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Expression of recombinant *Plasmodium falciparum* Cysteine-Rich Protective Antigen (PfCyRPA) fragment 26–181 in *Escherichia coli* BL21 CodonPlus (DE3) RIPL

Fifi Fitriyah Masduki[1,2*], Aurelia Galih Prameswari[1], Fernita Puspasari[1], Ihsanawati[1], Dessy Natalia[1,2]

[1]Biochemistry and Biomolecular Engineering Research Division, Faculty of Mathematics and Natural Sciences, Institut Teknologi Bandung, Jl. Ganesa No. 10, Bandung 40132, Indonesia

[2]Biosciences and Biotechnology Research Centre, Institut Teknologi Bandung, Jl. Ganesa No. 10, Bandung 40132, Indonesia

[*]Corresponding author: Jl. Ganesa No. 10, Bandung 40132, Indonesia. Email: f.masduki@itb.ac.id

ABSTRACT

Background: Malaria is a life-threatening infectious disease caused by *Plasmodium* parasites, transmitted through infected female *Anopheles* mosquitoes. PfCyRPA (*Plasmodium falciparum* Cysteine-Rich Protective Antigen) has emerged as a promising vaccine candidate due to its ability to elicit inhibitory antibodies against parasite growth.

Objective: This study aimed to construct recombinant plasmids encoding PfCyRPA fragment 26–181 and express this fragment in *Escherichia coli* BL21 CodonPlus (DE3) RIPL for cost-effective antigen production.

Methods: The PfCyRPA gene fragment (~480 bp) was amplified from *P. falciparum* genomic DNA (Jayapura isolate) by PCR. Recombinant plasmids pGEM-T-PfCyRPA 26-181 and pET-16b-PfCyRPA 26-181 were constructed and confirmed via colony PCR, restriction analysis, and sequencing. The pET-16b-PfCyRPA 26-181 was transformed into *E. coli* BL21 CodonPlus (DE3) RIPL. Protein expression was induced with 0.5 mM IPTG at 37°C, and analyzed by SDS-PAGE and Western blotting.

Results: SDS-PAGE and Western blot analysis demonstrated successful expression of recombinant PfCyRPA fragment 26–181 with a molecular mass of approximately 21.53 kDa, corresponding to the predicted size. The protein was predominantly expressed as inclusion bodies, typical for eukaryotic proteins in prokaryotic systems. Colony PCR and sequencing confirmed correct gene insertion and integrity.

Conclusion: Recombinant PfCyRPA fragment 26–181 was successfully expressed in *E. coli* BL21 CodonPlus (DE3) RIPL, providing a cost-effective platform for large-scale antigen production. This work establishes a foundational protocol for further immunogenicity research and supports development of this antigen as a potential blood-stage malaria vaccine candidate.

Keywords: *E. coli* BL21 CodonPlus (DE3) RIPL, malaria, malaria vaccine, PfCyRPA, protein expression, recombinant protein

Introduction

Malaria is an infectious disease caused by *Plasmodium* parasites that remains a significant global health concern, particularly affecting tropical and subtropical regions. The parasite is transmitted to humans through the bites of infected female *Anopheles* mosquitoes. Among several *Plasmodium* species affecting humans, *P. falciparum* is the deadliest, causing severe clinical manifestations such as anemia, cerebral malaria, and even death [1]. In 2022, approximately 443,530 malaria cases were reported with Papua and West Papua provinces (Indonesia) accounting for nearly 94% of national malaria incidences [2]. These areas represent persistent endemic regions where effective prevention and control strategies are critically necessary.

Although significant progress has been made in malaria prevention, ongoing challenges persist due to factors such as parasites developing resistance

to antimalarial drugs and mosquitoes building resistance to insecticides. Traditionally, malaria prevention strategies have relied on vector control methods, such as insecticide-treated bed nets, indoor residual spraying, and preventive chemotherapy. However, these approaches alone are insufficient to eradicate the disease, underscoring the urgent need for additional, more effective measures, including vaccines [3]. Vaccines offer a complementary, sustainable solution by inducing long-lasting immunity and reducing parasite transmission. They are particularly vital for protecting high-risk populations such as children under five and pregnant women [4]. The RTS,S/AS01 vaccine—the first to receive a WHO recommendation—demonstrated moderate efficacy in reducing severe malaria and hospitalizations, paving the way for broader implementation [5].

Vaccines are cost-effective, can be integrated into routine immunization schedules, and require less daily compliance than chemoprophylaxis. As part of an integrated strategy, vaccines are essential tools for achieving malaria elimination and supporting global health goals. Thus, vaccine development targeting various stages of the *Plasmodium* life cycle, including pre-erythrocytic, blood-stage, and transmission-blocking phases, has become a research priority [4]. Among the promising candidates for malaria vaccines, blood-stage antigens are of significant interest due to their direct role in disease pathogenesis and their potential to prevent parasite proliferation within erythrocytes.

One such candidate is the Cysteine-Rich Protective Antigen (CyRPA) from *P. falciparum*. CyRPA is a highly conserved component of the RH5-Ripr-CyRPA invasion complex, playing a crucial role in parasite attachment and invasion of erythrocytes [6–10]. Antibodies generated against CyRPA effectively inhibit parasite growth both in vitro and in vivo, indicating its suitability as a candidate for blood-stage vaccine development [8, 11–14]. In previous studies investigating the reactivity of monoclonal anti-PfCyRPA antibodies with various fragments of the PfCyRPA protein, it was found that PfCyRPA fragment 26–181 contains a greater number of epitopes recognized by these antibodies. Based on this observation, fragment 26–181 was selected for the present study with the expectation that it would serve as a more effective antigen. When delivered as a vaccine, this fragment is anticipated to induce the production of antibodies in humans capable of inhibiting *P. falciparum* erythrocyte invasion [15].

Moreover, the recombinant PfCyRPA fragment 26–181 antigen offers potential use in immunogenicity studies. To evaluate the success of the vaccination, the recombinant PfCyRPA 26–181 produced in this study can be used as a tool to measure anti-PfCyRPA antibody titers after immunization. Such studies are critical for the continued development of malaria vaccines targeting the blood stage of infection, where PfCyRPA serves as a key antigen candidate. The choice of this fragment thus supports both fundamental and translational research objectives in the quest for an effective malaria subunit vaccine.

However, producing recombinant CyRPA presents several practical challenges, especially regarding the expression and purification of recombinant antigens. Recombinant protein production in prokaryotic systems, such as *Escherichia coli*, is frequently utilized due to its cost-effectiveness and speed [16]. Nonetheless, these systems often face considerable hurdles such as codon usage bias, improper protein folding, and the formation of inclusion bodies [17]. These issues significantly impair the ability to obtain sufficient yields of correctly folded and functional antigen, highlighting the need for optimized expression conditions and systems to address these production challenges effectively.

Previous research has demonstrated various strategies to mitigate these challenges, including the use of specialized *E. coli* strains and optimization of expression conditions. Engineered *E. coli* strains such as BL21 CodonPlus, which offers enhanced codon usage compatibility, have shown promise in recombinant protein production [16,18]. Additionally, expression conditions, including inducer concentration and temperature optimization, as well as fusion tags or co-expression systems with

molecular chaperones, have been employed to improve protein folding and solubility. Despite these efforts, optimization of conditions for efficient expression of PfCyRPA fragment 26–181 has been insufficiently explored. Notably, the recombinant expression of CyRPA has predominantly been achieved in eukaryotic systems, providing properly folded proteins but typically at high production costs and low yields, limiting practical applications for large-scale vaccine development.

Literature specifically addressing the recombinant expression of PfCyRPA fragment 26–181 in optimized *E. coli* strains such as BL21 CodonPlus remains limited, which represents a significant research gap. Previous literature on other malaria antigens has successfully employed *E. coli* systems to generate functional recombinant proteins, supporting the potential feasibility of this approach [19]. To address this gap, the present study aims to construct recombinant plasmids encoding PfCyRPA fragment 26–181 and express this fragment in *E. coli* BL21 CodonPlus (DE3) RIPL. This research investigates optimal conditions for gene amplification, plasmid construction, and recombinant protein expression. By utilizing the BL21 CodonPlus strain, this study provides critical insights into its efficiency and capability for achieving high-yield production, thereby establishing a foundational protocol for recombinant PfCyRPA production. Ultimately, the findings from this research are expected to significantly contribute to further immunological investigations and advance the development of effective and economically feasible malaria vaccine candidates.

Methods

This research utilized materials for four main procedures: gene amplification, recombinant plasmid construction, host cell transformation and gene expression, and protein analysis.

Gene amplification materials: Genomic DNA from *P. falciparum* isolate Jayapura, PCR buffer (10× DreamTaq Green Buffer, Thermo Scientific), dNTP (KAPA dNTPs 10 mM, Kapa Biosystems), forward and reverse primers (Integrated DNA Technologies), ddH_2O, DNA polymerase (DreamTaq DNA Polymerase, Thermo Scientific), $MgCl_2$ (Fermentas), agarose, Tris base, glacial acetic acid, and Gel/PCR DNA Fragments Extraction Kit (Geneaid).

Recombinant plasmid construction materials: Cloning vector pGEM-T Easy (Promega), expression vector pET-16b, T4 DNA ligase buffer (Promega), T4 DNA ligase (Promega), restriction enzymes XhoI and BamHI (FastDigest Value Pack, Thermo Scientific), restriction buffer (10× FastDigest Buffer, Thermo Scientific), and Presto™ Mini Plasmid Kit (Geneaid).

Host cell transformation and gene expression materials: *E. coli* TOP10F', E. coli BL21 CodonPlus (DE3) RIPL, tryptone, yeast extract, NaCl, bacto agar, $CaCl_2$, ampicillin, tetracycline, chloramphenicol, gentamicin, IPTG, X-Gal, and 70% ethanol.

Protein analysis materials: 30% acrylamide solution, Tris-SDS buffer 2.5× (pH 8.8), Tris-SDS buffer 5× (pH 6.8), APS, TEMED, SDS, running buffer, loading buffer, staining and destaining solutions, protease inhibitors, transfer buffer, TBS, TBST, Ponceau red solution, skim milk, anti-His-Tag mouse antibodies (MyBioSource), HRP-conjugated anti-mouse IgG antibodies (Promega), and TMB substrate.

Primer design for amplification of *PfCyRPA* gene

Primers for amplifying the PfCyRPA gene were designed based on nucleotide sequences published in the *Plasmodium* database (PlasmoDB, accession number PF3D7_0423800). The design process began with a comprehensive literature review to identify the most potent inhibitory amino acid region recognized by monoclonal anti-PfCyRPA antibodies [14]. Based on this assessment, the gene fragment encoding amino acids 26–181 of PfCyRPA was selected for amplification. Restriction enzyme recognition sites *Xho*I and *BamH*I were incorporated into the forward and reverse primers, respectively, with an additional stop codon introduced at the C-terminal end to terminate translation. The primers synthesized for this study were

PfCyRPA-F (5'-CTCGAGATAAATTGTGATAGTCGTC-3') and PfCyRPA-R (5'-GGATCCTCAACCACATATTA GAAAGTACTC-3').

Amplification of gene fragment encoding amino acids 26–181 of PfCyRPA

The gene fragment encoding amino acids 26–181 of PfCyRPA was amplified using the polymerase chain reaction (PCR) method with genomic DNA of *P. falciparum*, isolated from a patient with a single *P. falciparum* infection in Jayapura, as the template as previously described [20]. Prior to PCR, the quality of the genomic DNA was verified by agarose gel electrophoresis, and its concentration was measured using a NanoDrop spectrophotometer.

The PCR reagent mixture was prepared in a 1.5 mL microtube with the following composition: 79.5 µL ddH_2O, 10 µL Green PCR DreamTaq 10× buffer, 2 µL forward primer (20 µM), 2 µL reverse primer (20 µM), 2 µL dNTPs (10 mM), 1 µL DreamTaq DNA polymerase, and 4 µL DNA template. The PCR protocol consisted of an initial denaturation step at 95°C for 3 minutes, followed by 30 cycles of denaturation at 95°C for 30 seconds, annealing at a gradient range of 46–68°C for 30 seconds, and extension at 72°C for 1 minute. A final elongation step was conducted at 72°C for 5 minutes, followed by storage at 4°C.

Construction of recombinant plasmids pGEM-T-PfCyRPA 26-181 and pET-16b-PfCyRPA 26-181

The amplified gene fragment encoding amino acids 26–181 of PfCyRPA was ligated with the pGEM-T Easy cloning vector using T4 DNA ligase. The ligation mixture consisted of T4 DNA ligase buffer (10×), pGEM-T (50 ng/µL), T4 DNA ligase (1 U/µL), the PfCyRPA DNA fragment, and ddH_2O. The composition of this reaction mixture included 1 µL of T4 DNA ligase buffer (10×), 1 µL of pGEM-T, 1 µL of T4 DNA ligase, 4.6 µL of PfCyRPA (13.45 ng/µL), and 2.4 µL of ddH_2O for the pGEM-T plasmid. The molar ratio between the pGEM-T vector and the PfCyRPA gene was 1:8. The reaction mixture was incubated for 24 hours at 4°C.

The recombinant plasmid pET-16b-PfCyRPA 26-181 was constructed with a similar composition. This included 1 µL of T4 DNA ligase buffer (10×), 2.25 µL of pET-16b (22.25 ng/µL), 1 µL of T4 DNA ligase, 1 µL of PfCyRPA (13.45 ng/µL), and 4.75 µL of ddH_2O. The molar ratio between the expression vector pET-16b and the PfCyRPA gene was 1:3. The reaction mixture was incubated for 24 hours at 4°C.

Preparation of competent *E. coli* cells using $CaCl_2$ treatment method

E. coli strain TOP10F' was used for cloning purposes, and strain BL21 CodonPlus (DE3) RIPL was used for gene expression. *E. coli* strains were grown in 5 mL of LB liquid medium containing the appropriate antibiotic at 37°C for 16-18 hours with shaking at 150 rpm. The culture was then diluted 1:100 into 20 mL of fresh medium supplemented with the corresponding antibiotic. *E. coli* TOP10F' cultures contained tetracycline (5 µg/mL), while *E. coli* BL21 CodonPlus (DE3) RIPL cultures contained chloramphenicol (50 µg/mL).

The culture was incubated until the optical density at 600 nm (OD_{600}) reached 0.2-0.4. Once the desired cell density was achieved, the inoculum was transferred to a 50 mL falcon tube and incubated on ice for 30 minutes. The cells were then centrifuged at 4°C for 10 minutes at 2,700 × g. The supernatant was discarded, and the pellet was washed with 6 mL of cold 0.1 M $CaCl_2$, followed by a 10-minute incubation on ice and another centrifugation at 4°C for 10 minutes at 2,700 × g. The pellet was resuspended in 800 µL of cold 0.1 M $CaCl_2$ and incubated for at least 2 hours on ice.

Transformation of *E. coli* with recombinant plasmids

The transformation of *E. coli* was performed using the heat shock method. A total of 5 µL of the recombinant plasmid ligation mixture was added to 100 µL of competent cells under aseptic conditions. As a positive control, the empty vector (circular pGEM or pET-16b) was also included

in a separate ligation mixture. The mixture was incubated on ice for 30 minutes and then subjected to heat shock in a water bath at 42°C for 90 seconds, followed by immediate cooling on ice for 2 minutes.

Subsequently, 900 µL of LB liquid medium was added to the microcentrifuge tube containing the mixture, which was then incubated for 1 hour at 37°C with shaking at 150 rpm. The transformed cells were centrifuged at 12,000 rpm for 2 minutes, and 900 µL of the supernatant was discarded. The remaining sample was resuspended and spread onto LB agar plates containing ampicillin (100 µg/mL). For samples containing the recombinant plasmid pGEM-T-PfCyRPA 26-181, 20 µL of 1 M IPTG and 20 µL of 50 mg/mL X-gal were added to the LB agar plates. The LB agar plates were incubated for 16-18 hours at 37°C.

Screening and confirmation of recombinant clones

The screening of the resultant colonies was carried out using blue and white colony selection for *E. coli* TOP10F' containing pGEM-T-PfCyRPA 26-181. *E. coli* TOP10F' harboring pGEM-T-PfCyRPA 26-181 appeared as white colonies. Moreover, direct colony PCR using PfCyRPA-F and PfCyRPA-R primers was also performed to confirm the presence of the PfCyRPA 26-181 gene in both *E. coli* TOP10F' containing pGEM-T-PfCyRPA 26-181 and *E. coli* BL21 CodonPlus (DE3) RIPL containing pET-16b-PfCyRPA 26-181.

The method for colony PCR was similar to that previously described, but the template was a bacterial colony instead of genomic DNA. From the positive transformants, the recombinant plasmids were isolated using the Presto™ Mini Plasmid Kit (Geneaid) and were sequenced by Macrogen Inc. (Korea).

Recombinant PfCyRPA 26-181 expression

Expression of rPfCyRPA 26-181 was initiated by preparing an overnight culture of a positive colony. This culture was then inoculated (1% v/v) into LB broth containing ampicillin (100 µg/mL) and incubated at 37°C with shaking at 150 rpm for approximately 2 hours until OD_{600} reached 0.6. The bacterial culture was induced by adding IPTG to a final concentration of 0.5 mM and further incubated at 37°C with shaking at 150 rpm for 3 hours.

Centrifugation at 2,800 × g at 4°C for 10 minutes was performed to harvest the culture. The cell pellet was resuspended in lysis buffer (100 mM Tris-HCl pH 7.5, 50 mM NaCl, protease inhibitors) and sonicated for 30 minutes with an amplitude of 40%, using cycles of 30 seconds on and 30 seconds off (Sonics VCX 130 Vibra-Cell Ultrasonic Liquid Processor). To separate soluble fractions from inclusion bodies, the lysates were centrifuged at 16,000 × g for 10 minutes at 4°C.

Analysis of protein expression by SDS-PAGE and Western Blot

Protein expression was assessed via SDS-PAGE and Western blot analysis. Loading dye containing SDS (2% w/v), glycerol (10% v/v), bromophenol blue (0.1% w/v), and β-mercaptoethanol (5% v/v) was added to samples of the soluble fraction and inclusion bodies. The samples were denatured by boiling for 5 minutes, and SDS-PAGE was conducted as described by Schägger [21].

For Western blot analysis, proteins were transferred onto a nitrocellulose membrane using the wet blotting method. The membrane was then blocked with 5% (w/v) skim milk in TBST overnight at room temperature. To remove excess blocking solution, the membrane was washed three times with TBST. The membrane was further incubated with the primary antibody (anti-His-Tag mouse monoclonal antibody, MyBioSource, USA) at a 1:5,000 dilution for a minimum of one hour at room temperature, then washed three times with TBST.

The membrane was then incubated with HRP-conjugated goat anti-mouse IgG secondary antibody (Promega) at a 1:10,000 dilution for at least one hour at room temperature, followed by three washes with TBST. Finally, to visualize the interaction between protein and antibody, 5 mL

of ChromoSensor™ One solution TMB substrate was added, and the membrane was shaken at low speed at room temperature until color developed.

Results

Construction of plasmid recombinant pET-16b-PfCyRPA 26-181

The PfCyRPA gene fragment was successfully amplified using optimized PCR conditions. An annealing temperature of 54.5°C was selected based on prior optimization studies, resulting in a clear amplicon with an approximate size of 480 bp as expected (Figure 1). The amplified 480 bp PfCyRPA 26-181 gene was first cloned into the pGEM-T Easy cloning vector, resulting in pGEM-T-PfCyRPA 26-181. Plasmid DNA was isolated and verified by sequencing. Sequence comparison with other available PfCyRPA sequences in GenBank confirmed its identity (100%).

The pET-16b plasmid was chosen as the expression vector due to its key features, including a T7 promoter, N-terminal His-tag for protein detection, lac operator, factor Xa cleavage site, and a ribosome-binding site. Prior to insertion of the PfCyRPA 26-181 gene, the plasmid was linearized with restriction enzymes BamHI and XhoI. The purified linear vector was subsequently ligated with the PfCyRPA 26-181 gene fragment isolated from the recombinant plasmid pGEM-T-PfCyRPA 26-181. The expression vector pET-16b-PfCyRPA 26-181 is depicted in Figure 2A using SnapGene 7.2.0 [22].

Restriction enzyme digestion with *XhoI* and *BamHI* further validated the plasmid construct integrity. The restriction analysis revealed expected fragments corresponding to the linearized plasmid (~5,700 bp) and the PfCyRPA 26-181 gene insert (~480 bp) (Figure 2B). The presence of additional bands suggested incomplete digestion or partial star activity of both enzymes, which can lead to non-specific cleavage under certain conditions, such as high enzyme concentrations and low ionic strength buffers [23]. This issue could be resolved by using high-fidelity enzymes that are less prone to star activity for critical applications.

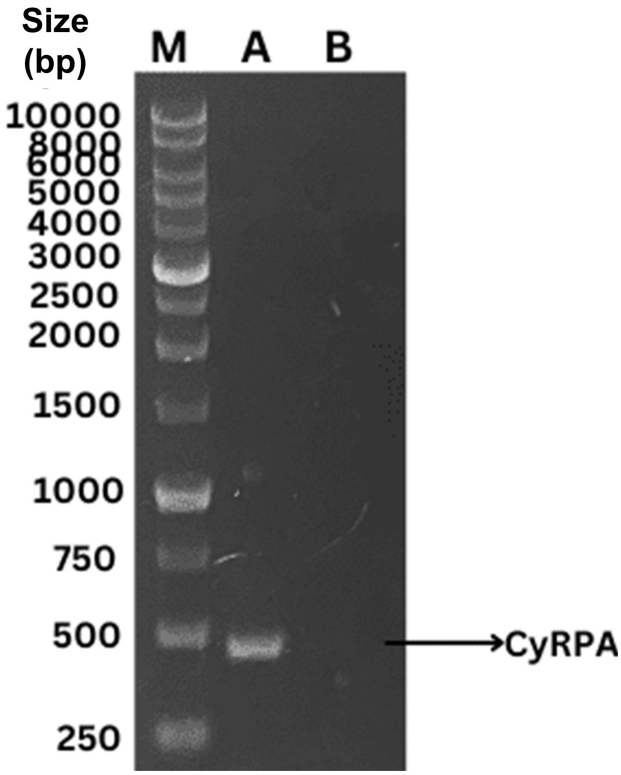

Figure 1. PCR amplification of PfCyRPA fragment 26-181. Amplification of PfCyRPA fragment 26-181 (480 bp) is shown in lane A, and the negative control is shown in lane B.

However, the major bands clearly aligned with the anticipated fragment sizes, reinforcing construct validity.

Sequencing analysis of the recombinant plasmid pET-16b-PfCyRPA 26-181 showed that the construct aligned perfectly with the PfCyRPA reference sequence (3D7 strain), exhibiting 100% identity. Translation of the nucleotide sequence into the corresponding amino acids verified the successful inclusion of the N-terminal His-tag and the integrity of the PfCyRPA 26-181 gene fragment, which is critical for downstream protein detection and purification steps.

Analysis of rare codons in the PfCyRPA fragment 26–181 gene and selection of *E. coli* BL21 CodonPlus (DE3) RIPL

Analysis of the PfCyRPA gene fragment 26–181 revealed the presence of multiple rare codons, totaling 38 occurrences, which were identified manually as detailed in Table 1. Among the rare codons identified, the most frequent was the AUA

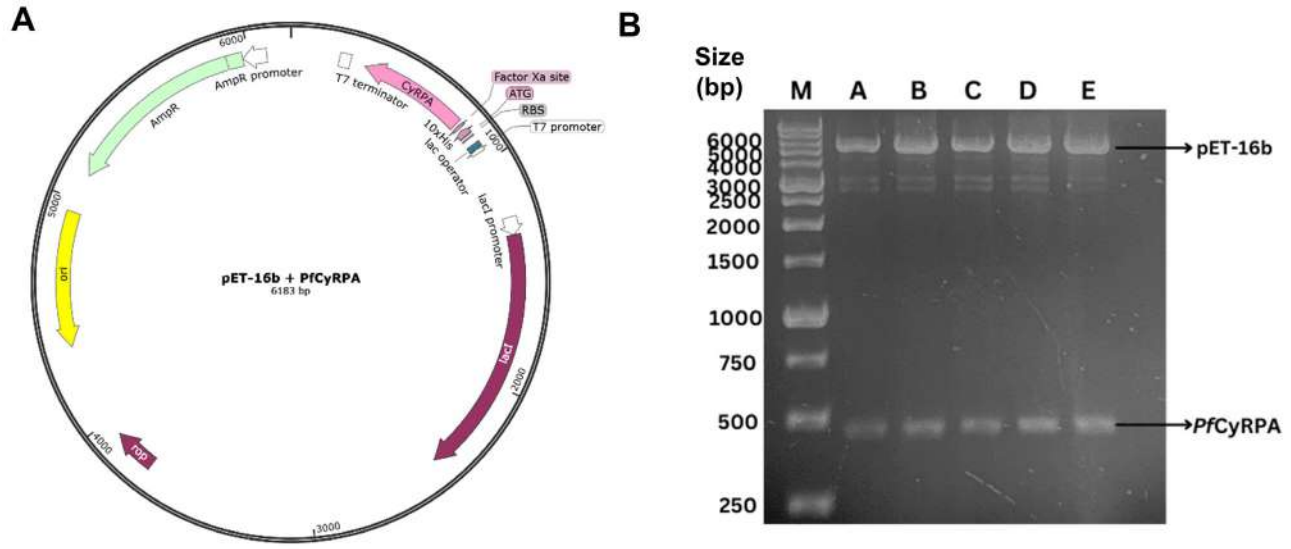

Figure 2. Construction and confirmation of recombinant plasmid pET-16b-PfCyRPA 26-181. (A) Schematic representation of recombinant plasmid pET-16b-PfCyRPA 26-181 construct carrying the PfCyRPA 26-181 gene with a 10× His tag at its N-terminus. The figure was created using SnapGene 7.2.0 [22]. **(B)** Restriction analysis of recombinant plasmid pET-16b-PfCyRPA 26-181 using *Xho*I and *BamH*I, resulting in plasmid pET-16b (approximately 5,700 bp) and PfCyRPA 26-181 fragment (480 bp).

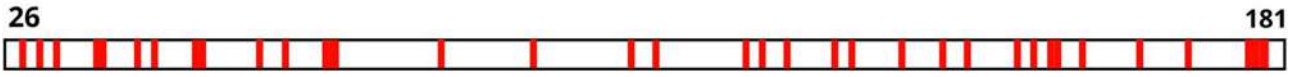

Figure 3. Rare codon distribution analysis of PfCyRPA fragment 26-181. Graphical representation of the rare codon distribution along the PfCyRPA fragment 26-181. The red lines represent rare codons that may affect expression efficiency in *E. coli*.

Rare Codon	Number	Rare Codon	Number
AGG	3	CCU	3
AGA	2	UCA	2
CUA	1	GGA	1
AUA	13	AGU	4
UGU	6	UCG	1
ACA	1	CUC	1

Table 1. Number of rare codons in the PfCyRPA gene Fragment 26–18

codon, observed 13 times, followed by UGU (6 occurrences), AGU (4 occurrences), and AGG and CCU (3 occurrences each). Other rare codons included AGA, UCA, CUA, GGA, UCG, ACA, and CUC, with lower frequencies ranging from one to two occurrences each. The graphical representation of the rare codon distribution along the PfCyRPA fragment is depicted in Figure 3.

The presence of such codons in the target gene significantly affects heterologous protein expression in common laboratory bacterial hosts due to inefficient recognition by the native bacterial translational machinery. To address this challenge, the *E. coli* BL21 CodonPlus (DE3) RIPL strain was selected as the expression host. This strain was specifically engineered to enhance the production of heterologous proteins containing rare codons. *E. coli* BL21 CodonPlus (DE3) RIPL harbors chromosomal copies of genes encoding additional rare tRNAs, including argU (AGA, AGG), ileY (AUA), leuW (CUA), and proL (CCC), which are commonly used in eukaryotic cells but rare in standard *E. coli* strains [24, 25]. Thus, the additional tRNAs within this engineered strain facilitate efficient translation by accurately recognizing the rare codons, thereby minimizing amino acid misincorporation and improving overall translational fidelity.

The *E. coli* BL21 CodonPlus (DE3) RIPL strain contains a chromosomal copy of the T7 RNA polymerase gene controlled by the lacUV5 promoter, enabling tightly regulated expression of proteins under the control of the T7 promoter

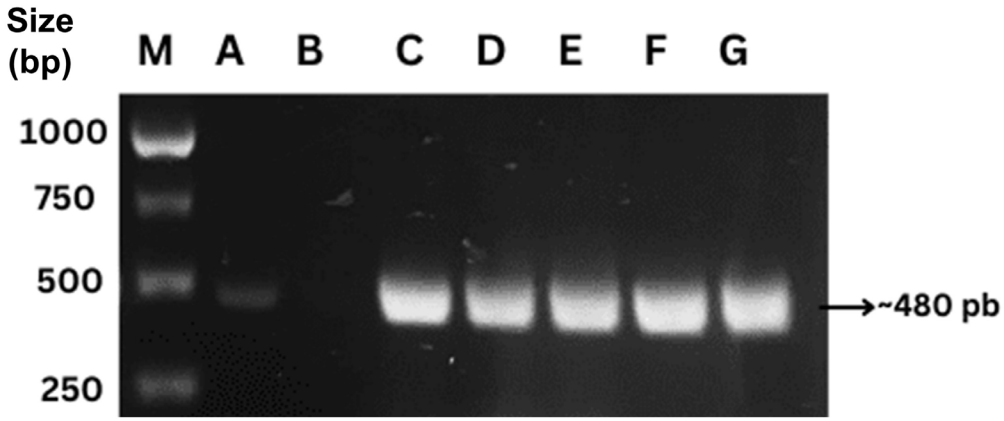

Figure 4. Colony PCR confirmation of transformant *E. coli* BL21 CodonPlus (DE3) RIPL. Colony PCR verification of *E. coli* BL21 CodonPlus (DE3) RIPL transformants carrying pET-16b-PfCyRPA 26-181. Lane assignments: M, DNA ladder (1 kb); A, positive control; B, negative control; C–G, transformant *E. coli* BL21 CodonPlus (DE3) RIPL containing pET-16b-PfCyRPA 26-181.

[26]. In this study, the expression plasmid pET-16b, which utilizes a T7 promoter, was chosen for its compatibility with the host strain. The T7 promoter is a strong, specific promoter recognized by T7 RNA polymerase, making it suitable for high-level gene expression in *E. coli*. The host strain must harbor the T7 RNA polymerase gene, which is integrated into the genome in DE3 strains. When the T7 promoter is activated by IPTG induction, efficient transcription of the target gene occurs, resulting in high protein yields. Therefore, using BL21 CodonPlus (DE3) RIPL was expected to significantly enhance the expression yield of recombinant PfCyRPA protein.

Recombinant PfCyRPA fragment expression in *E. coli* BL21 CodonPlus (DE3) RIPL

E. coli BL21 CodonPlus (DE3) RIPL was selected based on its suitability for protein expression involving codon optimization. This strain provides supplementary tRNAs to accommodate rare codons identified within the PfCyRPA gene fragment. The pET-16b-PfCyRPA 26-181 recombinant plasmid was successfully transformed into this strain, as verified by selective antibiotic resistance (ampicillin and chloramphenicol) and colony PCR (Figure 4).

Induction of PfCyRPA expression using 0.5 mM IPTG at 37°C was successful, with SDS-PAGE analysis clearly revealing prominent protein bands at ~21.53 kDa, matching the predicted molecular weight (Figure 5A). These bands were absent in control samples, clearly attributing their presence to recombinant protein expression from the plasmid construct. The expression experiments were performed independently three times with consistent results, demonstrating the reproducibility of the experiments.

Induced wild-type *E. coli* BL21 CodonPlus (DE3) RIPL was used as a negative control, and recombinant PfLDH containing a His-tag was used as a positive control for Western blot analysis using anti-His-tag antibodies as the primary antibody. The observed ~21.53 kDa band in the sample lanes confirms successful expression of rPfCyRPA 26–181, primarily in the insoluble fraction. No bands were observed in the negative control, validating the specificity of recombinant expression. The detection of bands at the predicted molecular weight confirmed the identity of the recombinant protein rPfCyRPA 26–181 and His-tag incorporation, which is critical for subsequent purification procedures (Figure 5B).

The expressed recombinant PfCyRPA fragment 26–181 predominantly localized as inclusion bodies within the bacterial cells. To reduce inclusion body formation, we attempted to express the PfCyRPA fragment in another host, *E. coli* Arctic Express (DE3), which offers advantages for expressing difficult proteins. This strain constitutively expresses chaperonins cpn10 and cpn60, which facilitate protein folding and refolding at low temperatures (4–12°C) [16]. These chaperones were expected to

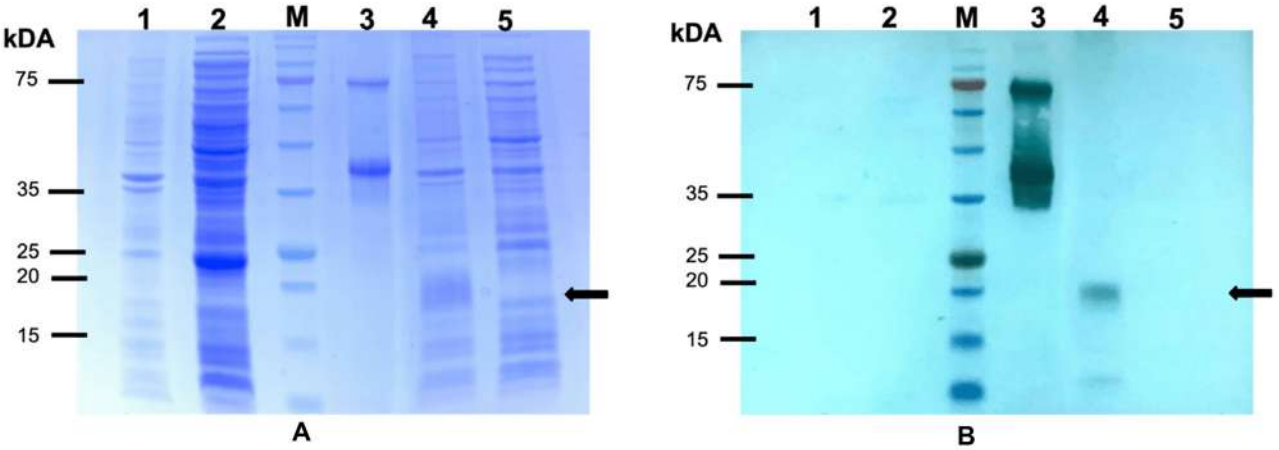

Figure 5. Expression analysis of recombinant PfCyRPA 26-181 by SDS-PAGE and Western Blot. (A) SDS-PAGE analysis and **(B)** Western blot analysis of rPfCyRPA 26-181 expression in *E. coli* BL21 CodonPlus (DE3) RIPL. Lane assignments: Lanes 1 and 2 represent the insoluble (debris) and soluble (supernatant) fractions, respectively, from induced wild-type *E. coli* BL21 CodonPlus (DE3) RIPL (negative control); Lane M contains molecular weight markers; Lane 3 contains recombinant PfLDH with His-tag (positive control for Western blot); Lanes 4 and 5 contain the insoluble (debris) and soluble (supernatant) fractions, respectively, of induced *E. coli* BL21 CodonPlus (DE3) RIPL transformed with pET-16b-PfCyRPA 26-181. The observed ~21.53 kDa band in Lanes 4 and 5 confirms successful expression of rPfCyRPA 26-181, with the protein predominantly found in the insoluble fraction. No specific bands were observed in Lanes 1 and 2, validating the specificity of recombinant expression.

assist nascent PfCyRPA polypeptides in achieving correct conformations and preventing aggregation [27]. Moreover, induction at reduced temperatures slows bacterial metabolism and translation rates, thereby reducing inclusion body formation and improving soluble protein yield [19]. However, the expression of the PfCyRPA fragment in *E. coli* Arctic Express (DE3) was significantly lower than that observed in *E. coli* BL21 CodonPlus (DE3) RIPL (data not shown).

Inclusion body formation likely resulted from the high expression rate, limited capability of *E. coli* to form disulfide bonds, and absence of necessary post-translational modifications. The PfCyRPA fragment 26–181 contains two disulfide bonds, specifically at positions 48↔64 and 119↔133, which are crucial for proper folding and functional activity. In the reductive cytoplasmic environment of *E. coli*, these disulfide bonds are difficult to form, resulting in protein misfolding and aggregation into inclusion bodies [26, 28]. Additionally, PfCyRPA undergoes post-translational modifications, notably N-glycosylation at residues such as N145 within the fragment expressed in this study [7]. The inability of *E. coli* to perform such glycosylation processes further contributes to improper protein folding and accumulation of inclusion bodies.

Potential factors influencing inclusion body formation include rapid protein synthesis outpacing folding kinetics and aggregation of partially folded intermediates. Despite these limitations, the significant expression level achieved in *E. coli* BL21 CodonPlus (DE3) RIPL provides a robust foundation for subsequent protein refolding and purification steps, enabling further investigation into immunogenic properties and vaccine potential.

Discussion

The main finding of this study is the successful expression of recombinant P. falciparum PfCyRPA fragment 26–181 in *E. coli* BL21 CodonPlus (DE3) RIPL with a molecular mass of approximately 21.53 kDa. This corresponds to the expected theoretical size calculated from the amino acid sequence plus the 10× His-tag, without post-translational modifications that are absent in prokaryotic systems. The native parasite protein contains one predicted N-glycosylation site (N145) as a post-translational modification, which would alter the molecular size in the natural context.

The choice of PfCyRPA fragment 26–181 for this study was strategically based on earlier investigations revealing that this fragment contains several

epitopes recognized by anti-PfCyRPA monoclonal antibodies, thus indicating strong potential as a vaccine candidate [8, 14]. Such antigenic epitopes are critical as they can induce protective antibody responses that inhibit erythrocyte invasion by the malaria parasite, a crucial step in the parasite's life cycle that makes this antigen a pivotal candidate for vaccine development [9].

Our results indicated pronounced expression of recombinant PfCyRPA fragment predominantly as inclusion bodies in *E. coli* BL21 CodonPlus (DE3) RIPL. This outcome aligns with typical observations in prokaryotic expression systems, especially when expressing eukaryotic proteins such as PfCyRPA that inherently possess complex tertiary structures reliant on disulfide bond formation and post-translational modifications such as glycosylation [19, 29]. Inclusion body formation in our study could be attributed to the reductive cytoplasmic environment of *E. coli*, where disulfide bond formation is challenging, thus leading to improper folding and aggregation [17].

Furthermore, the PfCyRPA fragment 26–181 includes two critical disulfide bonds, specifically at positions 48↔64 and 119↔133, which are essential for proper antigenic structure and immunological efficacy [7]. *E. coli*'s inherent limitations in supporting disulfide bond formation likely compromised correct protein folding, leading to aggregation. Additionally, this fragment contains predicted N-glycosylation sites, particularly at residue N145, which are crucial for the antigenicity and stability of the protein in its native context within *Plasmodium falciparum*. The absence of N-glycosylation machinery in *E. coli* further contributed to improper protein folding and inclusion body formation.

Similar results were observed in previous studies expressing PfCyRPA fragment 26–352 using *E. coli* BL21 Star (DE3), where the recombinant protein was expressed as inclusion bodies. Soluble recombinant PfCyRPA fragment 26–352 was obtained when HEK293 cells were used as the host [14]. This result was expected since HEK293 cells provide post-translational modification systems for eukaryotic proteins. Other substantial efforts to produce soluble recombinant PfCyRPA were also performed using the insect cell baculovirus expression vector system (IC-BEVS) [30]. Insect cells feature several advantages over mammalian and human cells, including ease of culture and high production yields of antigens with human-like folding and post-translational modifications, achieved in short time frames and at low cost [31,32].

Notably, the high-yield expression of recombinant PfCyRPA fragment 26–181 observed in BL21 CodonPlus (DE3) RIPL underscores the advantage of codon optimization and tailored tRNA availability in facilitating recombinant protein synthesis in prokaryotic systems. Codon optimization is a well-documented strategy to enhance protein expression levels, particularly for eukaryotic proteins that contain rare codons which can severely limit translation efficiency in standard *E. coli* strains [16,24]. BL21 CodonPlus (DE3) RIPL, specifically engineered to harbor additional tRNAs for rare codons, provides superior translational fidelity and efficiency, as confirmed by our findings. This is consistent with previous reports documenting enhanced protein expression using codon-optimized hosts, demonstrating that overcoming translational bottlenecks can significantly impact protein yield and cost-effectiveness of production [25].

Moreover, the use of recombinant protein technology in prokaryotic systems offers substantial advantages in terms of scalability and cost-effectiveness. Compared to eukaryotic systems, *E. coli*-based expression platforms exhibit rapid growth rates, simplicity of culture conditions, and significantly reduced production costs, making them particularly advantageous for large-scale antigen production necessary for widespread vaccine implementation [16,19]. Our successful expression of PfCyRPA fragment 26–181 thus provides an essential foundation for scalable antigen production and supports its application in immunogenicity testing and further preclinical and clinical trials.

Conclusion

This study has successfully demonstrated the recombinant expression of the *P. falciparum* Cysteine-Rich Protective Antigen (PfCyRPA) fragment 26–181 in *E. coli* strain BL21 CodonPlus (DE3) RIPL. The results underscore the critical role of host strain selection, codon optimization, and controlled expression conditions in achieving high-yield recombinant protein production. Specifically, expression in BL21 CodonPlus (DE3) RIPL resulted predominantly in inclusion bodies, confirming the strain's efficiency in high-level protein synthesis due to enhanced translation capabilities for rare codons.

The predominant formation of inclusion bodies highlights challenges related to the cytoplasmic reductive environment in *E. coli*, where essential disulfide bonds and post-translational modifications such as N-glycosylation are absent, thereby affecting proper protein folding. Despite this limitation, the high yield and robust detection of recombinant PfCyRPA through Western blot and SDS-PAGE analyses validate its successful production and confirm the protein's identity through His-tag recognition.

Importantly, this research contributes significantly to the field of malaria vaccine development by providing a foundational protocol for cost-effective, scalable antigen production. Utilizing bacterial expression systems such as *E. coli* offers substantial economic advantages due to rapid growth, simple culture conditions, and reduced production costs compared to eukaryotic systems. Such a scalable and affordable platform is essential for large-scale vaccine production, particularly in resource-limited, malaria-endemic regions where vaccine accessibility is crucial.

Future research should focus on optimizing protein refolding and purification methods to recover functional and immunologically active recombinant PfCyRPA from inclusion bodies. Detailed immunogenicity assessments and structural validation studies are essential to further evaluate the antigen's protective efficacy and its suitability as a blood-stage malaria vaccine candidate. Additionally, exploring alternative expression systems, such as eukaryotic hosts or periplasmic expression in bacteria, could overcome current folding and post-translational modification limitations. Through these concerted efforts, recombinant PfCyRPA holds significant promise as a viable candidate in the global strategy to control and eventually eliminate malaria.

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Declaration of interest

The authors declare that none of them has any conflict of interest with any private, public or academic party related to the information contained in this manuscript.

Author contributions

FFM: Conceptualization, Methodology, Writing – Original Draft. AGP, FFM: Data Curation, Formal Analysis, Visualization. AGP, FP, IH: Investigation, Resources, Validation. FFM, AGP, FP, IH, DN: Supervision, Writing – Review & Editing.

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