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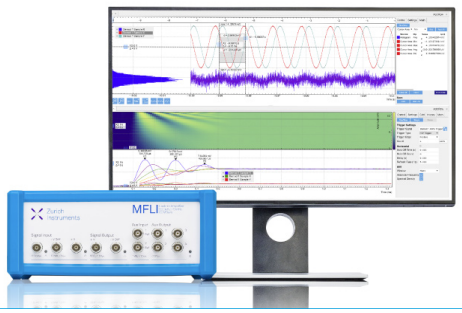

Cloning of SLPI Gene containing HM-1 Signal peptide in *Saccharomyces cerevisiae*

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Abstract. SLPI is a non-glycosylated protein and it composed of 107 amino acid secreted by epithelial cells, macrophages, and neutrophils. This protein has multiple functions i.e. anti-inflammatory, anti-microbial, and wound healing. SLPI is involved in wound healing by protecting epithelial tissues from serine protease degradation during inflammation. Our previous study has successfully cloned SLPI gene generated from the amniotic membrane into the pET-101/D-TOPO vector. However, *E. coli* is not a safe host for recombinant protein production because its cell wall contains toxic pyrogens. To overcome the limitation, we cloned SLPI gene into the pYHM1 vector for expression in *Saccharomyces cerevisiae*. The American Food and Drug Administration (FDA) categorized *S. cerevisiae* as a Generally Recognized as Safe (GRAS) microorganism. The pYHM1 vector contains HM1 signal peptide to mediate protein secretion. SLPI gene from amniotic membrane was amplified from pET-*ESLPI*. The PCR product was digested by *Sac*I and *Eco*RI and cloned into a pYHM1 vector to produce pY_*SLPI* recombinant plasmid in *E. coli* TOP10. The recombinant plasmid was then transformed into *S. cerevisiae* BJ1824 using lithium acetate method. The *SLPI* gene was successfully amplified with size ~339 bp and cloned into the pYHM1 vector in E. coli TOP10. The restriction and nucleotide sequence analysis showed that transformant number 31 contained SLPI gene. The pY_*SLPI* recombinant plasmid has been successfully transformed into *S. cerevisiae* BJ1824. SLPI recombinant was detected in intracellular and cell-associated by SDS PAGE.

Keywords: SLPI, pYHM1 vector, HM-1 Signal Peptide, *Saccharomyces cerevisiae.*

INTRODUCTION

Secretory Leukocyte Protease Inhibitor (SLPI) is a serine protease inhibitor belongs to Whey Acidic Protein family (WAP) which has a molecular weight of 11.7 kDa. It is expressed by different tissues, including trachea, bronchus, oral mucosa, salivary gland, lung, and vagina[1]. The protein also inhibits elastase and cathepsin G from human neutrophile, trypsin and chymotrypsin derived from pancreatic proteases, and chymase and tryptase from mast cell [2,3].

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Originally SLPI is discovered as protease inhibitor, yet recent studies showed that it has numerous functions such as anti-inflammatory, wound healing, antimicrobial and antiviral[3–5]. It contributes to accelerate the wound healing on inflammation, proliferation and maturation phase[3]. Its another role is reducing inflammatory response by inhibiting of neutrophil elastase, inhibiting nuclear factor-kB (NF-kB) activation, and increasing antiinflammatory cytokine production (interleukin 10 (IL-10) and tumor nuclear factor -β (TNF-β). Inhibition of NF-kB activation renders macrophages unable to release of TNFα, IL-1β and nitrite oxide. Additionally, SLPI also leads to an elevated level of vascular endothelial growth factor and platelet derived growth factor[3,6,7].

Several studies reported that SLPI has antibacterial activity against gram positive and gram negative bacteria. The inhibition activity of SLPI on skin associated bacteria including *Eschericia coli*, *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *S. epidermidis* indicates that SLPI controls microbial bioburden in the skin [8,9]. Besides having antibacterial activity, this protein also demonstrates antifungal activity against *Aspergillus fumigatus*, and *Candida albican*[3]. Antimicrobial mechanism is employed by SLPI through membrane disruption. A high positive charge could trigger interaction between SLPI and lipid membrane of bacteria which result in membrane damage[3]. As an antiviral, it prevents transmission of HIV-1 by blocking interaction between HIV-1 virus with receptor of the host cell. SLPI binds to the annexin II on the macrophage surface, thus interfering with the binding of the HIV viral phosphatidyl serine to host receptors[4]. Based on its multifunction, SLPI represents a promising protein therapeutic candidates to be used in post gingival recession therapy[10].

Based on X ray crystalography, SLPI has two highly identical domain (N terminal and C terminal domain) that each contains four disulfide bridges formed from eight cysteine residues. N terminal domain of SLPI consists of Ser1-Pro54 residues while C terminal domain consists of Asn55-Ala107 residues. These disulfide bridges stabilize and maintain the compact structure of SLPI[11].

The gene encoding SLPI has been widely isolated and engineered to obtain recombinant SLPI. A cDNA of SLPI from amniotic membrane was successfully cloned into the plasmid pET-101/D-TOPO in *Escherichia coli* BL-21. A full-length recombinant SLPI has successfully expressed by IPTG induction although at a low level[12]. Amniotic membranes have been widely used for treatment of burns, chronic ulcers, dural defects, peritoneal and genital reconstruction, and plastic surgery[13]. Different research groups have been expression SLPI in *E. coli* as inclusion bodies that requiring refolding of SLPI to correct its native conformation[14]. Although *E. coli* is preferred as a host for protein expression, the lipopolysaccharide (LPS) of the outer membrane can induce pyrogenic response and trigger septic shock. To overcome the limitation, We have chosen yeast *Saccharomyces cerevisiae* to express the full-length *SLPI* gene. *S. cerevisiae* is recognized as a safe microorganism. *S. cerevisiae* is also capable of modifying post-translation, including disulfide bond formation. In addition, Heterologous protein can be expressed extracellularly by using *S. cerevisiae* while in *E. coli* can not[15].

Stetler (1989) has cloned gene encoding SLPI isolated from human parotid tissue and secreted in *S. cerevisiae* using two secretion system i.e α-factor and invertase signal sequences. SLPI is only successfully secreted by α factor signal sequence, although incompletely processed and misprocessed form of SLPI. Invertase system generated a mixture of an active full-length SLPI and inactive fusion SLPI with invertase in cell-associated[16]. The choice of a signal sequence that is fused with proteins is an important factor for obtaining a secreted proteins. There is no systematic approach to predict which signal sequence is best for secretion a certain protein. A signal sequence from *Hansenulla mrakii* IFO 0895 killer toxin (HM1) was successful to secretion of α-L-arabinofuranosidase[17]. In this study, we cloned *SLPI* gene into the pYHM1 vector for expression in *S. cerevisiae.*

MATERIALS AND METHODS

Materials

All chemicals were purchased from Sigma (St. Louis, MO–USA) or thermo fisher scientific except when specifically mentioned. *E. coli* strain BL21 Star (DE3) containing pET-ESLPI recombinant plasmid, *E. coli* TOP10, and *E. coli* TOP10 containing pYHM-1 plasmid derived from Airlangga University collection. *Saccharomyces cerevisiae* BJ1824 (MATα, ura3, leu2, his3, trp1, pep4-3) and plasmid pAT425 are obtained from Yeast Genetic Resource Center (YGRC) collection, Osaka University, Japan. All oligonucleotides were synthesized by IDT. Pfu DNA polymerase, dNTP, T4 DNA ligase, restriction enzyme (*Sac*I, *Eco*RI), plasmid purification kit and gel extraction kit were used for DNA manipulation. Luria Berthani (LB) broth and agar containing 100μg/mL ampicillin were used for bacterial growth and selection of bacterial transformants. YPD (2% Bactopeptone, 1% yeast extract,

and 2% glucose) was used for yeast growth. YNB medium (0.67% yeast nitrogen base without uracil and 2% glucose) was used for yeast selection. YNB medium with 2% galactose (YNBG) was used for yeast expression.

Methods

Construction of pY-SLPI Recombinant Plasmid

Plasmid pET-*ESLPI* that harboring a full-length SLPI coding sequence from amniotic membrane was used as a template for PCR amplification. PCR amplification using the following primer pairs: primer 5'-GCGAGCTCATGTCTGGAAAGTCCTTCA–3' (forward) and GCCG-GAATTCTCAAGCTTTCACAGGG -3' (reverse). Forward primer and reverse primer contain artificial *Sac*I and *Eco*RI recognition sites (underline), respectively. PCR reactions were performed using 0.02 U/μL units of Pfu DNA polymerase, 0.2 μM dNTP, 1 X Pfu buffer with MgSO4, 0.5 μM primers and 5 ng/μL DNA template in final volume 50 μL. The annealing temperature was optimized to obtain desired target. The reactions were subject to 30 cycles. Purified PCR product (~339 bp) and pYHM1 were each digested with SacI and EcoRI restriction enzyme, then ligated at 4°C for 24h to generate pY_*SLPI*. This recombinant plasmid was introduced in *E. coli* TOP10 using bacterial transformaid kit and the resulting transformants were selected on ampicillin resistance. pY_*SLPI* was verified by restriction analysis and sequencing. Nucleotides sequence analysis was done commercially by IDT using primer pair T7 promoter as forward primer and cyc terminator as reverse primer.

Subcloning pY_SLPI into S. cerevisiae

The correct pY_*SLPI* recombinant plasmid was sub-cloned into *S. cerevisiae* BJ1824 using lithium acetate method. Briefly, A single colony of *S. cerevisiae* BJ1824 was inoculated in YPD medium broth overnight. At the mid-log phase (OD_{600} = 1), yeast was harvested by centrifugation, washed with water and resuspended with 0.1 M LiAc. After centrifugation, yeast was added 254 μL of 50% PEG 4000, 36 μL 1 M LiAc, 5 μL plasmid and 79 μL sterile water. This mixture was then incubated at 30 °C for 30 min and heat shocked at 42°C for 25 min. Yeast pellet was resuspended with 100 μL aquabidest and plated on YNB medium without uracil. Colony PCR was used to confirm the presence of SLPI gene in transformants.

Expression of SLPI

A single colony of *S. cerevisiae* BJ1824 carrying pY_SLPI was grown overnight at 30 °C, 150 rpm in 5 mL of YNB medium without uracil. The inoculum was then centrifugated at 1500 rpm for 5 minutes at 4 °C. Pellet cells were washed with distilled water and centrifugated again. The pellet cells were then resuspended in 1 mL YNBG medium, inoculated into 30 mL of YNBG medium, and incubated for 1 day at 30 °C, 150 rpm. The yeast cells were separated from culture medium by centrifugation. Protein secreted in culture medium was precipitated by ethanol, while intracellular and associated cells protein was collected after ultrasonication treatment of cells. All of protein fraction was then characterized by SDS PAGE.

RESULTS

Construction of pY-SLPI Recombinant Plasmid

The optimization of annealing temperatures to amplify the full-length and mature forms of *SLPI* gene can be seen in Fig. 1. The annealing temperature for the PCR amplification of genes encoding SLPI was 62.5, 62.3 and 62.2 °C. The PCR produced a single band in size about ~339 bp, which is close to the theoretical size (Fig. 1).

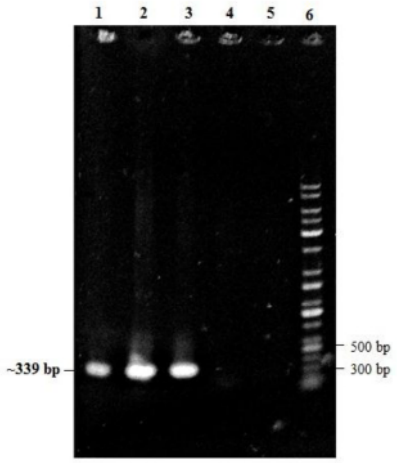
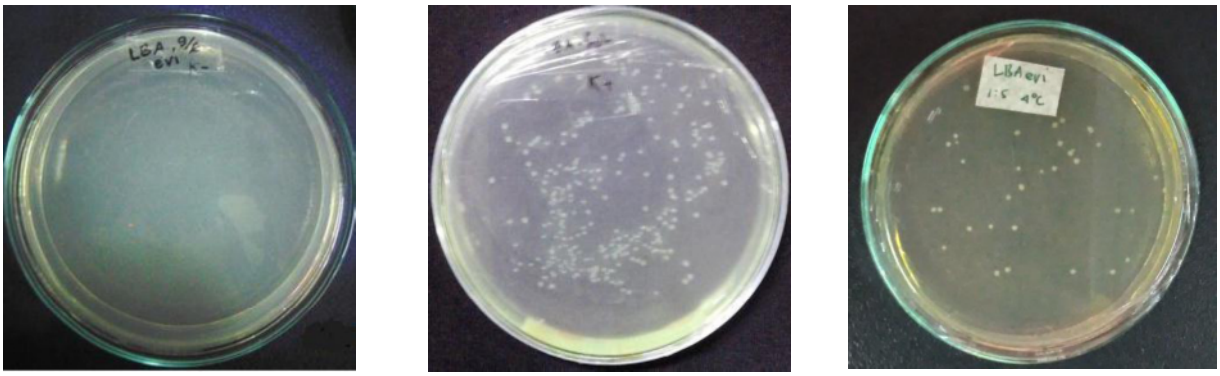


FIGURE 1. Optimization of annealing temperatures to amplify SLPI gene. Lane 1-5: annealing temperature at 62.5 °C, 62.3 °C, 62.2 °C, 61.9 °C, 61.7 °C, respectively, lane 6: 1 Kb DNA ladder.

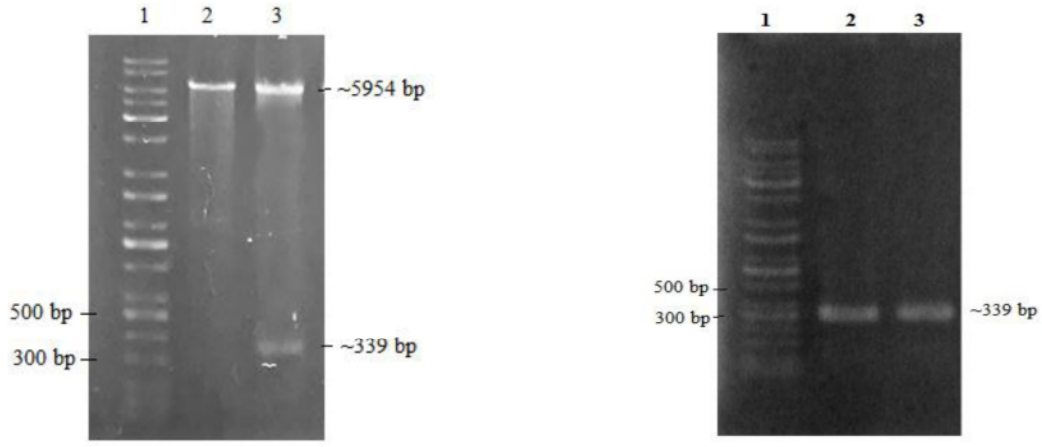
This amplicon was ligated in-frame with HM1 signal peptide in a pYHM1 vector to created recombinant plasmid pY_*SLPI*. pY_*SLPI* was transformed into *E. coli* Top10. Thirty-two (32) transformants were obtained on LB medium containing ampicillin (Fig. 2).



(a) (b) (c)

FIGURE 2. Transformant in *E. coli* TOP10. (a) negative control, (b) positive control (pYHM1), (c) Sample (pY_*SLPI*).

The recombinant plasmid of transformants was isolated and analyzed by single and double digestion and PCR amplification to confirm the presence of *SLPI* gene in the plasmid. Single band in size about 6 kilo bases (kb) were obtained after single digestion of recombinant plasmid of transformant no 31. Double digestion of recombinant plasmids of transformant 31 gave two DNA bands consist of vector 5.9 kb (pYHM1) and insert 339 bp (SLPI) (Fig 3a). Amplification of recombinant plasmid of transformant no 31 generated a single band size approximately 339 bp which corresponds to the theoretical size of the *SLPI* gene (Fig. 3b). These data indicated that transformant 31 carrying pY_*SLPI*

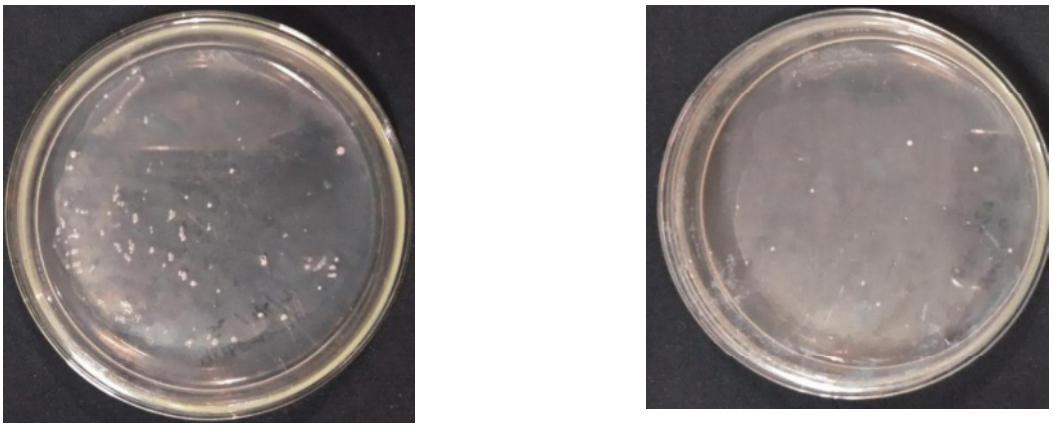


(a) (b)

FIGURE 3. Restriction analysis and PCR amplification of recombinant plasmid of transformant 31. (a). Restriction analysis, lane 1: 1 kb DNA ladder, lane 2: pY_SLPI digested by EcoRI, lane 3: pY_SLPI digested by SacI and EcoRI. (b). PCR amplification, lane 1: 1 kb DNA ladder, lane 2: positive control, lane 3: pY_SLPI.

The nucleotide sequence of a recombinant plasmid of transformant no 31 was determined to ensure the correct insert. An open reading frame of *SLPI* gene from ATG to TGA was found and in-frame with a HM1 signal peptide. Nucleotides alignment using BLASTn NCBI showed 100% similarity of identity with SLPI from NCBI accession number EU116331.

The correct pY_*SLPI* recombinant plasmid was introduced into *S cerevisiae* BJ1824 to produce SLPI. Fig. 4 shows that the pY_*SLPI* has been successfully subcloned into *S. cerevisiae* BJ1824 and generated four colonies.



(a) (b)

FIGURE 4. Transformant in *S. cerevisiae*. (a) positive control (pYHM1), (b) Sampel pY_SLPI.

Four yeast transformants were selected and characterized by colony PCR to confirm the presence of SLPI gene. The PCR produced a single band in size about ~339 bp, which is close to the theoretical size (Fig. 5). This result indicated that four yeast transformant harboring pY_*SLPI*.

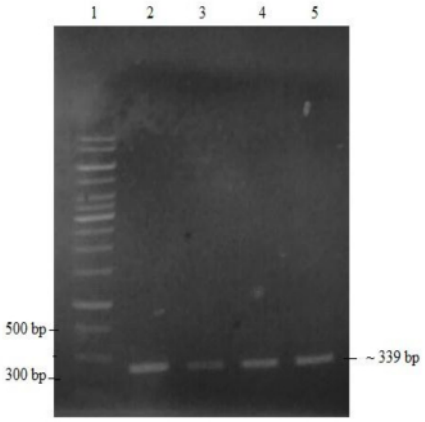


FIGURE 5. Colony PCR of yeast transformants. Lane 1 : 1 Kb DNA ladder, lane 2-5: tranformant no 1-4.

Expression of SLPI

An SDS PAGE analysis of the total intracellular and associated cells proteins showed the presence of protein band at ~16 kDa which close to the theoretical size of SLPI with additional HM1. However, no protein band at the same position was observed in extracellular protein (Fig. 6). It is indicated SLPI is expressed but not secreted.

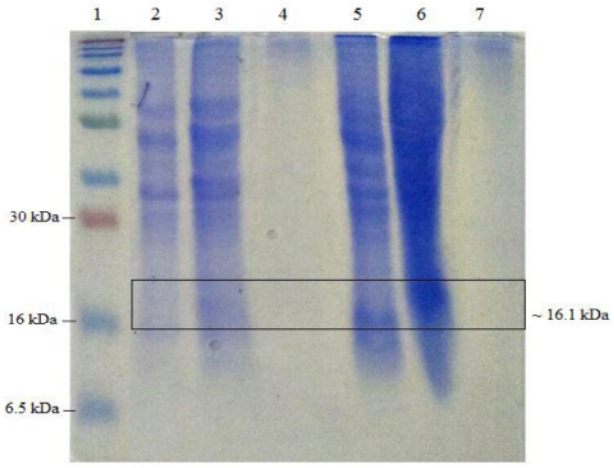


FIGURE 6. Coomasive stained 15 % SDS PAGE of SLPI expression. Lane 1: protein ladder, lane 2: associated cells proteins at 0 h, lane 3: intracellular protein at 0 h, lane 4: extracellular protein at 0 h, lane 5: associated cells proteins at 24 h, lane 6: intracellular protein at 24 h, lane 7: extracellular protein at 24 h.

DISCUSSION

S. cerevisiae is a widely used for the production of heterologous proteins. Besides known as safe microorganisms, yeast can also produce secreted proteins and modify heterologous protein. The secretion of recombinant proteins is desirable since a minimal amount of endogenous secreted protein, thus simplifying the protein purification process. Nevertheless, the secretory expression in yeast is bottleneck process that limits the yield

of recombinant protein. The factors that affected the secretion of recombinant protein i.e signal peptide, host, promoter and culture conditions[17]. Signal peptide mediates cotranslational translocation of protein into the endoplasmic reticulum (RE). Several signal peptides have been utilized to secreted recombinant protein. However, several recombinant proteins could not be secreted. It is difficult to predict which signal peptide is best for efficient secretion of protein[15].

In this study, we used HM1 signal peptide to secrete recombinant SLPI. Like α factor, HM-1 signal peptide has pre-structure at M1-A19 and pro structure at L20-R30[17]. The result of SDS PAGE showed the presence of recombinant SLPI only in cells (Fig. 6). The absence of recombinant SLPI in medium culture, suggesting that HM1 signal peptide fused to SLPI was not recognized by signal recognition particle (SRP), thus preprotein can not enter into the lumen of the endoplasmic reticulum. Rapid folding of polypeptide from ribosome may also lead to protein aggregation prior to translocation. Fusion protein of SLPI and HM1 has entered into the RE lumen but is not recognized and cleaved by signal peptidase. An amino acid residue of mature SLPI began with MSGKS, whereas the amino acid residues of mature α-L-arabinofuranosidase began with MATKK. This condition suggests that signal peptidase could not recognize and not cleave the different amino acid in the junction between signal peptide and recombinant SLPI. However, this possibility ought to be proved with Western blotting analysis.

This result was similar to Stetler (1989) who used with two different signal peptide i.e invertase and α factor for SLPI secretion. α factor system generated incompletely processed and misprocessed of secreted SLPI, while invertase system result in unprocessed fusion protein and processed protein that remain in cells[16].

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CONCLUSION

In conclusion, we have successfully cloned SLPI gene into pYHM1 plasmid in *S. cerevisiae*. SLPI was expressed as a fusion protein with HM1 signal peptide and remain in cells.

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