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Expression and Purification of Recombinant Envelope (rE) Protein of Dengue Virus in *Escherichia coli* BL21(DE3) with Computational Analysis

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ABSTRACT

Dengue hemorrhagic fever (DHF), caused by the dengue virus (DENV), has become an endemic issue in tropical and subtropical regions, making it a significant global health challenge. One molecular biology approach to DHF prevention is through vaccination. This study aims to evaluate the heterologous expression of the recombinant E (*rE*) protein of DENV using the *Escherichia coli* expression system with the pET-15b vector. The DENV envelope protein (E) is a crucial target for vaccine development due to its ability to elicit neutralizing antibodies (NAbs). This study optimized the gene sequence encoding the rE protein, followed by *in silico* testing of protein characteristics and structure modeling. The results showed that the rE protein had an instability index, aliphatic index, and isoelectric point (pI) of 32.14, 75.08, and 7.17, respectively. Ramachandran plot analysis revealed that 95.4% of amino acid residues were in allowed regions, with 4.7% in disallowed regions, indicating accurate protein modeling for rE. The *in silico* analysis demonstrated that the rE protein had a stable and high-quality structure. The *rE* gene was subsequently inserted into the pET-15b vector and expressed in the *E. coli* BL21(DE3) host system. Positive *E. coli* colonies carrying the *rE* gene were induced with 1 mM IPTG, and SDS-PAGE analyzed the expression. The rE protein was purified using a Ni-NTA column and further analyzed by SDS-PAGE and western blotting. The results confirmed the successful insertion of the recombinant pET-15b-rE plasmid into *E. coli* BL21(DE3), with the rE protein, approximately 50.68 kDa in size, successfully expressed, as shown by the SDS-PAGE analysis with a band in the 50-60 kDa range. In conclusion, this study successfully achieved the expression and purification of the recombinant DENV envelope (rE) protein in *E. coli* BL21(DE3).

Keywords: dengue virus, DENV envelope, dengue vaccine, *Escherichia coli* BL21(DE3)

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INTRODUCTION

The dengue virus (DENV) belongs to the Flavivirus genus within the Flaviviridae family and is the primary cause of dengue hemorrhagic fever (DHF). DENV infections are primarily transmitted by female mosquitoes of the species *Aedes aegypti* and, to a lesser extent, *Aedes albopictus* (Medeiros et al., 2018; Murugesan & Manoharan, 2019; Sabir et al., 2021). Between 1960 and 2010, global DENV infections experienced a remarkable 30-fold increase. In Indonesia, DENV infections have surged 700-fold over the past 45 years. Approximately 400 million cases are reported annually, with specific regions facing a mortality rate of 5-20%. The rise in DENV infections can be attributed to various factors, including population growth, uncontrolled urbanization, global warming, inadequate healthcare facilities, and the emergence of insecticide-resistant mosquitoes (Hasan et al., 2016; Utama et al., 2019). DENV infections have been documented in over 100 countries, with tropical and subtropical nations being endemic areas. This widespread occurrence has led the World Health Organization (WHO) to recognize DENV infections as a global health challenge (Sabir et al., 2021). Furthermore, Indonesia's environmental and climatic conditions favor the breeding of *Ae. aegypti* mosquitoes, making almost all regions in the country endemic to DHF, necessitating urgent and effective control measures (Mustafa et al., 2015).

The DENV genome comprises approximately 11,000 nucleotides and consists of a single-stranded positive-sense RNA (+ssRNA). This genome contains a solitary open reading frame (ORF) that encodes a polyprotein, which is further divided into three structural proteins—capsid (C), precursor membrane (prM), and envelope (E)—as well as seven non-structural proteins, NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5 (Diamond & Pierson, 2015; Murugesan & Manoharan, 2019). The E protein is the viral membrane fusion protein and comprises three elongated domains (EDI–III) that play a crucial role in the virus replication cycle. These domains bind to target cells, mediate membrane fusion, and facilitate the assembly of new virions. Consequently, the E protein can activate the host cell's immune response, aiding in the defense against the virus (Diamond & Pierson, 2015; Fahimi et al., 2016). Currently, there are no dedicated antiviral treatments for DENV infections, leading vector control to emerge as the primary strategy for prevention. However, the effectiveness of vector control methods is suboptimal. Developing a safe, effective, and affordable vaccine presents a promising approach to preventing DENV transmission and serving as a control measure in regions endemic to DHF. Dengvaxia® is a tetravalent vaccine that comprises live-attenuated strains containing *prM* and *E* genes from four DENV serotypes, along with non-structural genes from the yellow fever vaccine (chimeric yellow fever dengue - CYD) strain 17D (Thomas & Yoon, 2019). Endemic countries use Dengvaxia®, targeting individuals aged 9-16 years (Halstead, 2016). However, this vaccine is only suitable for seropositive individuals (Imai & Ferguson, 2018; Wibowo et al., 2020). Despite its availability, Dengvaxia® exhibits limited efficacy in preventing DENV transmission, particularly against DENV-1 and DENV-2 (Halstead, 2016). Developing a vaccine against DHF presents various challenges; an ideal vaccine should be tetravalent, offering lifelong protection while concurrently inducing protective immune responses against all four DENV serotypes (Ghosh & Dar, 2015). As a live-attenuated vaccine, Dengvaxia® carries the risk of potential secondary mutations that may revert to virulent properties and cause disease (Yadav et al., 2013).

In recent years, recombinant protein technology has emerged as a promising and viable option for vaccine development. Recombinant protein-based vaccines are considered safer because they cannot replicate independently and do not contain infectious components present in viral particles. Additionally, their cost-effectiveness is noteworthy, given their capacity for large-scale production using commonly available expression systems, such as bacteria or other host systems. Furthermore, the mild side effects associated with vaccination enhance their appeal. Consequently, growing interest is in further exploring recombinant protein vaccine technology (Pollet et al., 2021). In a notable study, Fahimi et al. (2016) successfully engineered a multivalent recombinant protein derived from domain III of the E protein in DENV. Sequences encoding the EDIIIF protein were synthesized using *E. coli* codons, and *in silico* analysis demonstrated that the EDIIIF protein exhibited structural stability, making it a potential vaccine candidate. The protein was expressed using a secretion system in *E. coli* to elicit an immune response in humans. To date, no research has investigated the expression of recombinant envelope (*rE*) genes in *E. coli* using the pET-15b vector. Consequently, the current study aims to assess the successful expression of the rE

protein from DENV using the *E. coli* expression system and the pET-15b vector.

MATERIALS AND METHODS

Sequence Selection and Codon Optimization

The sequence of the recombinant domain III of the DENV E (rE) protein, as conducted by Fahimi et al. (2016), was retrieved using the GenBank accession number JN985899.1. Adjustments were made, including deleting the 6× C-terminal His-tag and codon optimization (Newark, USA). Codon optimization was performed primarily to enhance the gene or protein expression in a heterologous prokaryotic host system, specifically *E. coli* BL21(DE3).

Recombinant Protein Bioinformatics Analysis

The online ProtParam tool (https://www.expasy.org/resources/protparam) was utilized to determine the essential physicochemical characteristics of the recombinant domain III of the DENV E protein. I-TASSER (https://zhanggroup.org/I-TASSER/) was employed to predict the stability of the tertiary structures of the model based on the distribution of amino acids observed in the Ramachandran plot. The online PDBsum server (http://www.ebi.ac.uk/thornton-srv/databases/pdbsum/) generated the Ramachandran plot.

Recombinant Protein Computational Immunology Analysis

To predict B-cell epitopes, web-based algorithm servers were employed, including SVMTriP (http://sysbio.unl.edu/SVMTriP/) and the Immunomedicine Group (http://imed.med.ucm.es/Tools/antigenic.pl).
The allergenicity of the recombinant protein was predicted using AllergenFp v.1.0 (https://ddg-pharmfac.net/AllergenFP/). The immunogenicity of the tetravalent fusion protein was assessed using the VaxiJen v.2.0 web server (http://www.ddg-pharmfac.net/vaxijen/VaxiJen/VaxiJen.html).

Gene Cloning

The recombinant plasmids were transformed into competent *E. coli* BL21(DE3) cells subjected to heat shock treatment at 42°C for 45 seconds (Chan et al., 2013). The cells were then inoculated on LB medium containing ampicillin (50 µg/mL) and incubated at 37ºC for 16 hours. Colony PCR was employed to confirm the presence of the inserted genes within the colonies, followed by plasmid isolation using the FavorPrep™ Tissue Genomic DNA Extraction Mini Kit (FAVORGEN). The *EcoRI* restriction enzyme was utilized to digest the plasmids, and both whole and digested plasmids were analyzed by electrophoresis in 1.0% agarose gel.

Expression of Recombinant E Protein by *E. coli* BL21(DE3) Cells

The recombinant *E. coli* BL21(DE3) cells were cultivated in LB broth supplemented with ampicillin (25 µg/mL) at 37°C with agitation at 150 rpm. Upon reaching an optical density of OD600: 0.5–0.6, cell induction was initiated by adding 1.0 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) and incubating at 37°C for 4 hours. The cell was harvested by centrifugation at 5,000 rpm and 4ºC for 10 minutes. The cell pellets were resuspended in phosphate-buffered saline (PBS) at pH 7 and subjected to sonication in an ice bath. The supernatants and cell pellets were separated via centrifugation at 5,000 rpm and 4ºC for 10 minutes. Variations were introduced in induction duration (4 and 16 hours) and IPTG concentration (0.5 and 1.0 mM) to optimize protein production. The optimization of induction duration was conducted using 1.0 mM IPTG, while the IPTG concentration was optimized with a 4-hour induction duration. To ascertain the molecular weight and solubility of the recombinant E protein, the supernatant protein and the cell pellet were separated through SDS-PAGE, utilizing a stacking gel of 5% and a resolving gel of 12%. The PageRuler Prestained Protein Ladder (Thermo Scientific) served as the protein marker, and Coomassie Brilliant Blue R250 (Sigma) was used as the staining solution to visualize protein bands.

Purification of Recombinant E Protein

The protein purification process employed the His-tag Protein Purification Kit (Ni-NTA) (Abbkine). Buffer lysis, wash, and elution solutions were supplemented with imidazole at 10 mM, 25 mM, and 250 mM concentrations. The cell pellets were resuspended in binding/washing buffer and centrifuged at 2,000 rpm for 2 minutes. The Ni-NTA magnetic silica resin was equilibrated with 1 mL of PBS, discarding the flow-through. The flow-through (unbound fraction, UF) was collected after introducing the protein sample into the column. Then, 12 mL of wash buffer was added to the column, and the resultant flow-through was collected (W1). 500 µL of elution buffer was introduced into the column to attain the purified

protein, repeated twice. The eluents were collected separately as E1 and E2.

SDS-PAGE Followed by Western Blotting

The recombinant domain III of the DENV E protein (rE) was visualized through a sequential process involving SDS-PAGE followed by western blotting. Initially, all samples underwent denaturation at 95°C for 5 minutes. The denatured samples were then loaded onto a 5% stacking gel and separated on a 12% resolving SDS-PAGE gel. The SDS-PAGE gel was allowed to run at 80 volts for 90 minutes. Following electrophoresis, the SDS-PAGE gel was transferred onto a polyvinylidene difluoride (PVDF) membrane to detect the recombinant protein.

For the western blotting preparation, the PVDF membrane was initially blocked with a solution containing bovine serum albumin (BSA) dissolved in TBS-Tween. Specifically, 0.4 grams of BSA were dissolved in 40 mL of TBS-Tween for this blocking solution. The PVDF membrane was incubated on a shaker at room temperature for 1 hour. After three washing procedures with a solution of 0.05% Tween in TBS, the membrane was probed with a mouse monoclonal antibody against the His-tag (ABclonal) diluted to 1:2,000. This incubation process occurred at 4°C for 48 hours. Following another round of triple washing with the washing solution, the PVDF membrane was incubated with a secondary antibody, specifically Alkaline Phosphatase (AP)-conjugated goat anti-mouse IgG antibody (ABclonal) diluted to 1:5,000. The membrane was then incubated at room temperature for 1 hour with agitation. After a two-time wash with the washing buffer, the immunological reaction was visualized by applying a BCIP Red/NBT Solution B substrate solution onto the PVDF membrane.

RESULTS AND DISCUSSION

Codon Optimization

The genetic template of the rE gene from the fourth serotype of DENV (GenBank accession number JN985899.1) was acquired from the National Center for Biotechnology Information (NCBI), encompassing 472 amino acids. Codon optimization is critically important for enhancing GC content, thereby elevating the stability of the RNA. This process not only increases RNA stability but also augments protein production. Furthermore, codon optimization is conducted to mitigate the risks associated with codon bias and the formation of inclusion bodies (Sørensen & Mortensen, 2005, as cited in Angov, 2011). The Codon Adaptation Index (CAI) evaluates a gene's codon bias, offering insights into its anticipated expression level within a specific organism (Puigbò et al., 2008). Upon meticulously evaluating the outcomes derived from codon optimization, a consistent CAI value of approximately 0.95 was noted within the *E. coli* host expression system (supplement Table I). The CAI value operates on a scale from 0 to 1, where a higher CAI value indicates a well-suited adaptation of the gene's codon usage to the host organism, suggesting the potential for substantial levels of expression (Sharp & Li, 1987, as cited in Jansen et al., 2003).

Recombinant Protein Modeling Prediction

The finalized sequence obtained was subsequently subjected to an initial evaluation to predict the designated protein's structural attributes, modeling characteristics, and stability. This process was conducted using the I-TASSER web server. The assessment of the three-dimensional structure of the protein models (Figure 1) can be scrutinized by examining the distribution of C-score values. C-score values range from -5 to 2, with higher values indicating a greater reliance on the precision and quality of the model (Roy et al., 2010). Model 1 exhibits the highest C-score among the three predicted models, quantified at -2.64 (supplement Table II). Further assessments are necessary to evaluate the accuracy of the designated protein. A commonly employed method for assessing a protein's quality and stability involves using Ramachandran plots.

Figure 1. Visualization of tertiary structure modeling of the rE DENV protein.

Upon examining the Ramachandran plot visualization for the rE protein derived from DENV, it is evident that the proportions of amino acid residues allocated to the most favored, additionally allowed, generously allowed, and disallowed regions are 51.9%, 38.0%, 5.5%, and 4.7%, respectively. The arrangement of amino acid

residues in structurally restricted regions plays a crucial role in determining the quality of a protein's structure. Achieving commendable structural integrity is indicated when the fraction of amino acid residues in these restricted regions remains below 15% (Lovell et al., 2003). Furthermore, a model deemed high quality demonstrates a notable feature, with over 90% of its amino acids residing within allowed regions (Arumugam et al., 2020). Additionally, the prevalence of amino acid residues in the most favored regions substantially exceeds those in the disallowed regions (51.9% > 4.7%). Thus, the structural depiction of the rE protein from DENV exemplifies high quality and stability attributes.

Recombinant Protein Modeling Prediction

Another important aspect includes the isoelectric point (pI), instability index, and aliphatic index (supplement Table III). The pI delineates the pH at which a specific molecular entity reaches an electrically neutral state. The anticipated pI value for the rE protein is 7.17, indicating that at this pH, the rE protein has no net electrical charge, as the count of positively charged amino acid residues offsets the number of negatively charged ones. Tokmakov et al. (2021) indicated that a protein's pI value can provide insights into its stability, given that proteins generally exhibit enhanced stability in environments corresponding to their pI values. Ideally, human blood maintains a pH range of 7.34 to 7.45 (Brinkman & Sharma, 2023). With a pI value of 7.17, the rE protein is predicted to remain stable within the physiologically relevant pH range of the human body.

An instability index exceeding 40 indicates a protein's susceptibility to instability (Gamage et al., 2019). The rE protein of DENV demonstrates an instability index of 32.14, confirming its stability. Additionally, the aliphatic index contributes to predicting a protein's thermal stability. Proteins with elevated aliphatic index values exhibit greater thermal stability due to a higher proportion of aliphatic amino acid residues (Nehete et al., 2013). The rE protein of DENV, with an aliphatic index value of 75.08, can be classified as thermally stable. The *in silico* approach (Wibowo et al., 2021, 2022; Widyarti et al., 2023) employed to construct the rE protein of DENV emphasizes its confirmed stability and appropriate physicochemical properties, positioning it as a potential candidate for an efficacious recombinant protein vaccine.

Recombinant Protein Computational Immunology Prediction

Analysis using the AllergenFp v.1.0 web server suggests that the rE protein of DENV is anticipated to lack allergenic properties in humans. Regarding sequence similarity, the rE protein demonstrates an 84% Tanimoto similarity index with the BAH and coiled-coil domain-containing protein 1 (BAHC1) (UniProt accession code Q9P281). A higher index value signifies enhanced structural or chemical resemblance, implying shared characteristics (Seo et al., 2021).

The protein sequence utilized in this investigation has an antigenicity score of 0.5196, exceeding the threshold of 0.5, indicating its potential as an antigen. Doytchinova & Flower (2007) reported that the Vaxijen 2.0 server's protein and compound antigenicity prediction boasts an accuracy rate ranging from 70% to 89%—moreover, the antigenicity evaluation involved using alternative web servers such as SVMTriP. The results of antigenicity prediction reveal ten projected epitopes within the target protein, with the highest antigenicity score (supplement Table IV). These epitope positions are visually represented in Figure 2. A score of 1.000 indicates a substantial probability that the scrutinized sequence contains antigenic epitopes capable of eliciting an immune response. Epitope prediction using the SVMTriP server yields a sensitivity value of 80.1% and a precision rate of 55.2% (Yao et al., 2012).

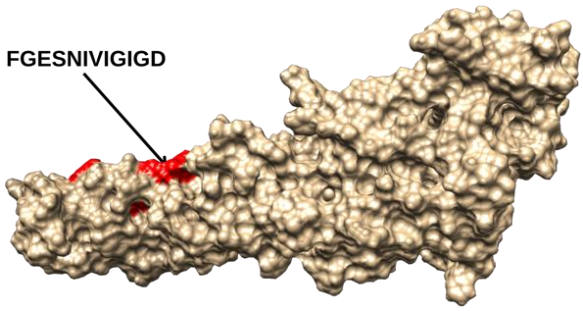

Figure 2. Visualization of epitope prediction in the DENV rE protein

Following establishing the target protein's stability, the distinct *rE* gene sourced from the four DENV serotypes was subsequently integrated into the pET-15b cloning plasmid using the *NdeI*

and *XhoI* restriction enzymes. This process culminated in the creation of the recombinant pET-15b-rE plasmid. The 5708 bp pET-15b cloning plasmid was conjoined with the 1419 bp Gene of Interest (GOI), resulting in a merged product measuring 7127 bp.

Production and Expression of the Recombinant Protein

The transformation procedure utilized *E. coli* BL21(DE3) as the host cells. The transformed cells were subsequently cultivated in an LB agar medium supplemented with ampicillin, serving as the selectable marker. This study's spreading technique involved introducing 40 µL of competent cells onto an agar medium supplemented with ampicillin (Figure 3). Post-transformation of *E. coli* on agar medium supplemented with ampicillin revealed the emergence of only two successfully growing transformant colonies (rE-1 and rE-2). This limited number of transformant colonies can be attributed to several factors. Weber and San (1989) demonstrated the dynamic nature of *E. coli* populations harboring plasmids and highlighted subpopulations with varying abilities to form colonies at different ampicillin concentrations over time. Limited *E. coli* transformant colony growth may not always indicate a negative outcome. In iron-limited environments, *E. coli* exhibits adaptive responses prioritizing overflow metabolism over biomass production, resulting in diminished yields. Furthermore, mutator populations of *E. coli* display a rapid accumulation of mutations, leading to a decline in fitness and the emergence of diverse defects despite exhibiting restricted colony growth (Dunn et al., 2021; Funchain et al., 2000).

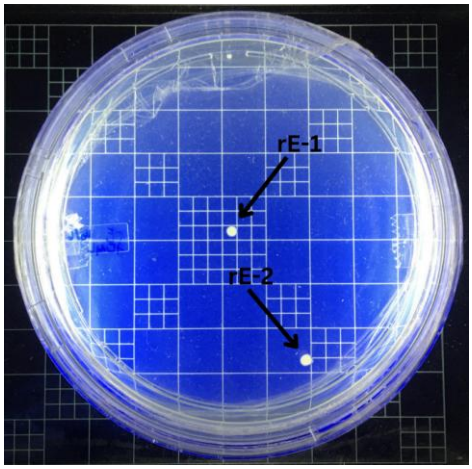

Figure 3. Transformation of *E. coli* BL21(DE3) with the recombinant plasmid pET-15b-rE

PCR assays were conducted on colonies to validate the presence of the recombinant pET-15b-rE plasmid. The primers employed in colony PCR were meticulously designed using the PrimerBLAST (https://www.ncbi.nlm.nih.gov/tools/primer-blast/) utility provided by NCBI (supplement Table V).

The results of the colony PCR were subsequently visualized using the electrophoresis technique with a 1% agarose gel concentration. Non-transformed *E. coli* BL21(DE3) colonies were the negative control, while the positive control included the synthetic *rE* gene. The illuminated bands within the agarose gel confirm the successful integration of the *rE* gene from all four serotypes of DENV into the recombinant plasmid. This assertion is supported by the close alignment of the amplified PCR product, measuring 197 bp, with the 200 bp marker (Figure 4A). Consequently, it can be deduced that the *E. coli* BL21(DE3) transformants have effectively hosted the recombinant pET-15b-rE plasmid. To ensure the accuracy and robustness of the transformants, further validation tests involving plasmid isolation are deemed essential.

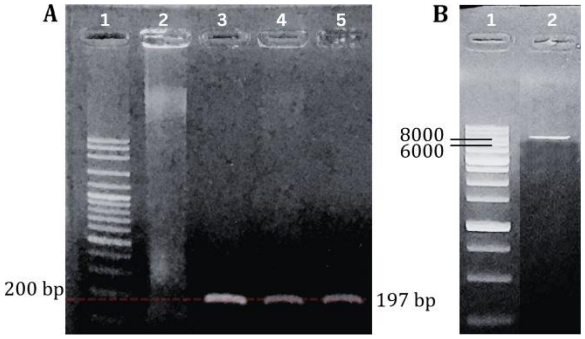

Figure 4A. Visualization of the transformed *E. coli* colonies in 1.0% agarose gel electrophoresis. Lane 1: DNA ladder 1 kb (Geneaid). Lane 2: Non-transformed *E. coli* BL21(DE3) colonies. Lane 3: Synthetic *rE* gene. Lane 4: Sample rE-1. Lane 5: Sample rE-2. Figure 4B. Visualization of the pET-15b-rE recombinant plasmid in 1.0% agarose gel electrophoresis. Lane 1: DNA ladder 1 kb (Geneaid). Lane 2: Sample rE-1.

The plasmid was isolated using the Tissue Genomic DNA Extraction Mini Kit (FAVORGEN). The obtained plasmid maintained its circular configuration, necessitating targeted restriction at the *EcoRI* recognition site, which avoids any disruption of the Gene of Interest (GOI) segment. Notably, the recombinant pET-15b-rE plasmid spans 7127 base pairs (bp) in length. Identifying a

distinct band signal within the range of 6000 bp and 8000 bp on the gel substantiates the effective integration of the DENV *rE* gene into the pET-15b vector (Figure 4B). The observation of band smearing during gel electrophoresis can arise from various factors, including insufficient plasmid DNA concentration, incomplete enzymatic digestion, or sample contamination. However, smeared bands do not definitively signify the absence of the plasmid or the recombinant DNA construct. While band smearing can sometimes indicate DNA degradation or impurities, it can also result from specific methodologies employed for visualization and analysis.

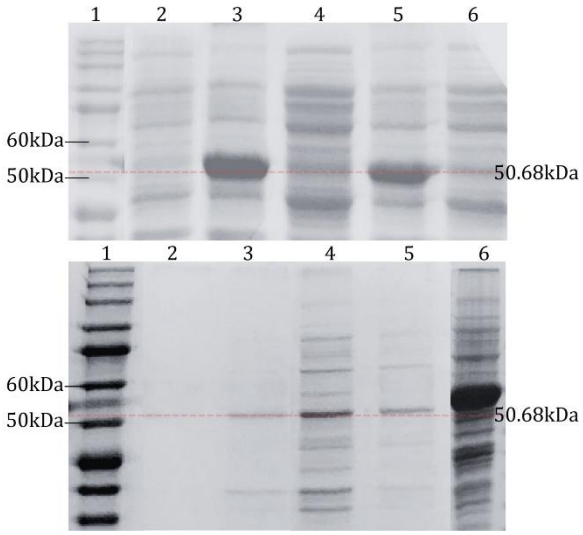

Figure 5A. SDS-PAGE visualization of recombinant E protein production optimization by variation of IPTG concentration. Lane 1: Protein marker. Lane 2: Cell without IPTG induction. Lane 3: IPTG concentration of 0.5 mM, induced for 4 hours, pellet fraction. Lane 4: IPTG concentration of 0.5 mM, induced for 4 hours, supernatant fraction. Lane 5: IPTG concentration of 1.0 mM, induced for 4 hours, pellet fraction. Lane 6: IPTG concentration of 1.0 mM, induced for 4 hours, supernatant fraction. Figure 5B. Recombinant E protein purification using Ni-NTA magnetic silica beads. Lane 1: Protein marker. Lane 2: Elution fraction (I). Lane 3: Elution fraction (II). Lane 4: Unbound fraction. Lane 5: Wash fraction. Lane 6: Crude protein.

The expression of the DENV rE protein is initiated by inducing transformed colonies of *E. coli* BL21(DE3) using IPTG. The visualization of the expression outcomes is achieved using the SDS-PAGE technique. The rE protein fragment exhibited substantial expression levels within BL21(DE3) cells (Figure 5); however, its predominant localization was observed within inclusion bodies, indicated by the solid white pellets obtained upon cell lysis.

Protein purification plays a pivotal role in obtaining a pristine protein product. The protein was purified in this study using the Antibody Purification Kit (Abbkine), employing Ni-NTA chromatography as a foundational technique. The eluted protein product was visualized using the SDS-PAGE method, followed by Western blot immunological analysis.

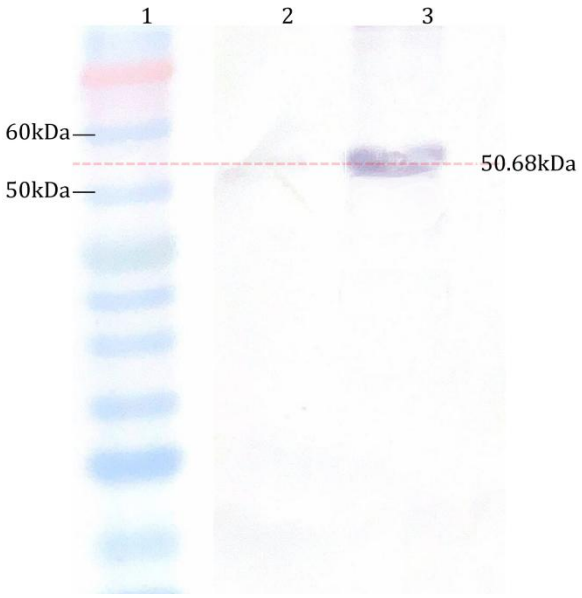

Figure 6. Visualization of the recombinant E protein of DENV by SDS-PAGE and western blotting. The recombinant E protein of DENV showed a single fragment size of 50.68 kDa. Lane 1: Protein marker; Lane 2: Elution fraction (I); Lane 3: Elution fraction (II).

Based on the characteristics of the rE DENV protein produced in the pellet, only samples from the pellet were advanced to the purification phase. The linear bands positioned between the 50 kDa and 60 kDa markers validate the purification of the protein without compromising the target protein (Figure 5B). The effectively refined protein has a molecular weight of 50.68 kDa; however, additional confirmation is imperative to establish the distinctiveness of the purified protein as the target one, achieved through the implementation of the Western blot technique. The results of the western blotting examination revealed the detection of a band with a violet hue corresponding to a molecular weight of 50.68 kDa (Figure 6).

These findings indicate the successful purification of the rE protein.

CONCLUSION

The computational *in silico* modeling assessment revealed that the rE protein has a molecular weight of 50.68 kDa and exhibits stability. Moreover, it does not possess allergenic traits while demonstrating antigenic attributes. The constructed *rE* gene, residing within the pET-15b plasmid, can be seamlessly integrated into the *E. coli* BL21(DE3) expression host system without mutational alterations. The rE protein can be effectively expressed within the *E. coli* BL21(DE3) expression host system, assuming an insoluble state.

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CONFLICT OF INTEREST

The authors declare no potential conflicts of interest regarding this article's research, authorship, or publication.

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