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EXPRESSION OF *SARCOPTES SCABIEI* EXON 5 TROPOMYOSIN GENE AS AN ATTEMPT TO DEVELOP RECOMBINANT ANTIGEN FOR SCABIES DIAGNOSIS

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ABSTRACT

Background: Scabies diagnosis is greatly influenced by the experience of the examiner, since the diagnosis rely on a complex diagnostic criteria. Antigen detection test is an alternative technique for diagnosing scabies in the future. Tropomyosin is a highly conserved protein which expressed by *Sarcoptes scabiei* mites. **Objective:** This research aimed to develop recombinant protein from exon 5 of the *S. scabiei* tropomyosin gene for further scabies diagnostic development. **Methods:** An exon from tropomyosin gene was cloned into pLATE-51 plasmid using ligation independent cloning methods. Protein overexpression was done using *Eschericia coli* strain BL21. Insoluble protein was solubilized using 5 mM dithiothreitol (DTT). Protein purification was done with Ni-NTA affinity chromatography principle. Analysis of the resulting recombinant protein was done using SDS-PAGE, immunodot blotting, and western blotting. **Results:** A 550-bp exon was successfully cloned into pLATE-51 plasmid. Overexpression result showed protein was mostly expressed in pellet (insoluble form). Confirmation using immunodot blotting showed the presence of recombinant protein from the overexpression. However, further confirmation using western blotting was unsuccessful to detect the intended recombinant protein. **Conclusion:** The exon of *S. scabiei* tropomyosin gene could be cloned in pLATE-51 plasmid and produced recombinant protein. However, the protein yield was low and the protein is mostly insoluble, preventing the success of purification of the intended recombinant protein.

BACKGROUND

Scabies is a skin disease caused by infestation of an ectoparasite, *Sarcoptes scabiei* mite.[1] Currently there are no definite data regarding the epidemiological burden of scabies. Indonesia is estimated as country with the highest scabies burden, with disability-adjusted life years (DALYs) reaching 153,86.[2]

Diagnostic criteria for scabies are complex. The diagnosis of scabies is based on a combination of physical examination results based on the history and visual examination.[3] The diagnostic technique is greatly influenced by the experience of the examiner. As a result, there are differences in the results of examinations by trained and untrained medical personnel in the diagnosis of scabies. The existence of diverse diagnostic criteria also makes it difficult to determine the epidemiological burden of scabies, due to differences in case definitions.[2,4]

The definitive diagnostic technique by identifying the presence of mites in skin scrapings using a microscope is time-consuming, operator-dependent, and requires a microscope which is not always available in the clinical setting.[2,3] Polymerase chain reaction (PCR) is an alternative method for definitive diagnosis of scabies. This method possesses a high sensitivity and specificity, which are close to 100%. However, PCR examination requires expensive equipment and materials as well as trained personnel.[5]

Antigen detection test is an alternative technique for diagnosing scabies in the future. However, this method still possess challenge especially in supply of antigen for the development of rapid diagnostic test.

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Antigen detection test shows promising development for scabies diagnosis.[5,6]

Tropomyosin is a highly conserved protein which expressed by *S. scabiei* mites. Tropomyosin is found in the mouthparts, muscles and legs of the mite. This protein is encoded by the tropomyosin protein coding gene which has a total length of 2,900 base pairs (bp). Previous studies report that recombinant *S. scabiei* tropomyosin is a soluble protein.[7,8]

Cloning is a method widely used to produce recombinant protein, including protein for diagnostic purpose. Specific target gene is inserted to expression vector, such as plasmid, and expressed using expression system. The pLATE-51 plasmid vector is a Ligation independent cloning (LIC) vector. LIC is a cloning method for inserting genes into plasmid vectors. This technique has the advantage that it can be carried out quickly because it does not require a restriction step with enzymes because the plasmid is already linear. In addition, LIC does not require a ligation procedure using the DNA ligase enzyme.[9]

Eschericia coli strain BL21 is a prokaryotic expression system commonly used in recombinant protein production. *E. coli* BL21 has a good protein expression capability, making it one of the expression systems that is widely used. The bacteria is a mutant with protease enzymes deficient, namely the ompE and lonT protease, thus making it possible to overexpress the protein with minimal protein degradation.[10]

This research aimed to develop recombinant protein from exon 5 (550 bp) of the *S. scabiei* tropomyosin gene for further scabies diagnostic development.

METHODS

Sample

Mites were obtained from skin scraping samples from *S. scabiei* in vitro culture using rabbits (*Oryctolagus cuniculus*) stored at the Parasitology Laboratory, Faculty of Medicine, Public Health, and Nursing, Universitas Gadjah Mada. Samples were stored in -20°C.

DNA extraction and PCR amplification

DNA extraction was carried out using the QiAMp DNA kit (Qiagen, Germany) using the method according to the manufacturer's protocol. Amplification of the exon 5 tropomyosin gene was carried out by polymerase chain reaction (PCR) using primers forward 5'– GGTGATGATGATGACAAG GAACGAGCCGAAACTGGAGAATCG -3' and reverse 5' GGAGATGGGAAGTCATTACTCTCGATTCTCA TCAAAGGCTGT -3'. PCR was carried out with Phusion High Fidelity DNA Polymerase (ThermoScientific, USA).

A PCR reaction was carried out with initial denaturation at 98°C for 30 seconds, and 35 cycles with denaturation at 98°C for 10 seconds followed by annealing at 67°C for 30 seconds, and extension at 72°C for 30 seconds, followed by final extension at 72°C for 10 minutes.

Electrophoresis, gel purification, and complementary overhang generation

The PCR products were viewed using electrophoresis on a 1.5% concentration agarose gel with fluorescence staining. Electrophoresis of the amplicon was carried out in Tris Borate EDTA (TBE) 1x. Extraction of the desired PCR products from gel electrophoresis was carried out according to the GeneJET Gel Extraction Kit protocol (Thermo Scientific, USA), by cutting the electrophoresis results at the desired band size.

The purified DNA was then stored at -20°C. Purified PCR products then treated using T4 DNA polymerase to create overhang at the 5-' and 3'- end. This procedure was done to create specific and complementary overhang, which is used to ligate the PCR product to pLATE-51 plasmid.

T4 DNA polymerase treatment was done by mixing 5 µl of gel purification of the PCR product with 2 µl of LIC buffer, 2 µl of nuclease-free water, and 1 µl of T4 DNA polymerase until the mixture volume reaches 10 µl. The reaction was then incubated at room temperature (20-25°C) for 5 minutes. A volume of 0.6 µl of 0.5 M EDTA then added to stop the reaction.

Cloning, transformation, and colony selection

Cloning of the desired gene was done using ligation independent cloning (LIC). The annealing reaction on the plasmid vector was carried out by adding 1 µl of LIC plasmid PLATE-51 to the mixture from the previous reaction and vortex for 3-5 seconds. Incubate at room temperature (20-25°C) for 5 minutes.

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The annealed PLATE-51 vector was transformed into competent bacteria *E. coli* BL21 using the heat-shock method. Add 1-5 µl of cloned pLATE-51 to the tube and mix gently. Incubate on ice for 30 minutes. Give heat shock treatment for 45 seconds at 42°C using a water bath then re-incubate the tube on ice for 2 minutes. Selection was carried out using antibiotic selection by growing bacteria on Luria Bertani (LB) agar with the antibiotic ampicillin at a concentration of 100 µg/ml. Confirmation of the presence of target genes in bacterial colonies is carried out by PCR using gene amplification primers.

Protein overexpression

Transformant colonies containing the pLATE-51 plasmid were grown in 100 ml of liquid LB medium at a temperature of 37°C for 4 hours or at a temperature of 20°C overnight (16h) in a shaker incubator. Induction of recombinant protein expression was carried out by adding isopropyl-1-thio-β-D-galactopyranoside (IPTG) at a concentration of 1 mM after reaching an optical density (OD) of between 0.5-0.6. The negative control used was *E. coli* strain BL21 bacteria which was not transformed.

Analysis of the presence of protein was carried out using SDS-PAGE at a concentration of 12%. The sample used for protein analysis is the bacterial lysate produced from the sonication process of the resulting bacterial colonies.

Culture harvesting and sonication

Harvesting of bacterial cultures is carried out by centrifugation. Harvesting of bacterial cultures is carried out by centrifugation. The protein extraction process from the culture results is carried out by sonicating the pellets obtained. Sonication was carried out at 300 watts (W). This procedure was carried out 10 times with a duration of 30 seconds each, interspersed with 30 second intervals.

Protein level quantification

Protein concentration was measured using nanodrop spectrophotometry. Two µl of sample was loaded into the cuvette. Absorbance level mas measured using 595 nm of wave length.

Immunodot blotting

The presence of the recombinant protein produced could be determined by immunodot-blotting. Immunodot-blotting was carried out by placing 5 µl of the induced protein solution on nitrocellulose paper. This procedure was carried out twice. First immunodot blotting was done to confirm the presence of recombinant protein using 6x-His tag monoclonal antibody (Invitrogen) as primary antibody, dissolved in a solution of 5% BSA in 0.1% TBS tween with a concentration of 1:2000. The secondary antibody used was goat anti-mouse IgG2b (Invitrogen) dissolved in 1% BSA in 0.05% TBS tween (concentration 1:15,000).

The second immunodot blotting was carried out to assess whether the recombinant protein could be identified by serum from scabies hosts. Scabies-infested rabbit serum was used as primary antibody. Serum was dissolved in a solution of 5% BSA in 0.1% TBS tween (dilution ratio 1:180). The secondary antibody used was goat anti-rabbit IgG whole molecule (Merck) dissolved in 1% BSA in 0.05% TBS tween (concentration 1:15,000).

Western blotting

Western blotting was done to identify the size of recombinant protein. Samples were transferred from SDS-PAGE gel into a nitrocellulose paper Scabies-infested rabbit serum was used as primary antibody. Serum was dissolved in a solution of 5% BSA in 0.1% TBS tween. The secondary antibody used was goat anti-rabbit IgG whole molecule (Merck) dissolved in 1% BSA in 0.05% TBS tween (concentration 1:15,000).

Protein solubilization

Solubilization was done using a solution of 5 mM dithiothreitol (DTT) in a buffer 50mM Tris, 1mM EDTA, 2% SDS, and 30% sucrose). Pellet was resuspended, then sonicated and centrifuged in 10.000 rpm (15 minutes) to obtain the supernatant.

Protein purification

Protein purification was done using PurkineTM His-Tag Purification Kit Ni-NTA (Abbkine) with affinity chromatography principle. Washing step was done using Tris-HCl, NaH2PO4, and Imidazole (10 dan 20 mM). Elution was done using Tris-HCl, NaH2PO4, dan Imidazole (350 mM and 550 mM).

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RESULTS

Amplification of exon 5 tropomyosin gene of *S. scabiei*

The exon 5 fragment of the tropomyosin gene from *S. scabiei* was amplified using Phusion High Fidelity DNA Polymerase (Thermoscientific, USA). PCR results showed an amplification band measuring 550 bp, which is consistent with the targeted gene segment (Figure 1).

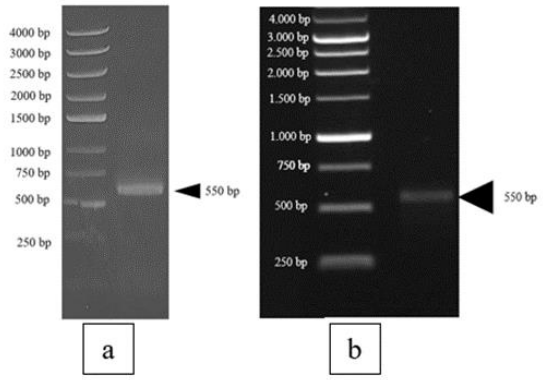

Figure 1. PCR result of exon 5 tropomyosin gene *S. scabiei* (a) and PCR result of confirmation cloning in pLATE-51 plasmid (b).

Cloning using Ligation-independent Cloning system

PCR products were inserted into plasmid with ligation-independent cloning methods. Clone products were then transformed into host, *Eschericia coli* strain B21, to producing the recombinant protein. Confirmation of inserted gene presence in the plasmid was done with PCR using the same primer pair in previous step. PCR showed a consistent band of 550 bp, which indicated the cloning step was successfully inserted targeted gene into the plasmid (Figure 1).

Protein overexpression

Recombinant protein in this study was estimated having molecular weight of 22 kilodalton (kDa). Analysis of protein products were done using SDS-PAGE of supernatant and pellet obtained from IPTG-induced *E. coli* BL21 culture (Figure 2). Thicker bands were observed from pellet counterparts. The finding also indicated that the recombinant protein is insoluble, thus needed a solubilization step using refolding buffer.

SDS-PAGE analysis from the pellet showed thicker bands compared to the supernatant counterparts. Results showed that the best results of protein induction with 1 mM IPTG at 37°C were obtained after 4 hours of induction, indicated with thickest band on SDS-PAGE results from supernatant and pellet. This finding showed that the protein yields were higher in pellet compared to supernatant. However, there was no single thick band observed in the SDS PAGE result.

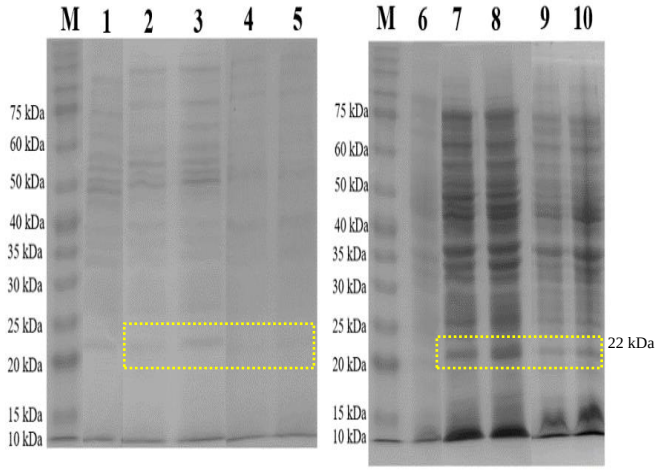

Figure 2. SDS-PAGE analysis of supernatant (1-5) and pellet (6-10) from *E. coli* BL21 culture. (M) Marker. (1) Supernatant from non-transformed E. coli BL21. (2) Supernatant from *E. coli* BL21 cultured in 20°C without IPTG. (3) Supernatant from *E. coli* BL21 cultured in 20°C with 1 mM IPTG. (4) Supernatant from *E. coli* BL21 cultured in 37°C without IPTG. (5) Supernatant from *E. coli* BL21 cultured in 37°C with 1 mM IPTG. (6) Pellet from non-transformed *E. coli* BL21. (7) Pellet from *E. coli* BL21 cultured in 20°C without IPTG. (8) Pellet from *E. coli* BL21 cultured in 20°C with 1 mM IPTG. (9) Pellet from *E. coli* BL21 cultured in 37°C without IPTG. (10) Pellet from *E. coli* BL21 cultured in 37°C with 1 mM IPTG.

Further step with nanodrop confirmed this finding. The protein concentrations were higher in pellet compared to supernatant products. Lower incubation temperature generates higher amount of protein (Table 1).

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Table 1. Recombinant protein concentration from the overexpression.

No.	Overexpression temperature (°C)	IPTG concentration (mM)	Recombinant protein concentration (mg/ml)	
			Supernatant	Pellet
1.	37°C	0	1,8	14,05
2.	37°C	1	2,323	22,44
3.	20°C	0	4,203	22,85
4.	20°C	1	5,03	24,963

Immunodot blotting

Confirmation of the resulting recombinant protein was done using immunodot blot. This step was done twice, using monoclonal antibody anti-Histidine tag and serum from scabies infested rabbit as primary antibody.

First immunodot blotting was done to confirmed the presence of recombinant protein using primary antibody 6x-His tag monoclonal antibody (Invitrogen). Positive results (indicated with blue dots) were found in transformed *E. coli* BL21, indicating the presence of recombinant protein. Results showed that IPTG-induced *E. coli* Bl21 cultured in 20°C overnight produced more protein compared to culture in 37°C for 4 hours (indicated with thick dots) (Figure 3).

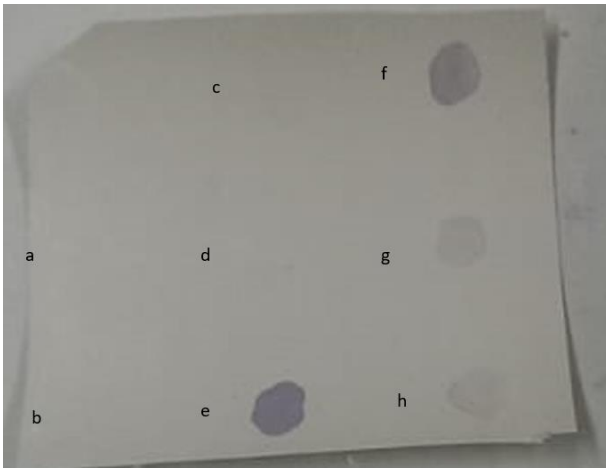

Figure 3. Immunodot blotting using anti-6-His-tag antibody as primary antibody. (a) Pellet from untransformed *E. coli* culture at 37°C (4h). (b) Supernatant from untransformed *E. coli* culture at 37°C (4h). (c) Pellet from untransformed *E. coli* culture at 20°C (16h). (d) Supernatant from untransformed *E. coli* culture at 20°C (16h). (e) Pellet from *E. coli* BL21 induced with IPTG at 20°C (16h). (f) Supernatant from *E. coli* BL21 induced with IPTG at 20°C (16h). (g) Pellet from *E. coli* BL21 induced with IPTG 1 M at 37°C (4h). (h) Supernatant from *E. coli* BL 21 induced with IPTG 1 M at 37°C (4h).

The second immunodot blotting was done after solubilization and purification of protein yielded from *E. coli* BL21 culture. The result indicated that recombinant protein was mostly insoluble, and despite effort to solubilize the protein the recombinant protein in the final product was not abundant (indicated with thinner dots in supernatant) (Figure 4).

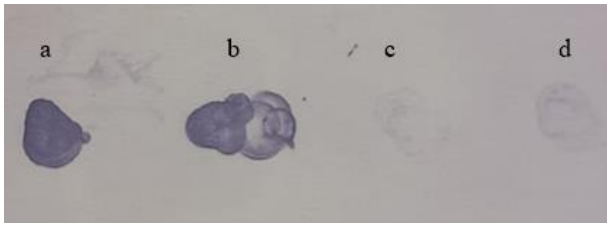

Figure 4. Immunodot blotting using scabies-infested rabbit's serum as primary antibody. (a) Pellet from untransformed *E. coli* culture at 20°C (overnight). (b) Supernatant from refolding product. (c)-(d) Purified product using Ni-NTA column. (c) Eluted with imidazole 350 mM. (d) Eluted with imidazole 500 mM.

Western blotting analysis

Analysis with Western blot using serum from scabies infested rabbit was done as confirmation of the recombinant protein size. As expected, multiple bands were found from unpurified products. Bands with the size around 22 kDa (expected size of the recombinant protein) were found in pre-purified products. No bands were found in purified products (Figure 5).

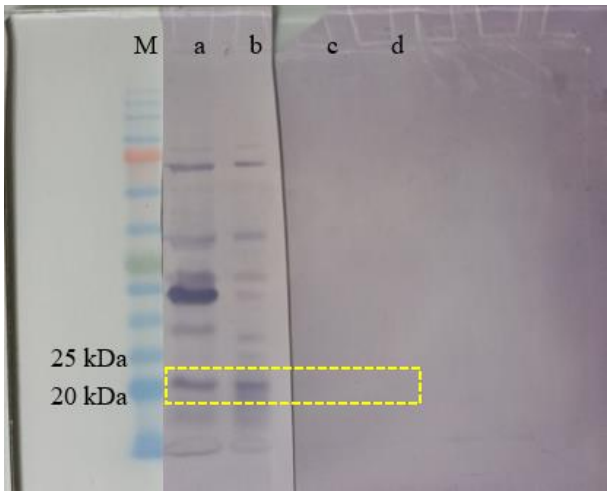

Figure 5. Western blotting using serum from scabies infested rabbit. (M) Marker. (a) Pellet from unpurified product. (b) Pellet from refolding product. (c) Purified product with imidazole 350 mM. (d) Purified product with imidazole 500 mM.

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DISCUSSION

High annealing temperature (67°C) was used in this study because the specific forward and reverse primer for pLATE-51 cloning had a melting temperature (Tm) that reached 79.0°C and 77.3°C. This is caused by the length of primer sequence. The forward and reverse primers are composed of quite long base sequences, reaching 42 base pairs, with a forward primer GC content of 42.85%, while the reverse primer has a GC content of 45.45%. Long primer sequence was needed because the primer is consist of specific primer to amplify target gene added with specific sequence which are complement with plasmid 5' and 3' end for ligation independent cloning purpose (LIC).[11]

The LIC procedure does not require a ligation step using a ligase enzyme, such as T4 DNA ligase, to insert the target gene into the vector. LIC utilizes the exonuclease activity of the T4 DNA polymerase enzyme to produce overhangs on the target gene to be inserted. This principle resembles gene recombination events that occur in vivo. The target gene inserted in this study is an amplicon that has gone through a gel electrophoresis purification procedure. The T4 DNA polymerase enzyme is used to open the double strand of amplicon DNA through 3'-5' exonuclease activity. The exonuclease activity of the T4 Polymerase enzyme can only occur without the presence of dNTPs in the reaction, so amplicons or PCR products resulting from gel purification are needed to ensure this condition. Without dNTPs, the T4 DNA polymerase enzyme will cut the double strand of DNA, allowing the overhangs at the 3' and 5' ends to be attached or annealed to the complementary end of the pLATE-51 plasmid.[12]

Recombinant protein in this study was estimated having molecular weight of 22 kilo Dalton (kDa). The SDS-PAGE profile of the overexpressed protein showed low yield of recombinant protein, with thicker bands in the pellet compared to the supernatant. The SDS-PAGE profile and nanodrop result showed that the best result of protein induction with 1 mM IPTG at 20°C were obtained after overnight (16 hours) induction. The protein produced is more abundant in the pellet compared to the supernatant. Recombinant proteins often found as inclusion bodies (IB) in *E. coli* cells, which means the protein are insoluble. IB is periplasmic aggregates which often occur in overexpression of recombinant proteins. IB occurs because the protein expressed from the insertion gene did not undergo appropriate post-translational modification, such as folding. The hydrophobic part of the protein in IB is located on the surface, thus making the protein exist in a water-insoluble form.[13]

The Luria-Bertani (LB) medium was used in this experiment. LB medium consists of tryptone, yeast extract, and NaCl, whish essential for bacteria growth. This medium has been widely used in terms of protein overexpression using various *E. coli* strains. LB medium is rich of amino acid, hence it could support the protein production by the bacteria.[14] *E. coli* cultured in LB medium underwent lower stress oxidative compared to other rich media, such as super broth (SB), terrific broth (TB), and 2× yeast extract-tryptone (YT). Lower oxidative stress rate contributes to long-term survival and mutation frequency of the bacterial colonies, therefore supporting a stable protein production.[15]

The optimum temperature for *E. coli* colonies growth is 37°C. In this research, the culture was initially done in this temperature. However, the protein obtained from the culture was not as expected, since the protein was mainly insoluble. For development of scabies diagnostic kit, soluble recombinant protein is preferred, since insoluble protein potentially has different biological activity compared to the soluble counterparts.[16]

Strategy applied to overcome the problem in this study was lowering the culture incubation temperature below 37°C (15-20°C). In this study, the incubation temperature was later reduced to 20°C, after the first attempt with optimum temperature was failed to produce soluble protein. Lower incubation temperatures for overexpressing recombinant proteins in this study aimed to reduce the growth speed of *E. coli* cultures. Slower culture growth results in a lower metabolic load, thus might minimize the formation of inclusion bodies by providing the opportunity for appropriate folding and post-translational modifications. Lower incubation temperatures of the cultures in this study did not affect protein overexpression, because the protein concentration in the pellet was higher compared to the supernatant.[13,17]

The immunodot blot results confirmed the presence of recombinant protein recognized by anti-Histidine antibody in the pellet from transformed *E. coli* culture which was incubated at 37°C and 20°C.

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Immunodot-blotting in this study was carried out for rapid detection of recombinant proteins in the harvested *E. coli* culture results. This procedure was chosen because it could provide confirmation of the presence of recombinant proteins in a short time, without an overnight incubation step. This method has a specificity that is close to western-blotting in protein detection, with specificities of 95.6% and 97.8%, respectively. In addition, this method has a higher sensitivity, which is 86.7%, compared to the specificity of western-blotting (76.4%).[18] However, this method has shortcomings because it cannot visualize the size of the protein recognized by antibodies.[18]

The result of SDS-PAGE indicated that the protein production was higher in pellet. Thus, in this study, we solubilize the pellet using dithiothreitol (DTT). DTT increases protein solubility by re-oxidizing disulfide bonds in proteins. This reaction prevents proteins from forming aggregates, thus increasing their solubility in water or solvents.[19] EDTA in the solubilization buffer prevents protein degradation by protease enzymes, because EDTA is an enzyme inhibitor.[20]

The recombinant protein binds strongly to Ni-NTA resin because it has 6 histidine amino acid residues. Imidazole is the most widely used compound as an affinity competitor for Ni-NTA resin, to elute recombinant protein. Optimizing imidazole concentration was done in this research. Increasing imidazole concentration was meant to increase the recombinant protein purity.[21] However, the resulting band showed from immunodot blotting and western blotting were thin despite optimization of the elution step of protein purification.

Recombinant proteins that are gene fragments have shorter amino acid sequences when compared to full-length sequences. The recombinant protein produced in the study was encoded by a 287 base pair long gene exon fragment (approximately consists of 95 amino acids), with a molecular weight of approximately 22.06 kDa. Protein measuring <230 amino acids have significantly lower number of epitopes or contact residue sequences compared to larger ones (≥230 amino acids), which might explained faint and no band each in immunodot blotting and western blotting.[22]

The lack of band recognized by antibody in western blotting might be explained by several factors. The first cause is that the overexpressed protein is insoluble or does not dissolve in water, as happened in this study. The protein expressed in inclusion bodies is more hydrophobic, making it difficult for the affinity chromatography purification process that requires protein in soluble form.[17]

In addition to causing the protein to be difficult to dissolve in water, protein misfolding could interfere with purification by affinity chromatography because the position of the poly-histidine tag may be on the hydrophobic side.[22] The position of the histidine tag causes the recombinant protein to be unable to bind to the metal ions on the affinity chromatography column, thus no band recognized by the serum on the purification result column.[23]

CONCLUSION

The exon 5 of *Sarcoptes scabiei* tropomyosin gene could be cloned in pLATE-51 plasmid and produced recombinant protein. However, the protein yield was low and the protein is mostly insoluble, preventing the success of purification of the intended recombinant protein. Further study is needed to produce soluble and abundant recombinant protein, in-order to develop a candidate for scabies diagnostic kit.

ETHICAL APPROVAL

This research has been approved by the Ethics Commission of the Faculty of Medicine, Public Health and Nursing, Gadjah Mada University with reference number KE/FK/0787/EC.

CONFLICT OF INTEREST

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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AUTHOR CONTRIBUTION

Conceptualization, AHD and TMP; methodology, WTA; software, TMP; validation, AHD and WTA; formal analysis, AHD; investigation, AHD; resources,

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TBT; data curation, WTA; writing—original draft preparation, AHD, TMP, TBT, and WTA; writing—review and editing, XXX; visualization, TMP; supervision, WTA; project administration, TBT; funding acquisition, TBT.

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