloning, *Escherichia coli* TOP10, optimasi kodon, SLPI domain C.

1. Introduction

Chronic obstructive pulmonary disease (COPD) is a group of lung diseases which characterized by airflow obstruction due to chronic inflammation of airways. It is a common cause of mortality worldwide including in Indonesia. WHO data in 2002, it is the third main cause of disease with high mortality in the world after cardiovascular disease and cancer.[1,2] By 2004, the prevalence rate of pain in people with COPD in Indonesia is 35% followed by asthma (33%) and lung cancer (30%). Based on Riskesdas data in 2018, chronic obstructive pulmonary disease prevalence in Indonesia is 3.7%.[3]

Cigarette smoking or exposure to noxious agents is the main cause of COPD and is thought to be responsible for 80-90% of cases. The long-term exposure of smoking induces chronic lung inflammation and increased levels of neutrophils, macrophages and dendritic cells. A Prolonged inflammation may damage lung parenchyma and narrowing of respiratory tract. Besides inflammation, oxidative stress and an imbalance between proteases and antiproteases also contributes in pathogenesis of COPD.[4,5] Neutrophils and macrophages release various proteases such as neutrophil elastase (NE), cathepsin G, and proteinase-3, as well as MMP-8 and MMP-9. NE, cathepsin G, and proteinase-3 play as potent mucus stimulants that can aggravate airflow obstruction in COPD patients. These enzymes are also destroying alveolar tissue. Excessive proteases due to accumulation of inflammatory cells result in protease-antiprotease imbalance and further trigger the process of lung destruction in COPD.[6,7]

Protease inhibitors play an important role in reducing chronic inflammation caused by protease activities. A molecule like secretory leukocyte protease inhibitor (SLPI) is also able to control excessive NE activity. Consequently, SLPI represents a potential candidate for use in the treatment of COPD. SLPI is a protein with a size of 11.7 kDa and consists of 107 amino acids with two Whey Acidic Protein (WAP) domains, namely the N-terminal domain and the C-terminal domain.[8,10] SLPI consists of two homologous domains that each domain has four disulfide bonds, and is responsible to maintain the structure of SLPI. A study of the domain structure-activity relationship of SLPI suggests that the inhibition of NE is located in the C-terminal domain.[8,9]

A previous study (Munadzhiroh, 2017) has been successfully expressed a full-length human SLPI (rhSLPI) in *Escherichia coli*. However, the expression level of recombinant full-length SLPI is ≤1.0 mg/L culture and not sufficient for industrial scale.[11] Other researchers successfully overproduced a recombinant full-length SLPI in *E. coli* using a codon-optimized synthetic gene. Unfortunately, rSLPI accumulated intracellularly in inclusion bodies and should be properly refolded into its biological active conformation.[12,13]

Protein fusion technology has been widely used to promote protein solubility in *E. coli*, help with protein purification, and increase protein immunogenicity as well. Fusion tags like Maltose binding protein (MBP), thioredoxin (Trx), Small ubiquitin-modifier (SUMO), and glutathione S transferase (GST) have commonly used for protein solubility fusion and are commercially available.[14,15] GST fusion partner, 26 kDa, can be used to increase the solubility of numerous proteins of mammalian origin in *E. coli* as an N terminal fusion.[15,16] GST has the additional benefit of functioning as an affinity tag with glutathione resin, thus helping the purification process. The number of dissolved protein fractions of G3PDH, DHFR,

DUSP14 fused with GST tag and his tag is higher than without fusion.[16] In this study, we designed and cloned the gene encoding of the C-terminal domain of SLPI (SLPIc) that is fused with the GST tag. To facilitate protein purification using a nickel-affinity column, we also inserted polyhistidine tag at C-terminal after SLPIc.

2. Materials and Methods

2.1. Materials

The bioinformatics tools used for the design and in silico analysis are OptimumGene™ (*www.genscript.com*), Rare Codon Analysis (*https://www.genscript.com/cgi-bin/tools/rare_codon_analysis*). SoDoPe proteins (https://tisigner.com/sodope/) were used for protein solubility prediction. The sequence of SLPI amino acids was obtained from the NCBI (Access code: EU116331). Luria Bertani (LB) media (tryptone 1% yeast extract 0.5% and NaCl 1%), LB media supplemented with ampicillin (100 µg/L), *E. coli* TOP10, DNA plasmid isolation kits from TianGene, bacterial transformation kits (Thermo scientific), LEW agarose, TAE 1x buffers, DNA ladder, pGEX 4T-2.

2.2. Methods

2.2.1. Design and codon optimization of SLPIc

The amino acid sequence of SLPI domain C (Arg55–Ala107) was codon-optimized using OptimumGene™(Genscript). Histidine tag was placed at the C terminus to facilitate protein purification. The codon adaptation index (CAI), GC content, and Codon Distribution Frequency (CFD) of optimized and non-optimized SLPIc were investigated by Rare Codon Analysis (*https://www.genscript.com/cgi-bin/tools/rare_codon_analysis*).

2.2.2. Prediction of SLPIc solubilization

The solubility of SLPIc with GST and without GST was performed by SoDoPe (https://tisigner.com/sodope) software. The GST tag was fused at terminus via thrombin site cleavable linker sequence, while histidine tag was fused at N terminus.

2.2.3. Construction of the gene encoding SLPIc on the pGEX 4T-2

The nucleotide sequence of optimized SLPIc followed by a His-tag was synthesized and inserted into the pGEX 4T plasmid vector by GenScript (NJ, USA) using *Eco*RI and *XhoI* restriction sites to produce pGEX-SLPIc recombinant plasmid (Figure1).

2.2.4. Transformation of pGEX-SLPIc in E. coli TOP10

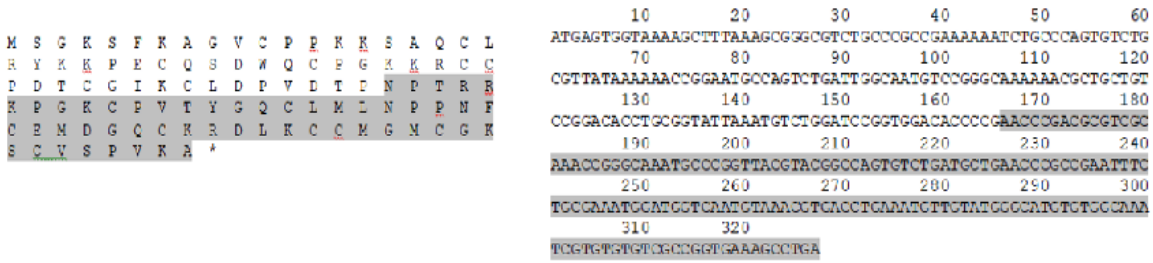
The plasmid recombinant of pGEX-SLPIc was transformed into *E. coli* TOP10 competent cells by heat shock method (45–60 seconds at exactly 42°C). Competent cells of *E. coli* TOP10 were prepared by using the TransformAid bacterial transformation kit (Thermo scientific). Transformed bacteria were cultured on LB media supplemented with 100 µg/ml ampicillin at 37°C. The presence of SLPIc into the recombinant vector was confirmed by restriction enzyme analysis and sequencing.

3. Results

3.1. Design and codon optimization of SLPIc

The amino acid sequence of SLPIc was back-translated and optimized considering *E. coli* as the expression host. The nucleotide sequence of the SLPIc after optimization by using OptimunGeneTM software can be seen in Figure 1. The results of Rare Codon analysis of genes encoding SLPIc before and after optimization can be seen in Table 1.

The CAI (codon adaptation index) value of the non-optimized SLPIc gene was calculated to be 0.58 outside the ideal value for expression in *E. coli*. The FOP (*frequency of optimal codon*) of nonoptimized SLPI gene value is 34% which is also outside the ideal value (Table 1). This result showed that non-optimized SLPIc contains a rare codon for E. coli; thus, ineffective translation might occur 17,18. Codon optimization using OptimumGeneTM software was used to overcome this problem. The optimized SLPIc gene exposed a CAI of 0.8, a GC content of 52.31 %, and an FOP of 60%. All parameter of optimized SLPIc is in the ideal range



(A) (B)

Figure 1. The amino acid sequence of SLPI (A) and the SLPI coding sequence with codon optimization for *E. coli* (B). SLPIc (gray).

compared with non-optimized SLPIc. The nucleotide sequence of optimized SLPIc can be seen in Figure 1.

Prediction of SLPIc protein solubility was performed by using SoDoPe tools. The Fusion protein of SLPIc with GST (SLPIc-GST) has a probability of solubility 0.4686 when expressed in *E.coli* (Table 2).

3.2. Construction of the gene encoding SLPIc on the pGEX 4T-2

The construction of the optimized SLPIc gene to be inserted into the pGEX 4T-2 vector plasmid is shown in Figure 2. The SLPIc gene was introduced at *Eco*RI and *XhoI* recognition sites. After the *Eco*RI recognition site, two nucleotides (AC) were added so that the SLPIc gene was inframe with a GST tag. SLPIc constructs on the pGEX 4T-2 sequentially were the EcoRI recognition site, nucleotides A and C, the SLPIc gene, His-tag, stop codon, and XhoI recognition site (Figure 2).

3.3. Transformation and characterization of pGEX-SLPIc in *E. coli* TOP10

The recombinant plasmid of pGEX-SLPIc that has been synthesized commercially was transformed into *E. coli* TOP10 using the heat shock method. There are 26 transformant colonies were obtained on screening media. A plasmid of selected transformant was isolated and performed restriction analysis using single and double digestion (Figure 3). The double digestion of selected recombinant plasmid using *Eco*RI and *XhoI* restriction enzymes showed the predicted size of the inserted gene (215 bp) and plasmid vector (4.9 Kbp). The result of nucleotide sequencing showed a coding sequence of the SLPIc gene in frame with a GST fusion partner and His-tag (Figure 3). These results indicated the selected plasmid carrying coding sequence of SLPIc.

4. Discussion

SLPI is a protein that plays an important role to control the activity of elastase and prevent excessive inflammatory processes in pulmonary epithelial cells.[17] It has been tested as a potential anti-elastase candidate drug for various diseases due to uncontrolled NE activity such as cystic fibrosis, and COPD[18,19]. The inhibitory activity of SLPI against NE, chymotrypsin, and trypsin is located in the C-terminal domain. Masuda (1995) reported that a recombinant half-sized SLPI containing the C-terminal domain active an inhibitor as full-length SLPI against NE. This data provides information that SLPIc is also promising as a candidate for anti-elastase drugs.[20]

Despite having full-length SLPI-like activity, very few studies have addressed the expression of recombinant SLPIs in *E. coli* and their pharmacological activity. Meckelein *et al*. (1990) reported that the C-terminal domain of SLPI can be expressed directly

Table 1. Rare Codon Analysis Results

Sequences	CAI	GC	FOP
Non-optimized SLPIc	0.58	43.06%	34%
Optimized SLPIc	0.81	52.31%	60%

Table 2. Results of Prediction Analysis of SoDoPe Analysis

Protein	Probability	Fleksibilitas	GRAVY
SLPIc	0,1248	0,9985	-0,4642
SLPIc-GST	0,4686	1,0006	-0,4296

in *E. coli*, however, the yield of 1/2SLPI expression was <1 mg/L culture.[9] An in-silico analysis by codon optimization and fusion with solubility-enhancing proteins is needed to increase the yield of recombinant SLPIc protein expressions in *E. coli*.

The result of rare codon analysis showed the native gene sequence of SLPIc contains rare codons for the *E. coli* expression system, thus protein translation inefficient might occur.[17,18,25] Human genes generally used codons AGG/AGA (arginine), GGA (glycine), and AUA (isoleucine) which are rare codons in *E. coli*. [21] Replacing rare codons or adding rare codon tRNAs to the host expression system can be used to overcome the problem of translational inefficiency. Recently, synthetic gene technology is widely used to replace rare codons due to cost-effectively than site-directed mutagenesis. [22]

In this research, the amino acid of SLPIc was back-translated and optimized considering *E. coli* as the expression host. Three parameters i.e codon adaptation index (CAI), GC content, and Codon Frequency Distribution (CFD) were computed using codon optimization tool and optimized based on the desired host. CAI is a value that indicates how frequently a favored codon is used in the desired organism. CAI can be applied for predicting the expression level of heterologous protein based on its codon sequence. CAI values range from zero to one, the gene with a CAI value of more than 0.8 is rated good for being expressed in the desired host. The GC content of the desired gene determines its transcription and translation efficiency. The ideal GC content value is between 30-70%. The codon with frequency distribution lower than 30% is likely to hamper the expression efficiency.[18,25] The optimized SLPIc gene has better CAI, GC, and CFD values than the native gene sequence of SLPIc (Table 1). The optimized CAI was 0.81, and the CFD value was about 2% revealing the high and stable expression

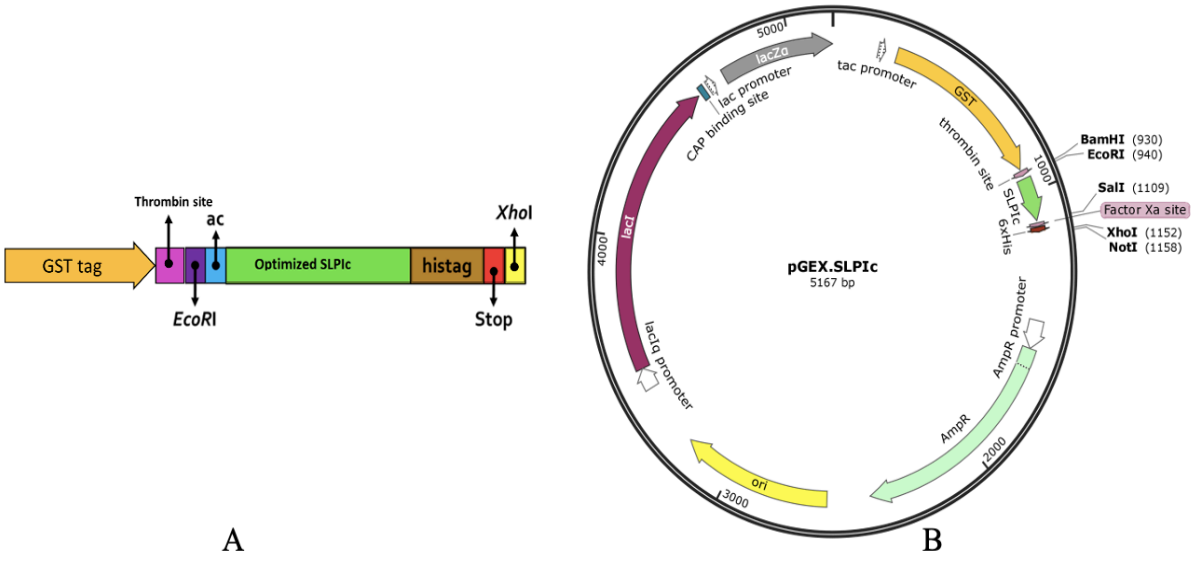


Figure 2. The schematic representation of the SLPIc construct (A) and recombinant plasmid pGEX-SLPIc (B). SLPIc: gene for the C-terminal domain of SLPI, *Eco*RI: restriction sites of *Eco*RI, Start: start codon, His-tag: histidine tag, Stop: stop codon, *XhoI*: restriction sites of *XhoI*, GST: GST Fusion partner, AmpR: ampicillin resistance.

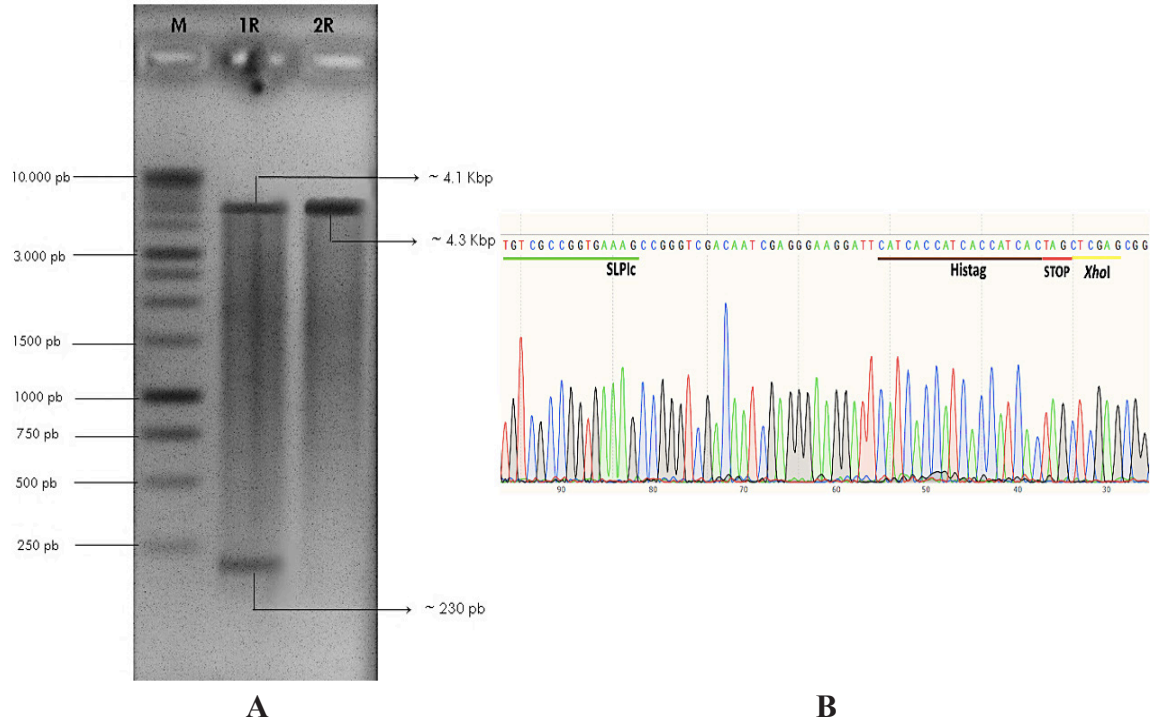


Figure 3. Restriction analysis of pGEX-SLPIc (A) and Nucleotide Sequencing of pGEX-SLPIc (B). P: undigested pGEX-SLPIc, 2R : pGEX-SLPIc digest with *Eco*RI and *XhoI* enzymes, M: DNA marker 1 Kb.

in bacterial cells.

Multiple publications have shown that codon-optimized genes increased the yield of expressed proteins in *E. coli* compared to native genes. The heterologous protein yields of optimized IL-2 with CAI 0.85 were increased 16 times than native IL-2 with a CAI value of 0.3318. These results are in line with Mignon *et al* (2018), who reported expression of optimized ecTRAP (the extracellular domain of thrombospondin-related adhesive protein from *Plasmodium vivax*) and scFv D1.3 (a murine anti-lysozyme antibody single-chain variable fragment) allowed better expression than the native protein. The CAI of unoptimized ecTRAP and scFv were < 0.6 while the CAI of optimized ecTRAP and scFv were > 0.826. Comparison of native gene expression and synthetic gene expression of 30 human short-chain dehydrogenase/reductase genes (SDRs) in *E. coli* showed 29 genes were improved when expressed from codon-optimized genes. Only one gene, (HSD11B2), was expressed at a higher level than the synthetic version.[22]

Solubility prediction of SLPIc using SoDoPe software was performed to estimate the solubility of SLPIc before and after fusion with GST. The SoDoPe software predicts protein solubility based on the *Solubility-Weighted* Index (SWI).[23] The solubility index of the SLPIc optimized gene was improved from 0.124 in wild type sequence to 0.468 (Table 2). The GST fusion partner is expected to increase soluble SLPI expression levels. Solubility of recombinant protein is influenced by molecular weight, hydrophobicity, isoelectric point, polarity, as well as by extrinsic factors including ionic strength, pH, and temperature.[24]

Considering *in silico* analysis, we used a commercial pGEX4T-2 vector for cloning and expression SLPIc. This vector is an expression vector that carries a GST tag. GST tag can be used for purification as well as for increasing the yield of soluble protein.[26] pGEX 4T-2 also carries a beta-lactamase gene which can be used for transformant selection. The E. coli TOP10 that carried pGEX-SLPIc plasmids can grow in LB ampicillin media,

while *E. coli* TOP10 that does not carry pGEX -SLPIc plasmids cannot grow on ampicillin LB media (Figure 2B).

Restriction analysis and nucleotide sequencing are routinely used methods to confirm successful cloning. Nucleotide sequencing confirmed the presence and sequence orientation of the desired gene in the vector. Restriction using *Eco*RI and *XhoI* showed the presence of two bands with sizes of 4955bp and 215 bp. This size corresponds to the theoretical size of the pGEX 4T-2 plasmid (4955 bp) and DNA insert (SLPIc = 159 bp) fused with Histag. The nucleotide sequencing result on recombinant plasmid showed the existence of recognition site of *Eco*RI (GAA TCC), nucleotide A and C, SLPIc, His-tag, stop codon (TAG), and recognition site of *XhoI* (CTC GAG) (Figure 3B). BLAST analysis on amino acid sequence showed 100% similarity with the amino acid of SLPI C-terminal domain from human This data indicated that the pGEX-SLPIc recombinant plasmid has been successfully transformed into *E. coli* TOP10.

5. Conclusion

The results of the analysis *in silico* showed that the optimized SLPIc gene has a high probability of being expressed in *E. coli.* The fusion of the SLPIc gene with GST was successfully designed and transformed into *E. coli* TOP10. Although testing in-silico predictions were good, the experimental studies are required to be verified this construct.

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