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Construction and Expression of Recombinant LL-37 as Histag-SUMO Fusion Protein with Factor Xa Cleavage Site

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ABSTRACT

LL-37 is an antimicrobial protein expressed by the CAMP gene in humans. This protein has various antibacterial, antiviral and anticancer effects. Expressing LL-37 as a heterologous protein in *E. coli* cells has its challenges. LL-37 is a peptide that is so small that it must be engineered with a fusion protein to increase its size solubility and prevent proteolysis of the target protein in cells. Factor Xa is the protease chosen to cleave LL-37 from its fusion protein in this research due to leucine binding factor Xa quite strongly. The aim of this study is, therefore, to express LL-37 as a fusion protein with Histaq_SUMO at the N-terminus linked to LL-37 at the C-terminus through Factor Xa cleavage site. Then, the fusion protein was cleaved by Factor Xa to obtain pure LL-37. In this study, LL-37 was produced by recombinant DNA technology, starting with the construction, expression, purification and cleavage of the fusion protein. The constructed genes consisted of 6xHis, SUMO, the factor X cleavage site, and LL-37, a total of 450 bp inserted in the pD451-SR vector plasmid. The results of this study yielded a SUMO_LL-37 protein with a molecular weight of approximately 17.34 kDa, which could be purified by Ni-NTA under native purification conditions. Based on ImageJ SUMO_LL-37 quantification, it was 1.65 µg/µL. LL-37 can be cleaved by factor Xa from SUMO with an enzyme-to-substrate ratio of 1:12.5 at 37°C with a 24-hour incubation time and results as much as 0.144 µg/µL.

1. Introduction

Antimicrobial peptides (antimicrobial peptides/AMPs) known as host defence peptides (HDPs) are small molecules that have broad-spectrum antibiotic activity against bacteria, fungi, and viruses and have cytotoxic activity on cancer cells and can work as anti-inflammatory and immunomodulator. Of the several known antimicrobial families, two large families have been identified in humans, i.e. defensins and cathelicidins (Kang *et al.* 2017). Currently, the only antimicrobial peptide cathelicidin class that has been identified in humans is LL-37 (Li 2011).

LL-37 is the only antimicrobial peptide from the cathelicidin family that is present in the human body and has a wide spectrum of antimicrobial activity, so that many research has been carried out in the pharmaceutical field for the further development of this peptide (Moon *et al.* 2006). Production of LL-37 by recombinant DNA is a protein production technique that uses host cells into which the gene encoding LL-37 has been inserted. AMP production in *E. coli* can be potentially toxic to host cells and susceptible to proteolytic degradation. This is due to the small size of the peptide and AMP, which are cationic. This problem can be overcome by fusing another peptide onto the expression so that the protein will form a fusion between LL-37 and the peptide (Li 2011). One example of a fused peptide is a small ubiquitin-associated modifier (SUMO). SUMO can increase the mass of expressed LL-37 so that the recombinant protein is larger and better protected from the proteolysis process (Li 2013).

SUMO peptides can reduce the effectiveness of LL-37. Therefore, it is necessary to cleave the tag

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(fusion peptide) to obtain LL-37 (± 5 kDa) (Moon *et al.* 2006). In this study, pD451-SR SUMO_LL-37 plasmid was designed with a cut point of the proteolytic enzyme factor Xa instead of SUMO protease. SUMO protease has a small active pocket, so when it is attached to the large leucine residue of LL-37, it reduces the ability of SUMO protease to cleave LL-37 due to steric hindrance (Zhang *et al.* 2022). However, another cleavage site, i.e. factor Xa has a better hydrolysis rate when it encounters a flanking leucine residue after the Isoleucine, Glutamate, Glycine, Arginine (IEGR) cleavage site. Leucine LL-37, located exactly after the factor Xa cleavage site arginine, increases kM and kcat (10)-1 (Bromfield *et al.* 2006). Until now, there has been no research reporting that the LL-37 gene was fused with a SUMO tag designed with a factor Xa cleavage site. In this study, recombinant LL-37 was constructed as pD451-SR_ SUMO_LL-37. pD451_SR is a plasmid type with a high copy number and a strong ribosome binding site. Research results for pD451-SR as a carrier vector showed the effects of temperature, incubation time and inducer concentration, which significantly affected the amount of protein in the soluble fraction (Razali *et al.* 2021). The recombinant gene construction consisted of the coding sequence His tag, SUMO, factor Xa cleavage site, and LL-37. The recombinant plasmid was transformed into BL21(DE3), expressed using IPTG inducer, purified by Nickel affinity chromatography, and purified LL-37 obtained by cleavage using Factor Xa.

2. Materials and Methods

2.1. Gene Construction and Plasmid Selection

The gene was synthetically constructed using pD451-SR with nucleotide base coding 6x His, followed by SUMO, factor Xa cleavage site, and LL-37 gene in downstream position. The coding base sequence of 6xHis, SUMO and Factor Xa cleavage site were obtained using the SnapGene software sequencing tool from the commercial plasmid. In addition, the LL-37 coding gene sequence was obtained from NCBI with optimized codons. Then, the similarity of the designed gene sequence was confirmed using the NCBI BLAST feature (https://blast.ncbi.nlm.nih.gov/Blast.cgi). The recombinant gene sequence was translated into amino acids using the website https://web.expasy.org/translate/, and then the translation results were analyzed using https://www.genscript.com/tools/peptide-molecular-weight-calculator to determine the molecular weight. The constructed gene was inserted into pD451-SR plasmid, which is available on ATUM website https://www.atum.bio/. The constructed recombinant plasmid was ordered and manufactured by ATUM Inc (Newark, CA).

2.2. Production of Competent *E. coli* BL21 (DE3) Cells

Transformation of the recombinant plasmid was performed by the heat shock method using competent *E. coli* BL21 (DE3) cells. Competent cells were started before by cultivating *E. coli* BL21 (DE3) in liquid LB medium overnight at 37°C. The culture was transferred as much as 1% to 50 ml of liquid LB media and cultivated again at 37°C at 200 rpm incubator shaker until it reached an OD_{600} of 0.35-0.4. The culture was then cooled by keeping the tube in a container filled with ice water at 0°C for 10 minutes. The culture was centrifuged at 5,700 g for 10 minutes at 4°C, the supernatant was discarded, and the pellet was collected. The cell pellet was then resuspended in a pre-cooled solution of 80 mM $MgCl_2$ + 20 mM $CaCl_2$ and incubated for 45 minutes in a container containing ice water at 0°C. The suspension was centrifuged, and the cell pellet was collected and resuspended as competent cells in 1 ml of cold 100 mM $CaCl_2$ and 1 ml of sterile 30% glycerol (Sambrook and Russell 2001).

2.3. Transformation of Recombinant Plasmids into Competent Cells

200 µL of competent cells in a 1.5 ml tube were added recombinant plasmid (20 ng/µL) and allowed to stand for 30 minutes in a dish containing ice water at 0°C. The tube was then placed in a water bath at 42°C and incubated for 60 seconds. The tube was then immediately transferred to a container containing ice water at 0°C and incubated for 1–2 minutes. Then 1 ml of liquid LB medium was added to the tube. The tube was incubated for 90 minutes in an incubator at 37°C at 200 rpm. The bacterial suspension was centrifuged again at 4°C at 5,700 g. The supernatant was then partially removed, and the cell pellet was resuspended, from which 100 µL were taken and plated on a solid agar medium containing 25 µg/mL kanamycin, spreading it evenly on the surface. The medium was incubated at 37°C for 18 hours. Subsequently, recombinant plasmids were isolated from growing colonies (Sambrook and Russell 2001; Chang *et al.* 2017).

2.4. Overexpression of the Gene Encoding SUMO_LL-37 and Overproduction of Its Protein

First, a starter culture of E. coli BL21(DE3) plasmid pD45-SR_SUMO_LL-37 or recombinant *E. coli* BL21(DE3) was prepared in 5 ml liquid LB medium containing 25 µg/ml of kanamycin incubated at 37°C on an incubator shaker at 200 rpm for 18 hours. Then, 1 ml of starter culture was re-incubated in a 100 ml liquid LB medium containing 25 µg/ml kanamycin on an incubator with the same temperature and speed condition (Maksum *et al.* 2017). Then, the optical density at 600 nm was checked every hour. At an OD_{600} of 0.6 to 0.8, IPTG inducer was added at a final concentration of 1 mM. The incubation process was continued for 5 hours at 37°C at 200 rpm according to induction protocol pD45-SR by ATUM Inc (Newark, CA).

2.5. Extraction of Crude Recombinant Protein

The bacterial culture obtained in the recombinant protein overproduction process was centrifuged at 10,000 g at 4°C for 20 minutes. The supernatant was discarded, and the cell pellet was lysed using B-PERTM (for 1 gram cell pellet, 4 ml B-PER TM buffer, 1 ml B-PER TM buffer and 2 µL lysozyme 50 µg/µL and 2 µL DNAse 2500 U/ml were used). The cell pellet was resuspended to homogeneity using a pipetting technique and then incubated at room temperature for 15 minutes. Then, the lysis results were centrifuged at 15,000 g at 4°C for 5 minutes. The supernatant was collected and then transferred to a new centrifuge tube. The pellet contains aggregated protein that can be solubilized with 1% sarkosyl buffer as an insoluble fraction (Massiah *et al.* 2016).

2.6. Purification of Recombinant SUMO_LL-37 Protein

The purification process used the kit and followed the protocol HisPur™ Ni-NTA Spin Columns Cat. No 88228 with 1 ml column volume (CV) capacity. First, the chromatography column was washed with 10 CV of distilled water and the distilled water was allowed to flow through the column. The column was then centrifuged at 700 g for 2 minutes. Then, 6 CV Equilibration buffers were added and allowed to flow through the column. Protein extracts were then added with equilibration buffer 1:1 (v/v), loaded onto the column, and left at cold temperature for 30 minutes, mixing as often as possible. The column was then centrifuged at 700 g for 2 minutes, and the fraction was collected as flow-through. The column was then washed with 2 CV wash buffers, repeating 4 times. Each wash fraction was collected by column centrifugation at 700 g for 2 minutes. The column was then eluted with 1 CV of elution buffer in 4-fold replicates. Each elution fraction was collected by column centrifugation at 700 g for 2 minutes.

2.7. Diafiltration and Quantification of SUMO_LL-37 Protein

Diafiltration was performed using the Amicon Ultra-4 Centrifugal Filter 10 kDa Cat. No UFC8010 with the procedure provided. First, 3.5 ml of diluted sample with sterile Milli-Q distilled water was used to create a final volume of 10 ml. The kit was activated before being used with 3 ml of sterile milli-Q and then centrifuged at 4°C 5,000 g for 10 minutes. 3.5 ml of the diluted sample was loaded into the Amicon kit and then centrifuged at 6,000 g for 10 minutes at 4°C. The solution remaining in the filter was then collected. This process was repeated until 10 ml of the sample had passed through the slide filter.

Protein quantification was performed using different concentrations of bovine serum albumin (BSA) i.e 0.5; 0.25; 0.125; 0.0625; 0.03125 µg/µL. The used BSA solution volume was 40 µL in the stacking gel well of SDS PAGE. BSA was then analyzed using SDS-PAGEs with MWCO. The area of the BSA band was then measured using ImageJ software.

2.8. Optimization of SUMO_LL-37 Protein Cleavage with Factor Xa at Temperature and Incubation Time Variation

6 µL of 0.055 µg/µL Factor Xa from Invitrogen (Cat. No RP-43114) was mixed with 6 µL of 0.69 µg/µL SUMO_LL-37 protein in 1.5 ml of reaction tube so the ratio of substrate to protein (1; 12.5). The tubes were then incubated at variations in temperature (4, 25, 37°C) and incubation time (4, 16, 24 hours). The result of incubation from all tubes was then characterized by SDS-PAGE.

2.9. Cleavage of SUMO_LL-37 by Factor Xa and Purification of LL-37

The optimal conditions for cleavage of SUMO_LL-37 were applied at 37°C for 24 hours with a factor Xa to sample ratio of 1:12.5 obtained from the previously described procedure. The substrate was 172.5 µg in the cleavage buffer. The cleavage products were then purified again using Ni NTA affinity

chromatography. At the post-cleavage purification stage, the fraction collected for activity testing was flowthrough. The flow-through fraction contained LL-37, while the elution fraction contained the SUMO tag still attached to the 6x His.

2.10. Western Blot

The western blot procedure was held using Bio Rad Mini Protean equipment. First, the protein was transferred from SDS-PAGE to a nitrocellulose membrane in a cassette consisting of a sponge, filter paper, gel, nitrocellulose membrane, filter paper and sponge. The cassette was soaked in towbin buffer (25 mM Tris, 192 mM glycine, 20% v/v methanol pH 8.3) for 15 minutes. The cassette was then inserted into the blotting module and then into the tank, and towbin buffer was added up to the limit mark. The transfer process was carried out with a current strength of 200 A for 30 minutes. Then, the blocking process was performed on a nitrocellulose membrane by soaking the membrane in TBS-T (20 mM Tris-HCl, 500 mM NaCl, 0.05% Tween 20 pH 7.5) for 5 minutes and then incubating in a 5% skim milk solution for 1 hour. The membrane was washed with TBS-T for 5 minutes three times. The membrane was then soaked overnight in the primary antibody, i.e. anti-Histag and anti-LL-37, at a cold temperature. The membrane was washed with TBS-T for 5 minutes three times. Then, the membrane was soaked in a secondary antibody (anti-mouse IgG-Hrp) for 1 hour. The membrane was then washed again for 5 minutes with TBS-T three times. Then, the ECL reagent was added in the dark, incubated for 10 min, and imaging was performed.

3. Results

3.1. Result of the LL-37 Coding Gene Construction

The results of the gene construction are shown in Figure 1, which contained 450 bp, and the number of amino acids produced was 149. Based on predictions https://www.genscript.com/tools/peptide-molecular-weight-calculator, the expressed protein size was 17.34 kDa with an isoelectric point of 8.02, and it was soluble.

3.2. Results of the DNA Transformation

The results of the transformation of the recombinant plasmid into competent *E. coli* BL21(DE3) cells and the results of the isolation of the plasmid from the transformed cells are shown in Figure 2. Several colonies grew spread out on the surface of the solid agar medium, indicating that the recombinant plasmid had successfully entered the *E. coli* BL21(DE3) cells. The results of recombinant plasmid isolation also showed the presence of recombinant plasmids in competent cells.

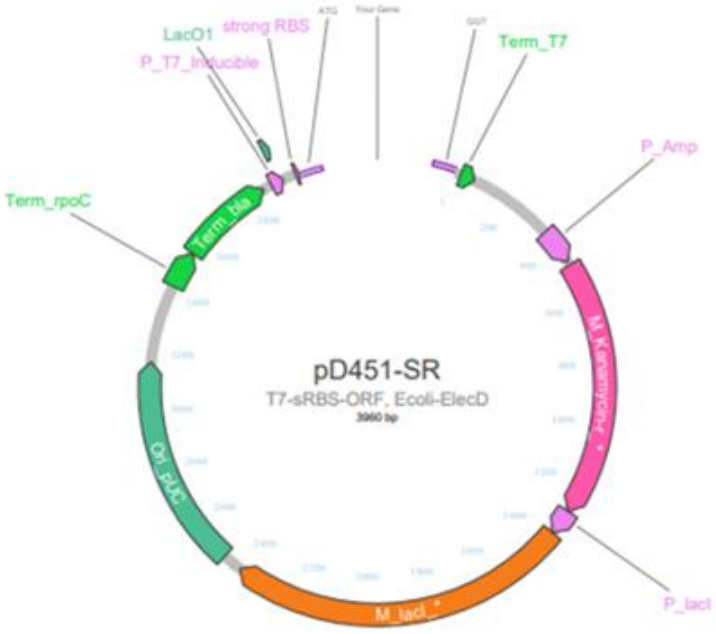
3.3. Results of Recombinant Protein Overproduction in Extraction and Purification Phase

The result of overexpression of the gene encoding SUMO_LL-37 in *E. coli* BL21 (DE3) cell was an intracellular protein. Proteins were extracted by cell lysis to determine the presence of recombinant protein. The extracted protein can be in two forms, i.e. soluble and insoluble protein. The dissolved protein then continued through the purification process. The presence of the recombinant protein because of overexpression of the target gene, followed by the extraction and purification process, can be seen in Figure 3.

From Figure 3, the recombinant proteins were in soluble and insoluble phases. Although, based on observations, the pellets obtained by centrifugation of cell lysis are almost invisible. However, the intensity of the protein band in the soluble protein fraction was higher than in the insoluble protein fraction. The column washing fraction showed that washing the column with washing buffer containing 25 mM imidazole was more effective in removing protein impurities compared to that containing 50 mM imidazole. The target protein was obtained from the elution fraction. The protein band between the marker 15-25 kDa predicted the size of the target protein, i.e. 17.34 kDa. The protein band in the elution fraction using 250 mM and 500 imidazole showed a thick band with the size of the target protein. Protein impurities were still present in this elution fraction.

3.4. Result of Diafiltration and Quantification of SUMO_LL-37 Protein

Based on Figure 4, the SUMO_LL-37 protein can be concentrated and separated from contaminating proteins using a diafiltration method. The concentration of protein was measured using the SDS PAGE band image analysis method based on ImageJ software. BSA as a standard protein was characterized by SDS PAGE with the samples. Based on the BSA regression results, the graphical equation was y = 7568.9x -79.17, the SUMO_LL-37 protein concentration was 1.65 µg/µL.



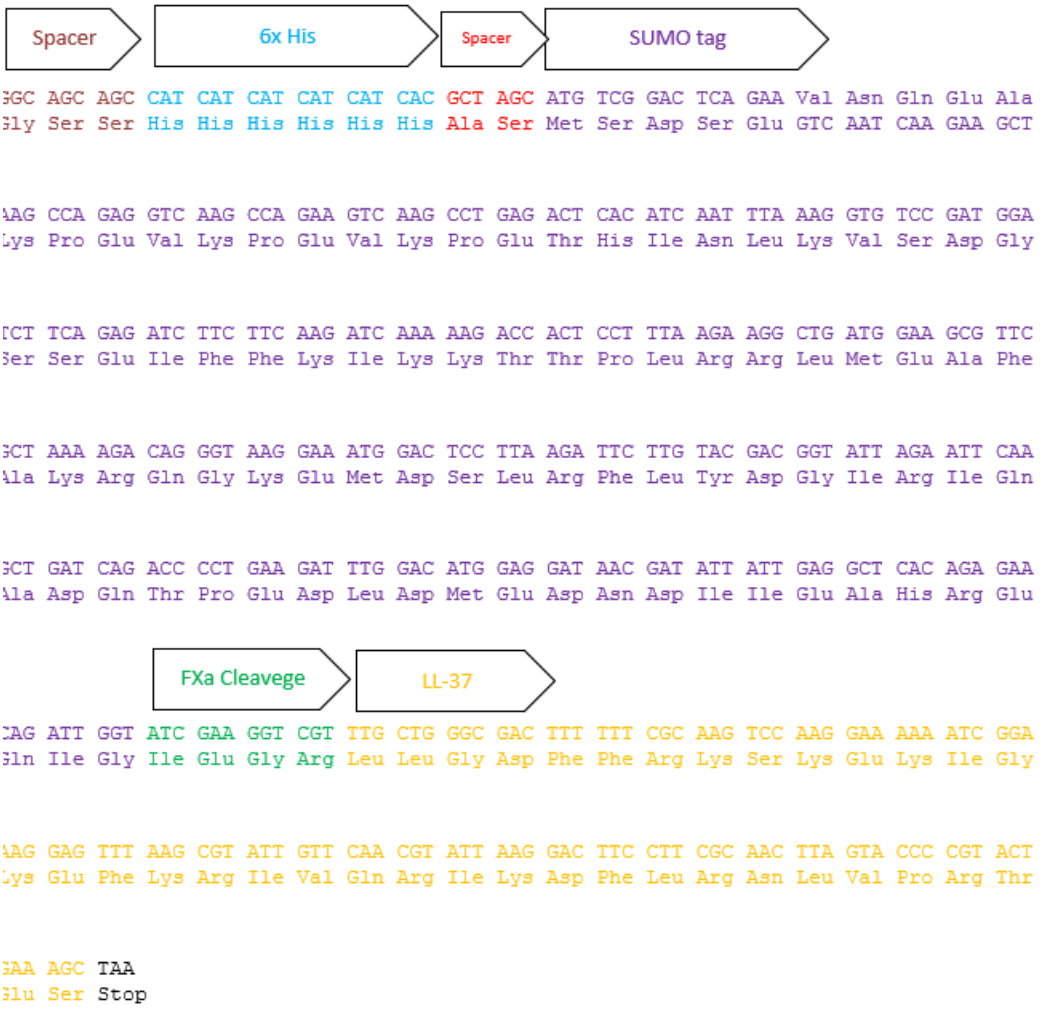
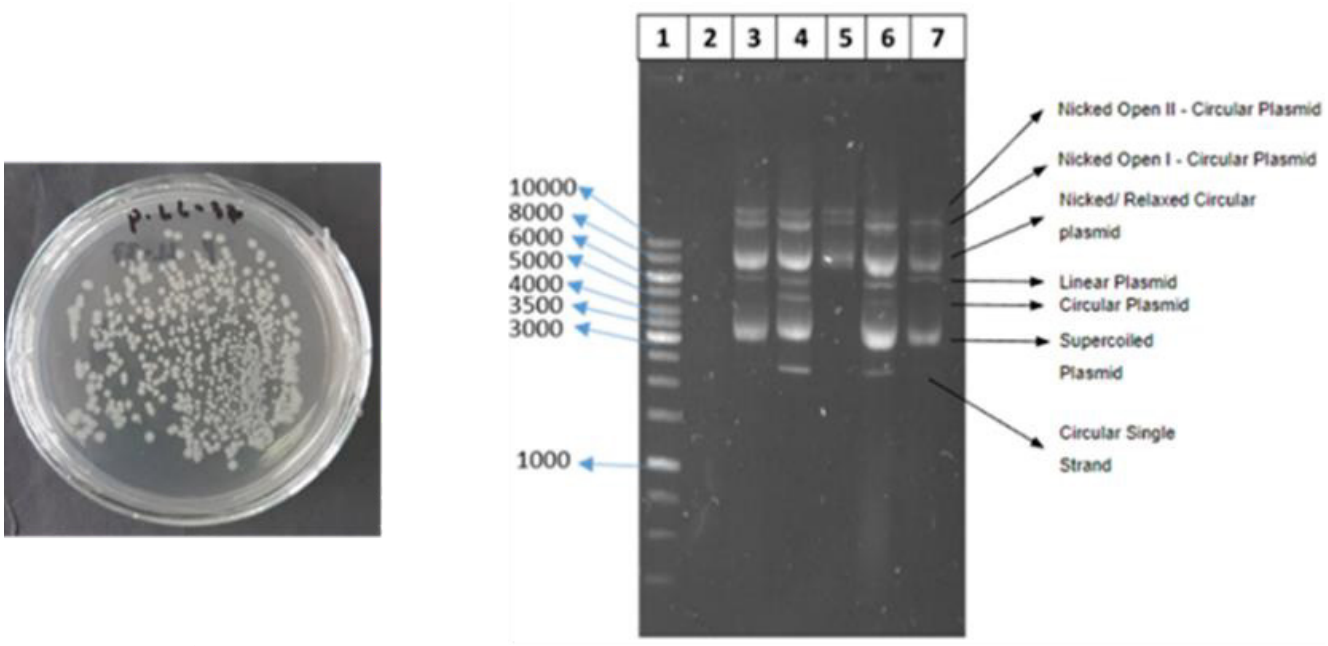


Figure 1. pD251-SR map and Construction of LL-37 recombinant in pD451-SR plasmid



A B

Figure 2. Results of recombinant plasmid transformation into *E. coli* BL21(DE3): (A) Transforman colonies grown on MHA media containing 25 µg/ml of kanamycin, (B) 1. Ladder DNA, 2. Isolated plasmid from non-transformant *E. coli* BL21(DE3), 3-7. Isolated plasmids from transformants *E. coli* BL21(DE3)

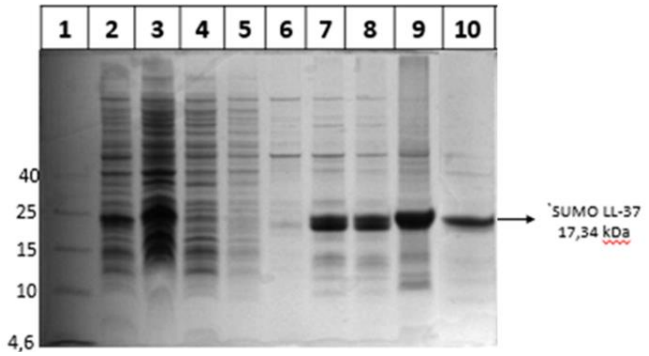


Figure 3. The presence of the recombinant protein as a result of overexpression of the target gene was characterized using tricine SDS-PAGEs with an acrylamide concentration of 10%: 1 4.6-40 kDa ladder; 2 insoluble fraction; 3 soluble fraction; 4 flowthrough fraction; 5 washing fraction (25 mM of imidazole); 6 washing fraction (50 mM of imidazole); 7-8 I and II elution fractions (250 mM of imidazole); 9-10 III and IV elution fractions (500 mM of imidazole)

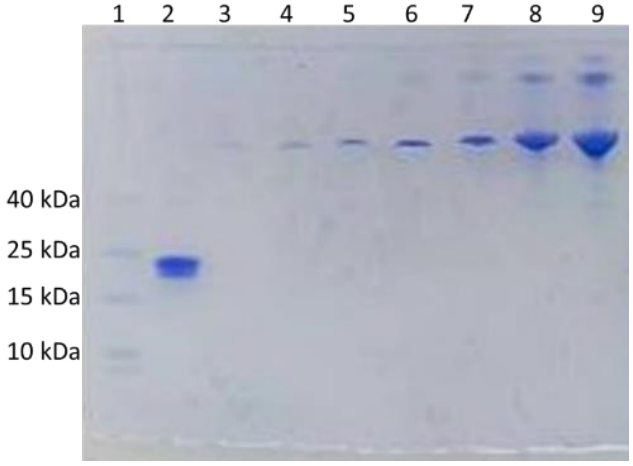


Figure 4. Result of Diafiltration and quantification of SUMO_LL-37 protein: 1 10-40 kDa Ladder; 2 SUMO_LL-37; 3-9 BSA concentration 0.03125, 0.0625, 0.125, 0.25, 0.5, 1, 2 (µg/µL)

3.5. Result of Optimization of SUMO_LL-37 Protein Cleavage with Factor Xa

The optimal results for factor Xa cleavage of SUMO_LL-37 can be seen in Figure 5. Based on Figure 5, factor Xa was capable of cleavage in the temperature range of 4°C to 37°C. Factor Xa activity at 25°C for 24 hours and at 37°C for 16 and 24 hours indicated that SUMO_LL-37 was completely cleaved compared to other conditions. The explanation was that the Histag SUMO_LL-37 band was no longer visible under these conditions. Column 2 showed SUMO_LL = 37 protein band with 15-25 kDa. Then, between 10-15 kDa, there was another protein, possibly lysozyme, of ~11 kDa, which was also concentrated during the process of concentrating the target protein with Amicon MWCO 10 kDa. In addition, there was more than one band in the band region suspected to be the SUMO_LL = 37 protein. It is possible that a contaminating protein can also bind to Ni^{2+}, which is similar in size to the target protein.

3.6. Result of Cleavage of SUMO_LL-37 by Factor Xa and Purification of LL-37

The results of cleavage of the SUMO_LL = 37 protein with factor Xa and purification of LL = 37 can be seen in Figure 6. LL-37 was successfully recovered from the flow-through fraction after cleavage of SUMO_LL-37 with factor Xa. His_SUMO was mostly bound to Ni. Thus, most of LL-37 could be separated from Histaq SUMO, although SUMO protein was still present in the flow-through fraction. Quantification with ImageJ, LL-37 resulted in 0.144 µg/µL.

3.7. Result of Western Blot

The results of the Western blot assay for SUMO_LL = 37 and LL-37 can be seen in Figure 7. Anti-HisTaq antibodies can bind to SUMO_LL-37 as the SUMO_LL-37 protein is a recombinant protein with the following

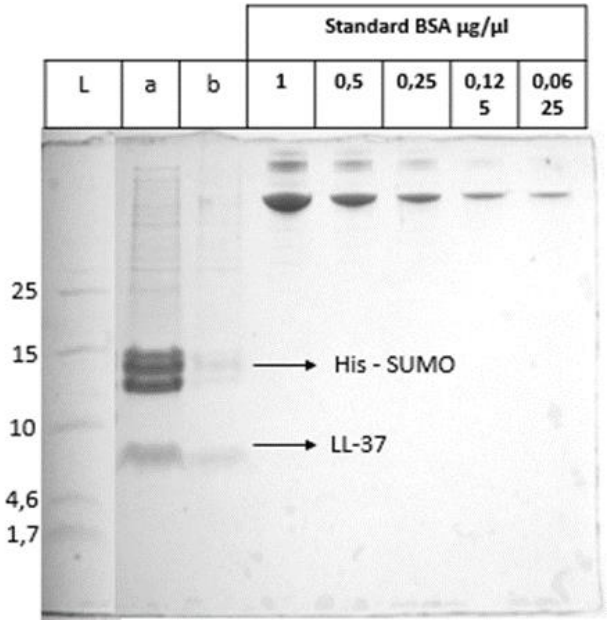


Figure 6. LL-37 purification using Ni NTA Chromatography of cleavage result of SUMO-LL-37 by factor Xa and its quantification using BSA as standard: L 1.7-40 kDa Ladder; a. the result of SUMO_LL-37 cleavage by factor Xa without continuing with the purification process; b. the result of SUMO_LL-37 cleavage by factor Xa continuing with the purification process as flowthrough fraction

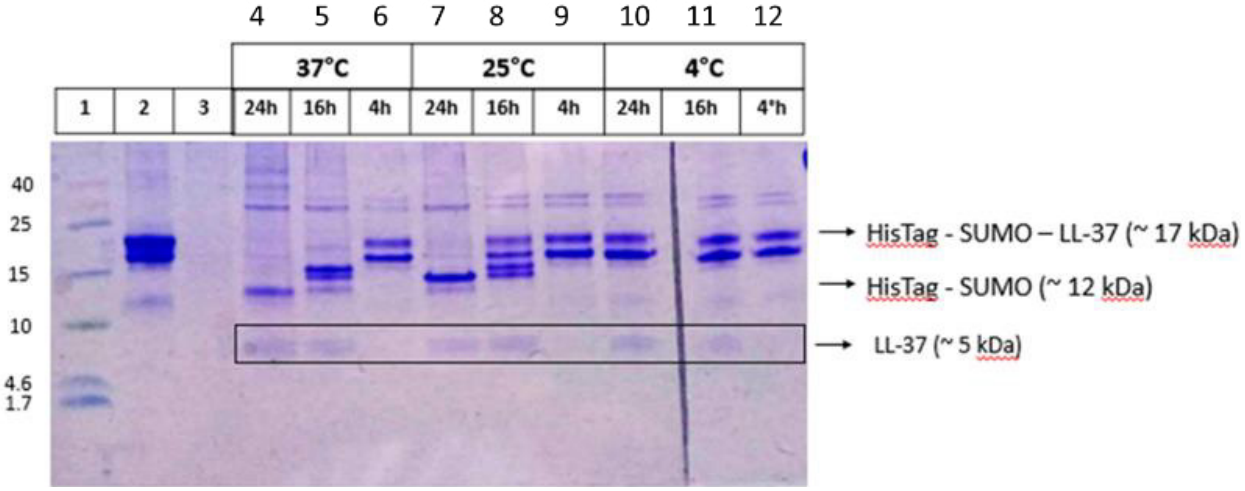


Figure 5. Result of optimization of SUMO_LL-37 protein cleavage with factor Xa: 1 1.7-40 kDa ladder; 2 uncleaved SUMO_LL-37; 3 filtrates of MWCO 10 kDa; 4-6 cleavage condition 4-24 hours at 37°C; 7-9 cleavage condition 4-24 hours at 25°C; cleavage condition 4-24 hours at 4°C

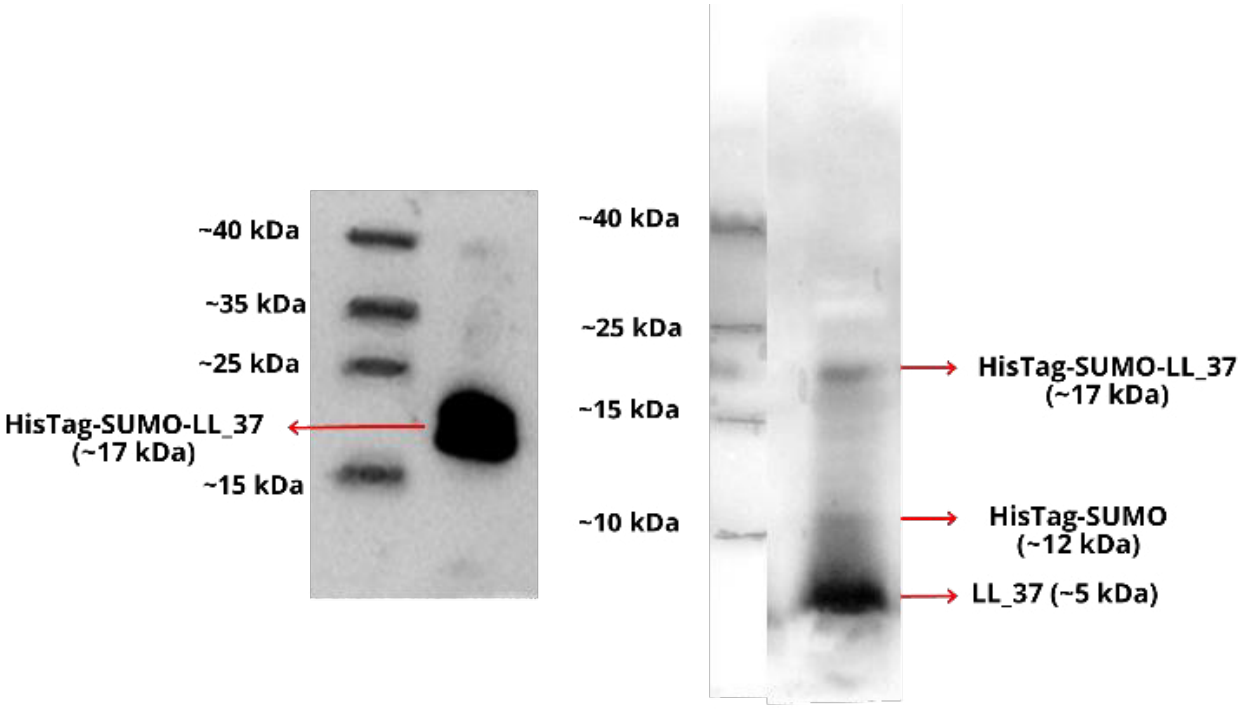


Figure 7. Western blot results using antibodies: (A) anti-histaq antibody bound to SUMO_LL-37, (B) anti-LL-37 antibody bound to LL-37

construction 6xHis_SUMO_Factor Xa cleavage site_LL-37. So, 6xHis(Histaq) can bind the anti-His antibody. This is indicated by positive western blot result,s which are in the size of SUMO_LL-37 with a size of around 17 kDa. This also indicates that the recombinant LL-37 coding gene construct has been successfully expressed using pD451-SR. Then, LL-37 can bind to anti-LL-37 antibodies. This was indicated by a positive western blot result for a target protein size of about 4.3 kDa (the figure showed the location of the target protein below 10 kDa). So, SUMO_LL-37 can be cleaved to result in LL-37.

4. Discussion

The recombinant LL-37 gene was constructed as follows: 6x His coding gene, SUMO tag, factor Xa cleavage site, and LL-37 coding gene. 6x Its purpose is to contribute to the purification process of Ni-NTA affinity chromatography. SUMO serves as a tag or fusion peptide for LL-37. SUMO helps stabilize proteins during overproduction by increasing solubility, preventing aggregation, and preventing host cell proteolysis. SUMO is thought to increase the transfer of target proteins from the cytosol to other compartments, thus reducing the concentration of recombinant proteins in the protease-rich cytosol (Butt *et al.* 2005; Wang *et al.* 2010a, 2010b). The results of overexpression and overproduction of the recombinant SUMO_LL-37 protein were more abundant in the soluble fraction, as indicated by the thicker band of the target protein in this fraction (Figure 3). Another study reported that LL-37 fused without SUMO had less soluble protein compared to LL-37 fused with SUMO in this study (Moon *et al.* 2006).

SUMO_LL-37 protein was well expressed under 1 mM IPTG, 6 hours induction, and 37°C conditions, as indicated by the thickness of the protein bands in the soluble and insoluble fractions. An IPTG concentration of 0.1 mM and an induction time of 24 hours at 18°C was the best condition for the production of recombinant protein vector pD451 SR (Nuryana *et al.* 2022). If the concentration of IPTG is too high and the incubation at 37°C is too long, it produces insoluble and aggregated protein (Razali *et al.* 2021). This may explain why the SUMO_LL-37 protein was also present in the insoluble fraction. This result was supported by other recombinant LL-37 fused to SUMO, which also had higher solubility (Moon *et al.* 2006; Shad *et al.* 2024).

Sumo_LL-37 protein was successfully purified by nickel affinity chromatography, although there was still a small amount of protein impurities in the elution buffer containing 500 mM imidazole. This showed that the protein can be purified under natural conditions. The protein fold does not cover the 6xHis residue to allow it to bind to Ni^{2+}. This protein was shown to be SUMO_LL-37, supported by Western blot data for SUMO_LL-37. In other studies, the expression of the target protein fused with SUMO Histaq can be increased and purified using Nickel affinity chromatography (Wang *et al.* 2010a, 2010b, Lamer and Vederas 2023).

LL-37 was successfully separated from SUMO residues by cleavage using factor Xa. This data was supported by LL-37 western blot (Figure 7). The sequence recognized by factor Xa was Ile-Glu-Gly-Arg inserted previously the amino acid encoding LL-37. No Arg or Gly sequences were found in the gene encoding LL-37, making the factor Xa cleavages at specific sites. The presence of leucine in the first amino acid sequence of LL-37 after arginine causes SUMO_LL-37 to bind strongly to factor Xa because leucine is hydrophobic. The position of leucine after arginine increases the rate of enzyme activity compared to other residues. The kinetics of factor Xa as a leucine substrate was kM 14.88 µM and kcat(10)-1 14.34 (Bromfield *et al.* 2006). In addition, temperature and incubation time affected the cleavage of LL-37 from SUMO_LL-37 by factor Xa. This LL-37 obtained by factor Xa cleavage process, showed a higher yield of 144 mg/L compared to another reported study where 2.4 mg/L of LL-37 was obtained from thioredoxin Sumo-fused LL-37 using the SUMO protease. SUMO protease is less optimal for cleaving LL-37 due to steric hindrance of the leucine residue (Zhang *et al.* 2022).

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