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JOURNAL INDEX

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Table of Contents

Articles

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Optimized Expression Condition of CIDRα-PfEMP1 Recombinant Protein Production in *Escherichia coli* BL21(DE3): A Step to Develop Malaria Vaccine Candidate

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KEYWORDS

CIDRα-PfEMP1; *E. coli* BL21(DE3); IPTG; incubation period; recombinant protein

Abstract Malaria is still an essential epidemiological disease worldwide, including in Indonesia. Several approaches are performed to control the disease, as well as vaccine development. The Cysteine-rich interdomain region α of *Plasmodium falciparum* erythrocyte membrane protein 1 (CIDRα-PfEMP1) is a pivotal domain in the malaria pathogenesis make it a malaria vaccine candidate. The development of the malaria vaccine is performed using recombinant technology. Recombinant protein production is an important step. The study aimed to determine the optimized condition for CIDRα-PfEMP1 recombinant protein expression in *Escherichia coli* BL21(DE3) expression system. Serial IPTG concentrations from 0.05, 0.1, 0.3, and 0.5 mM and two different incubation periods of 4 h and 8 h were optimized. The recombinant protein expression was visualized in SDS-PAGE, measured using the *Bradford* protein assay, and calculated using software Image J. SDS-PAGE visualization showed a 27 kDa band expressed CIDRα-PfEMP1 recombinant protein. The optimized condition for CIDRα-PfEMP1 recombinant protein expression was at 0.03 mM IPTG concentration and 8 h incubation period.

Introduction

Malaria is an infectious disease caused by *Plasmodium* and transmitted by female *Anopheles* mosquitoes. World Health Organization (WHO) reported 228,000,000 malaria cases in 2018, while 93% occurred in the WHO African region, followed by the WHO South-East Asia Region (3.4%) and WHO Eastern Mediterranean Region (2.1%) (WHO, 2019). An estimated 7.9 million cases in South-East Asia and Indonesia contributed to 30% of total cases by publishing 220,000 malaria cases and 21% death due to malaria in 2018. The three highest endemic areas in Indonesia are Papua, West Papua, and Nusa Tenggara Timur (Kemenkes RI, 2013; Riskesdas-Kemenkes RI, 2018).

Five types of *Plasmodium* cause malaria in humans, i.e., *Plasmodium falciparum*, *Plasmodium ovale*, *Plasmodium malariae*, *Plasmodium vivax*, and *Plasmodium knowlesi*. *P. falciparum* is the most common cause of malaria and resulted in the most severe malaria symptom even death (WHO, 2019). *P. falciparum* has the ability to modify the red blood cells infected by *falciparum* malaria into an adhesive phenotype (Emile *et al*., 2012), which caused by *Plasmodium falciparum* erythrocyte membrane protein-1 (PfEMP1). The PfEMP1 is a complex protein and consists of intra and extra-cellular part. The extra-cellular part of PfEMP1 is composed of several Cysteine-rich interdomain regions (CIDR) and the Duffy-binding-like (DBL) domain. Each domain consists of homologous groups (α, β, γ,

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δ, ε, ζ) and (α, β, γ) (Adams *et al*., 2014) and has specificity to bind host cell receptor and the binding lead to malaria pathogenesis and symptom. CIDRα-PfEMP1 mediates the binding to several host receptors such as Cluster of Differentiation 36 (CD36), CD31, and Chondroitin Sulfate A (CSA) which facilitates cytoadherence process as well as Immunoglobulin M (IgM) which mediates rosetting process ((Chen *et al*., 2000; Rowe *et al*., 2009). Furthermore, a crucial one is the binding of the CIDRα-PfEMP1 and the endothelial protein C receptor (EPCR) in the endothelial brain, which provokes coagulation, vascular inflammation, and increase endothelial permeability and leads to severe malaria pathogenesis especially in cerebral malaria symptoms (Bernabeu and Smith, 2017; Turner *et al*., 2013; Turner *et al*., 2015). The pivotal role of CIDRα-PfEMP1 makes the domain a malaria vaccine candidate.

The approach of malaria vaccine development is conducted using DNA recombinant technology, and the vaccine technology has led the peptide-based vaccine as the choice for several reasons (Draper *et al*., 2015). The process starts by identifying and characterizing immunogenic protein, a protein that can induce an immune response in host cells, followed by toxicity, mutagenicity, determination of best dose-response, and other pre-clinical trials before starting the clinical trial. All the pre-clinical studies will need bulk protein production followed by recombinant protein purification (Corradin, 2007; Draper *et al.*, 2018; McCullers & Dunn, 2008).

One of the studied immunogenic proteins is PfEMP1(Bull & Abdi, 2016), an essential protein for malaria pathogenesis by binding the host receptor, leading to malaria symptoms, such as the CIDRα domain, which binds EPCR (Kessler *et al*., 2017). The previous study has constructed the CIDRα-PfEMP1 recombinant and transformed it into *E. coli* BL21(DE3) expression system (Dewi *et al*., 2018). Further steps of CIDRα-PfEMP1 recombinant protein expression are crucial for the success of the malaria vaccine. The expression of CIDRα-PfEMP1 recombinant protein expression needs to be optimized based on several factors such as temperature and the concentration of isopropyl-β-D-thiogalactoside (IPTG) for expression induction, IPTG induction time, incubation periods, and centrifugation process (Chhetri *et al*., 2015; Gomes *et al*., 2020). This study aimed to determine the optimized condition of IPTG concentration and incubation period for the CIDRα-PfEMP1 recombinant protein expression in *the E. coli* BL21(DE3) expression system to support peptide-base malaria vaccine development.

Materials and Methods

Ethical Clearance

This was an exploratory descriptive study using CIDRα-PfEMP1 recombinant. The study has received ethical approval from the Ethical Committee of Faculty of Medicine University of Jember with reference number 1469/H25.1.11/KE/2021. The research was conducted based on good laboratory practice.

Production of the recombinant protein CIDRα-PfEMP1 in E. coli BL21 (DE3)

The CIDRα-PfEMP1 recombinant protein production procedure was conducted as previously reported (Dewi *et al*., 2018; Flick *et al*., 2004; Guerra *et al*., 2016). The previous study reported that CIDRα-PfEMP1 recombinant protein was expressed in the supernatant as a soluble protein and in the pellet as insoluble protein when were induced by low IPTG concentration (< 1 mM) for 4 – 16 hrs (Dewi *et al.*, 2018). The recombinant protein CIDRα-PfEMP1 production was started by culturing the CIDRα-PfEMP1 recombinant into 3 ml of liquid LB medium containing 50

E-ISSN 2355-9926

µg/ml kanamycin in an incubator shaker at 150 rpm at 37°C for 16 hours. The culture was then inoculated into 250 ml of liquid LB medium containing 50 µg/ml of kanamycin with a ratio of 1:50 and incubated at a shaker incubator of 150 rpm 37°C until $0D_{600}$ reached 0.6-0.8. The culture was induced with various IPTG concentrations for each sample, from 0.05 mM, 0.1 mM, 0.3 mM, and 0.5 mM. Each sample was then incubated at different incubation periods, i.e., 4 h and 8 h, followed by centrifugation at 4°C of 6.000 rpm for 15 min. The pellets were solubilized using a buffer (150 mM NaCl and 50 mM Tris HCl in pH 7,5) with a ratio of 1:2 for pellets: extraction buffer, then lysed with 1 mg/ml lysozyme in ice for 15 min before sonication for 5x18 cycles. The last step was centrifugation at 12.000 rpm 4°C for 20 min to separate the supernatant containing soluble protein and pellet containing insoluble protein.

Bradford protein assay

The protein concentration from the supernatant was measured using the Bradford protein assay. The Bradford assay measured the protein concentration by determining the color change in the sample from colorless to blue, due to reaction of amino acid residues such as arginine, lysine, and histidine in the sample. The density of blue color is proportional with the protein concentration. The procedure was started by preparing the standar protein and appropriate buffer to determine standard curve. As much as 3 ml of *Bradford* reagent was added into each tube containing supernatant protein and vortexed. The samples were then incubated at room temperature for 5-45 mins and transferred to cuvettes for measuring their absorbance at a 595 nm wavelength spectrophotometer. The absorbance values were used to determine the protein concentration by comparing it with the standard (Ernst & Zor, 2010; He, 2011).

Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

The supernatant containing soluble protein was visualized using SDS-PAGE. The method detects the protein based on the molecule movement in the gel matrix which makes protein separated according to their size. The high molecular weight protein will posed at the top, while a low molecular weight protein will locate at the bottom of the gel. The size of protein will be determined by the protein marker. The procedure was started by preparing running buffer and sample buffer followed by denaturated protein using a dry heat block (95°C) for 5 min. Separation gel was prepared using 15% acrylamide and placed at a lower gel, while stacking gel was prepared using 4.5% acrylamide and placed at the upper gel. As many as 10 µl denaturated sample was added on each well and 4 µl prestained marker was added on a well. The adjusted samples ran through an intermediate running buffer at 50 V for 60 min for the lower gel and 80 V for 90 min for the upper gel. The gel was then stained with a staining solution, followed by destaining to see the protein expression in the form of a band (Laboratories Bio-Rad, 2012; Manns, 2011).

Analysis using Image J

The CIDRα-PfEMP1 recombinant protein calculation based on its nucleotide showed 27 kDa. The recombinant protein concentration was also measured using Image J analysis. The procedure was conducted based on previous report (Reinking, 2007). Eight protein samples were diluted into three stages dilution, started from 0 dilutions, ¾ dilution, and ½ dilution. Diluted samples were running on SDS-PAGE gel together with BSA standard protein in various concentrations (1.25 µg/µl, 0.625 µg/µl, 0.3125 µg/µl, and 0.15625 µg/µl). The SDS-PAGE result was scanned and inserted into Image J software to be converted into 8-bit and binary formats. Each sample's and BSA's band were

E-ISSN 2355-9926

analyzed for their thickness using the area provided on the software. The protein concentration was measured based on the BSA standard curve (Reinking, 2007).

Results and Discussion

Protein concentration analysis using the Bradford protein assay method

This study optimized the CIDRα-PfEMP1 recombinant protein expression in the *E. coli* BL21 (DE3) expression system, using various IPTG concentrations and two different incubation periods. Optimation results were observed by calculating the protein concentration using the Bradford assay, visualization using SDS-PAGE, and analysis using Image J software. Table 1 showed the Bradford protein assay results in triplicates and the total protein concentration. Color-changing determines absorbance due to the bonding of dye with protein in a 595 nm spectrophotometer. The color change that occurs in the Bradford reagent confirms that the sample contains protein. The protein concentration was calculated based on the linear equation y = 0.04x + 0.0376 obtained from the standard BSA protein dilution as in Figure 1. (Pedrol and Ramos, 2001). This total crude protein concentration contains the whole protein expressed by *E. coli* BL21 (DE3). This protein concentration was used to calculate the load protein volume for SDS-PAGE analysis.

Table 1. The Bradford Assay and the Protein Concentration in Various Induction Conditions

Sample	Absorbance Replication			Absorbance Mean	Total Protein Concentration
	I	II	III		
1A	0.710	0.745	0.619	0.691	3.270 µg/µl
2A	0.746	0.915	0.811	0.824	3.935 µg/µl
3A	1.043	0.965	0.717	0.908	4.335 µg/µl
4A	0.972	0.884	0.804	0.887	4.250 µg/µl
1B	0.614	0.870	0.682	0.722	4.820 µg/µl
2B	0.971	1.002	0.641	0.871	3.425 µg/µl
3B	0.965	0.812	0.762	0.846	4.170 µg/µl
4B	0.613	0.618	0.780	0.670	4.045 µg/µl
Control	1.120	0.940	0.950	1.003	3.165 µg/µl

1A: protein expression using 0.05 mM IPTG induction and 4 h incubation period.
2A: protein expression using 0.1 mM IPTG induction and 4 h incubation period.
3A: protein expression using 0.3 mM IPTG induction and 4 h incubation period.
4A: protein expression using 0.5 mM IPTG induction and 4 h incubation period.
1B: protein expression using 0.01 mM IPTG induction and 8 h incubation period.
2B: protein expression using 0.1 mM IPTG induction and 8 h incubation period.
3B: protein expression using 0.3 mM IPTG induction and 8 h incubation period.
4B: protein expression using0.5 mM IPTG induction and 8 h incubation period.

E-ISSN 2355-9926

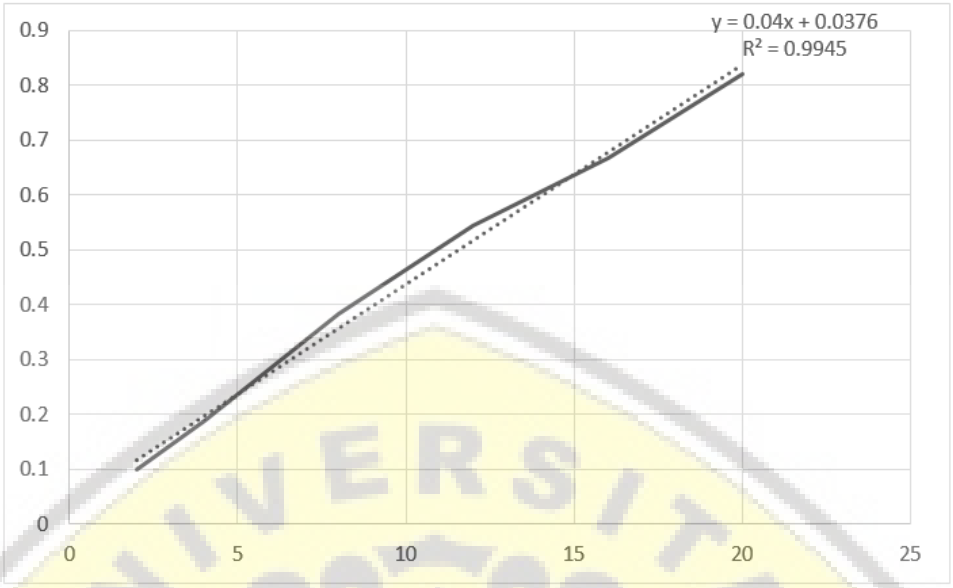

Figure 1. *Bradford* protein assay standard curve

Visualization of the CIDRα-PfEMP1 recombinant protein expression on SDS-PAGE

The expressed protein was expressed as soluble protein, which separated from insoluble protein by centrifugation. Centrifugation resulted in a supernatant containing soluble protein and a pellet consist of insoluble protein. As many as 15 µg soluble proteins of each sample were loaded into SDS-PAGE well. The previous study reported that the CIDRα-PfEMP1 recombinant protein was expressed more in the supernatant, and the calculation showed that the protein was 27 kDa in weight. The SDS-PAGE analysis confirmed this, as a 27 kDa band was not observed in the control sample containing *E. coli* BL21 (DE3) bacteria only.

The target protein band was expressed with various thicknesses based on the induction conditions, including incubation period and IPTG concentration. Visualization of SDS-PAGE in Figure 2 showed that the group B samples using 8 h incubation period produced a thicker and clearer band than group A with 4 h incubation period. This implicated that 8 h incubation period induces better CIDRα-PfEMP1 protein expression than 4 h incubation period. The growth of recombinant bacteria *E. coli* BL21 (DE3) is related to the length of the incubation period. The incubation period increases the number of cells related to the amount of protein produced. More cell growth will be proportional to the amount of recombinant protein produced (Hermana *et al*., 2015). A previous study by Dewi *et al*. (2018) showed that the CIDRα-PfEMP1 recombinant protein expression was better at 8 h or more incubation period than 4 h. However, a long incubation period could destroy the protein due to increased protease enzyme secretion in the medium, which can degrade protein (Lestari, 2010). Flick *et al*. (2004) reported that the very long incubation period of the PfEMP1 protein could activate the arginine, leucine, isoleucine, and proline codons, while these codons inhibit the translation process by reducing tRNA performance. Furthermore, the very long incubation period may produce a poor-quality protein due to decreased amino acid reserves in the PfEMP1 protein (Flick *et al*., 2004).

E-ISSN 2355-9926

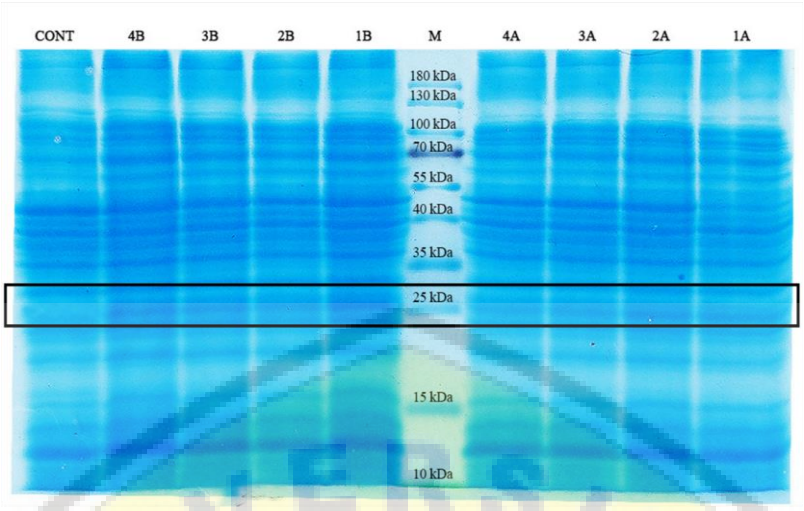

Figure 2. SDS-PAGE visualization results of soluble protein produced in 15% lower acrylamide gel. A single thickening band slightly above 25 kDa marker indicated the 27 kDa CIDRα-PfEMP1 recombinant protein in samples and could not be observed in the control. Cont: control sample containing *E. coli* BL21 (DE3) only; 4B: 0.5 mM IPTG induction and 8 h incubation period; 3B: 0.3 mM IPTG induction and 8 h incubation period; 2B: 0.1 mM IPTG induction and 8 h incubation period; 1B: 0.01 mM IPTG induction and 8 h incubation period; M: protein marker; 4A: 0.5 mM IPTG induction and 4 h incubation period; 3A: 0.3 mM IPTG induction and 4 h incubation period; 2A: 0.1 mM IPTG induction and 4 h incubation period; and 1A: 0.05 mM IPTG induction and 4 h incubation period.

Protein concentration analysis using Image J

The protein concentration was also measured using Image J software. The scanned gel images which was converted into 8-bit format and binary form was shown in Figure 3. Image J is a semiquantitative software for converting image data into numeric data. The protein concentration was calculated based on standard curve and the results were presented in Table 3. The measurement of the target protein concentration using Image J showed that the highest concentration is 0.054 µg/µl resulted from the 3B sample, induced by 0.3 mM IPTG.

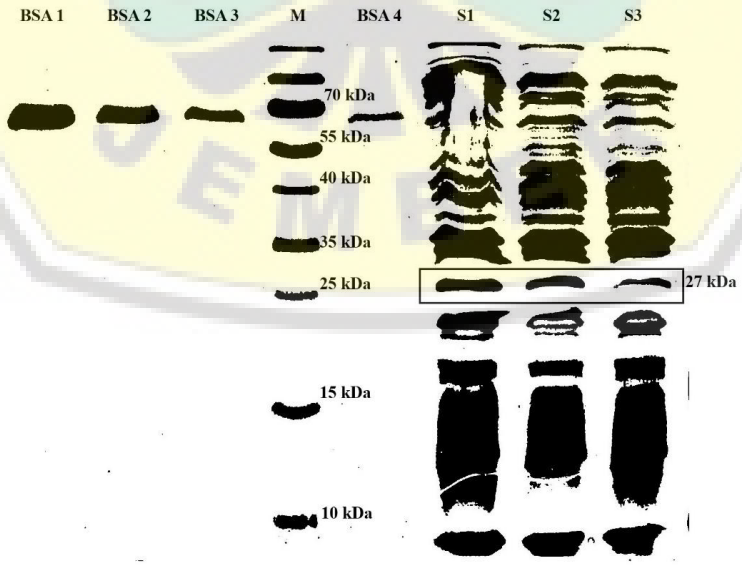

Figure 3. Result Image of a 3B sample in 8-bit binary format. BSA 1: 1.25 µg/µl; BSA 2: 0.625 µg/µl; BSA 3: 0.3125 µg/µl; BSA 4: 0.15625 µg/µl; M: protein marker; S1: sample without dilution; S2: sample with ¾ dilution; S3: sample with ½ dilution.

E-ISSN 2355-9926

Tabel 3. Result of Protein Concentration Measurement Using Image J

Sample	r^2	Equation	Protein Concentration
1A	0.949	y = 166850x + 17599	0.015 µg/µl
2A	0.979	y = 99556x + 43415	0.035 µg/µl
3A	0.978	y = 60230x + 37117	0.015 µg/µl
4A	0.964	y = 111064x + 35080	0.040 µg/µl
1B	0.974	y = 105861x + 34665	0.047 µg/µl
2B	0.978	y = 90000x + 26888	0.039 µg/µl
3B	0.976	y = 120705x + 35880	0.054 µg/µl
4B	0.952	y = 94704x + 28780	0.024 µg/µl

1A: protein expression using 0.05 mM IPTG induction and 4 h incubation period.
2A: protein expression using 0.1 mM IPTG induction and 4 h incubation period.
3A: protein expression using 0.3 mM IPTG induction and 4 h incubation period.
4A: protein expression using 0.5 mM IPTG induction and 4 h incubation period.
1B: protein expression using 0.01 mM IPTG induction and 8 h incubation period.
2B: protein expression using 0.1 mM IPTG induction and 8 h incubation period.
3B: protein expression using 0.3 mM IPTG induction and 8 h incubation period.
4B: protein expression using0.5 mM IPTG induction and 8 h incubation period.

Conclusions and suggestion

The optimal conditions of the CIDRα-PfEMP1 recombinant protein expression in *E. coli* BL21 (DE3) was 0.3 mM IPTG concentration and 8 h incubation period. The optimized expression condition could be used to study further the CIDRα-PfEMP1 recombinant protein, including the protein purification process and other research to support malaria vaccine research development.

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E-ISSN 2355-9926

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