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Cloning and Expression of a Recombinant NS1 Antigen from a Dengue 3 Serotype Viral Isolate in *Escherichia Coli*

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Abstract

Dengue is a dangerous infectious disease affecting more than half of the global population living in areas at risk of dengue. This disease is caused by infection with dengue virus (DENV) through the bite of infected female mosquitos. Detection of the dengue virus infection could be performed by virus isolation, molecular biology methods, or by immunology methods. Non-structural protein 1 (NS1) has been considered as a diagnostic marker for detection of dengue virus infection. In this study, the full length NS1 region from DENV serotype 3 was cloned in vectors to generate the recombinant constructs of pCR::DENV-3 ns1 and pET::DENV-3 ns1. The rNS1 protein was expressed successfully in E. coli BL21(DE3) and confirmed by Western blot. Optimal conditions for expression of rNS1 were established. The highest level of protein expression was achieved at induction conditions of 0.05 mM IPTG inducer, 2% ethanol, and 37 °C for 4 hours. The rNS1 protein was successfully purified by immobilized metal affinity chromatography. Obtained pure rNS1 could be used for further studies in the development of vaccine and diagnostic tools.

Keywords: Dengue virus, *Escherichia coli*, expression, NS1, recombinant

1. Introduction

Dengue is an acute infectious disease that is caused by the Dengue virus through the bite of an infected *Aedes* species (mainly *Ae. aegypti*). Severe dengue is a leading cause of illness and death in some Asian and Latin American countries [1]. More than 50% of the global population lives in areas at risk of dengue. Vietnam is one of the countries suffering the highest burden [2,3]. It was found that COVID-19 was significantly and positively associated with dengue in Indonesia and Vietnam [4]. Therefore, the resurgence of dengue during the COVID-19 pandemic has been raising concern for countries in Asia.

According to Ministry of Health (MOH) statistics on Sep. 2021, there were 49,113 cases of dengue fever in Vietnam, with 18 deaths. Particularly in Hanoi, 1031 cases of dengue fever were recorded, distributed in 27/30 districts and towns. The peak of the epidemic will be reached in October-November every year.

Dengue virus (DENV) is a single-stranded positive-sense RNA virus. The genome of dengue virus consists of approximately 11 kilobases. The dengue viral genome has a single open reading frame encoding a polyprotein. The polyprotein will be post-translationally cleaved into three viral structural proteins (the capsid - C, membrane - M, and envelope - E) and seven non-structural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5) [5]. There are four main different serotypes of DENV based on the differences in their viral structural and nonstructural proteins. For Dengue virus serotypes 3 (DENV-3), five different genotypes have been identified and are geographically distributed [6]. DENV-3 was identified as the most common serotype in Peru based on NS1 antigen and IgM co-detection [7].

Diagnosis of dengue infections is based mainly on serological detection of either antigen (NS1) in acute cases or antibodies (IgM and/or IgG) in both acute and chronic infections. Viral detection and molecular diagnosis (real-time PCR detection, LAMP) are helpful, but they are not feasible in cases of limited resources [8]. More effective and rapid diagnosis will play an important role in the control of dengue, reducing the severe status of patients.

The non-strutural protein (NS1) is a glycoprotein that is synthesized in a monomer, then forms a dimer in the endoplasmic reticulum lumen and a hexamer in the serum. This protein is present in the serum of patients in the early stage of infection [9]. Therefore, NS1 protein has been used as a diagnostic marker of dengue virus infection [10,11]. The NS1 protein has been included in immunological tests such as enzyme-linked immunosorbent assay (ELISA) and lateral flow immunoassay test strip (LFA). Furthermore, NS1 protein has been used as an antigen for the production of specific antibodies. The NS1 protein can be prepared by isolating it from DENV-infected mammalian cell tissue cultures. This approach was

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facing several limitations, such as unsafe, very low yield, and expensive. It is hard to produce antigens on a large scale.

In this study, the full length *ns1* gene of dengue virus serotype 3 (DEN-3) was cloned in cloning vector pCR2.1-TOPO and expression vector pET22b(+). Recombinant NS1 (rNS1) protein was successfully expressed in *E. coli* and purified. Obtained rNS1 could be used as an antigen for further investigation in the development of vaccines and diagnostic tools.

2. Materials and Methods

2.1. Materials

The forward and reverse primer were designed based on the NS1 encoding gene sequence with an accession number of KF385930.1 and shown as : 5'-CTTGGATCCTATGGGGTGTGTCATAAACT-3' and 5'-TCCGTCGACTAAAGACTTTACCATGTT-CTC-3', respectively. These primers were synthesized by IDT company, USA and used to PCR amplify the coding sequence of the *DENV-3 ns1* gene. Two restriction enzyme sites of *Bam*HI and *Sal*I were incorporated into the primers at the 5' ends, respectively. Dengue RNA was supplied by Dr. Nguyen Thi Thu Thuy, Arbo Laboratory, National Institute of Hygiene and Epidemiology. Vectors pCR2.1-TOPO (Invitrogen, USA) and pET22b(+) (Novagen, USA) were used for cloning and expression of *DENV-3 ns1* gene in *E. coli* BL21(DE3) (Novagen, USA), respectively. Rabbit anti-His tag antibody was purchased from Genscript, USA. Goat anti-rabbit IgG antibody HRP conjugated was purchased from Sigma-Aldrich, USA. All chemicals and reagents used in this study were molecular biology grade.

2.2. Methods

2.2.1. Reverse transcription polymerase chain reaction

The cDNA of the Dengue type 3 NS1 gene was synthesized using SuperScript III Reverse Transcriptase according to the producer's instructions (Invitrogen, USA). Briefly, 5 µl of total RNA extract was added to 20 µl of total reaction mixture containing 0.5 mM dNTPs, 5 µM DTT, 1X RNaseOUT, 1X reaction buffer, and 0.25 µM DENV-3 specific primers. The reaction was performed at 55 ºC for 60 min and then 70 ºC for 15 min.

The cDNA was used as a template for PCR by adding 1 µl to 25 µl of total reaction mixture containing 1X reaction buffer, 0.5 mM dNTPs, 0.5 µM DENV-3 specific primers, and 1 U of Taq DNA polymerase. The reaction was carried out at the following conditions: 94 ºC for 5 min; 35 cycles of 94 ºC for 30 s, 59 ºC for 30 s and 72 ºC for 90 s, and an additional cycle at 72 ºC for 5 min. The product was checked by 0.8% agarose gel electrophoresis.

2.2.2. Cloning of DENV-3 ns1 gene into pCR2.1-TOPO vector

The DENV-3 NS1 gene was cloned into the pCR2.1-TOPO vector according to the producer's instructions (Invitrogen, USA) with slight modification. The reaction mixture of PCR product and TOPO vector was incubated at room temperature for 5 minutes. The ligation product was transformed into competent *E. coli* BL21(DE3). The transformants were selected by spreading the transformed *E. coli* cells on the selective LB agar medium (100 µg/ml Ampicillin), clonal PCR using the DENV-3 specific primers and digestion of plasmid isolated from transformants with *Bam*HI/*Sal*I.

2.2.3. Cloning of DENV-3 NS1 gene into expression vector pET22b(+)

The recombinant construct of pCR*::DENV-3 ns1* was digested with *Bam*HI and *Sal*I. The *DENV-3 ns1* gene fragment was purified and sub-cloned into the expression vector pET22b(+) at *Bam*HI/*Sal*I sites. The recombinant vector was transformed into *E. coli* BL21(DE3). Recombinant colonies were selected by the selective medium (100 µg/ml Ampicillin) and confirmed by PCR, restriction digestion, and sequence analysis.

2.2.4. Expression of DENV-3 NS1 protein in E. coli

Recombinant isolates of *E. coli* BL21(DE3) harboring the pET*::DENV-3 ns1* construct were cultured in LB medium at 37ºC and induced to express recombinant NS1 protein by adding 0.1 mM isopropyl-β-D-thiogalactopyranoside (IPTG). Cells were harvested by centrifugation 4 hours after induction and the pellet was lysed in 1X PBS, pH 7.4 by sonification for 2 min. To collect the soluble protein fraction, the lysate was centrifuged at 8 000 rpm for 20 minutes at 4 ºC; the insoluble proteins were solubilized by sonicating the remaining pellet in lysis buffer (1X PBS pH 7.4, 20 mM -mercaptoethanol, 5 mM DTT, and 0.5% SDS) for 2 minutes. The soluble protein fraction was obtained by centrifugation at 8 000 rpm for 20 min at 4 ºC. The protein extracts were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) [12].

2.2.5. Western blot analysis

To confirm the expression of recombinant DENV-3 NS1 protein in *E. coli*, the protein extracts from recombinant clones harboring the pET::*DENV-3 ns1* construct and the control clone harboring the empty vector pET22b(+) were analyzed by Western blot. Briefly, the protein extracts were separated by 12.5% SDS-PAGE [12] and transferred to the nitrocellulose membrane for 90 min at 35 mA using a Trans-Blot® SD Semi-Dry Electrophoretic Transfer Cell (BioRad, USA). The membrane was ink-stained to get a protein pattern image and then blocked for

1 hour at room temperature with blocking buffer (1X PBS pH 7.4, 3% skim milk). After three washes, the membrane was probed with the primary rabbit anti-His tag antibody for two hours at room temperature. After incubation, the membrane was washed three times, for 5 min each, with 1X-PBS pH 7.4 and 0.05% Tween-20 solution. Then, the membrane was subsequently incubated with goat anti-rabbit IgG conjugated horse radish peroxidase (1/10 000) for 2 hours at room temperature. After three washes, the signals were developed by incubating the membrane with a solution of NBT/BCIP.

2.2.6. Optimization of recombinant protein expression

Expression experiments of the recombinant DENV-3 NS1 protein were carried out at different conditions. After culture for three hours at 37 ºC, recombinant protein expression was induced at different IPTG concentrations of 0.01, 0.05, 0.1, and 0.5 mM. Expression was performed at different temperatures of 10 ºC, 25 ºC, and 37 ºC and at different induction times of 4, 8, 20, and 24 hours.

3. Results and Discussions

3.1. Cloning of DEN-3 ns1 Gene

3.1.1. Cloning DENV-3 ns1 gene into the pCR2.1-TOPO vector

The *DENV-3 ns1* gene was amplified by reverse transcription polymerase chain reaction (RT-PCR) using RNA as a template. The theoretical size of the PCR product will be 1054 bp. The presence of a single DNA band of approximately 1,1 kb on the agarose gel pattern (Fig. 1, lane 1) indicated that the target gene of *DENV-3 ns1* was successfully amplified. Due to using Taq DNA polymerase in the amplification reaction, a single nucleotide Adenine (A) was added to the 3' end of amplified DNA fragments. Therefore, the PCR product was directly ligated to the cloning vector pCR2.1-TOPO and transformed into *E. coli* BL21(DE3).

Thirteen random transformants were checked by colony PCR using *DENV-3 ns1* specific primers. The result showed that four colonies were positive (Fig. 2). Plasmids from four positive colonies (5, 8, 9, 13) and one negative colony (1) were digested with *Bam*HI and *Sal*I restriction enzymes to confirm the presence of the target gene *DENV-3 ns1*. According to the digestion result, a DNA fragment of approximately 1,1 kb was released from the vector (approximately 4 kb) (Fig. 3) in all four positive colonies, whereas no DNA band was presented in the negative colony. The obtained results demonstrated that four positive colonies were recombinant clones carrying the target gene of *DENV-3 ns1*. We could conclude that the recombinant cloning vector was successfully constructed as pCR::*DENV-3 ns1*.

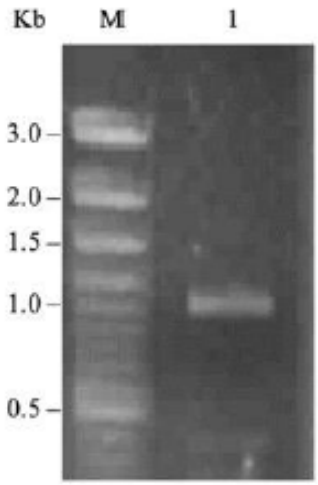

Fig. 1. RT-PCR of *DENV-3 ns1* gene. Lane M, DNA marker; lane 1, PCR product.

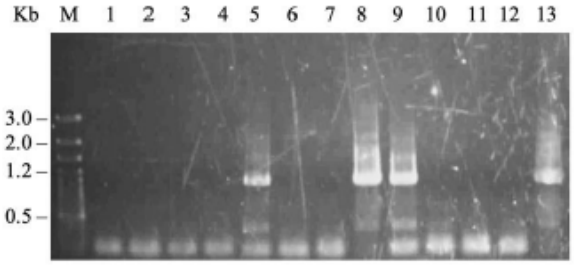

Fig. 2. Colony PCR of transformants. Lane M, DNA marker; lane 1-13, PCR products from 13 randomly selected colonies.

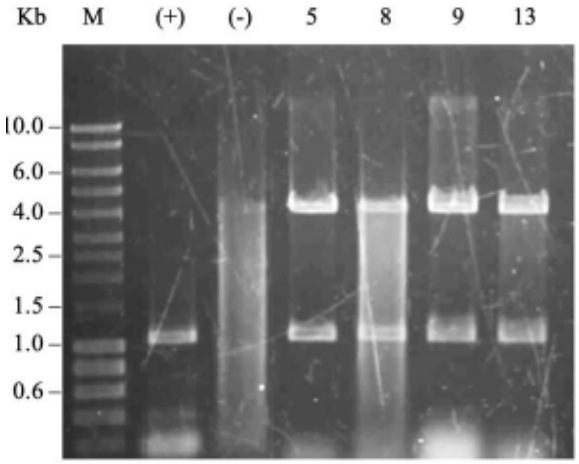

Fig. 3. Confirmation of recombinant pCR::*DENV-3 ns1* by restriction enzyme digestion. Lane M, DNA marker; lane (+), PCR product of target gene; lane (-), restriction enzyme digestion of plasmid isolated from clone 1; lane 5, 8, 9, 13; RE digestion of plasmid isolated from clone 5, 8, 9, 13, respectively.

3.1.2. Cloning DENV-3 ns1 gene into the pET22b(+) vector

To construct the recombinant expression vector, the target gene was purified from the recombinant construct of pCR::*DENV-3 ns1* after double digestion with *Bam*HI and *Sal*I and ligated with the *Bam*HI/*Sal*I-linearized pET22b(+) vector (Fig. 4). The ligation product was transformed into *E. coli* BL21(DE3). Eight random colonies were selected for screening recombinant clones by the colony PCR method using *DEN-3 ns1* specific primers. The result showed that all eight colonies were positive, possibly carrying the *DEN-3 ns1* gene (Fig. 5).

For further examination, the plasmid from clone 1 was double digested with both *Bam*HI and *Sal*I enzymes. The result of restriction enzyme digestion showed the presence of a DNA band of approximately 1,1 kb on the agarose gel electrophoresis pattern (Fig.6, lane 3). This DNA band size was equivalent to the theoretical size of the target gene, implicating the successful insertion of the *DEN-3 ns1* gene into the pET22b(+) vector. The recombinant plasmid was confirmed by gene sequencing using T7 promoter primer for the forward sequencing direction and T7 terminator primer for the reverse sequencing direction. These two sequencing primers were located at the two flanks of the inserted gene, permitting to detect the whole sequence of the insert. The sequencing result showed that the *DEN-3 ns1* gene was exactly incorporated into the pET22b(+) vector at *Bam*HI and *Sal*I sites. An open reading frame was determined from start codon to stop codon with the conjunction of a leading sequence, the *DEN-3 ns1* gene, and a 6x His-tag at the C-terminus (Fig. 7). Thus, we can conclude that the target gene of DENV-3 ns1 was successfully cloned in the expression vector to generate a recombinant construct as pET::*DENV-3 ns1*.

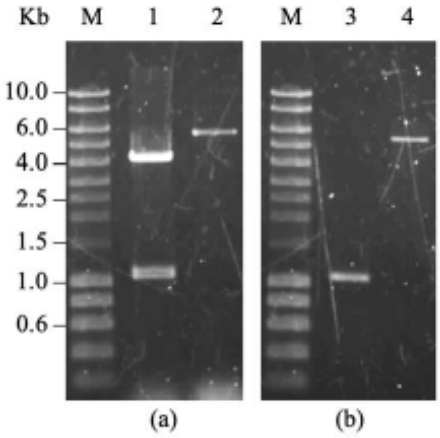

Fig. 4. Preparation of vector and target gene for ligation. Lane M, DNA marker; lane 1 and 2, pCR::*DENV-3 ns1* and pET22b(+), respectively digested with *Bam*HI/*Sal*I; lane 3, the target gene was purified by gel purification from pCR::*DENV-3 ns1/Bam*HI/*Sal*I; lane 4, purified pET22b(+)/*Bam*HI/*Sal*I.

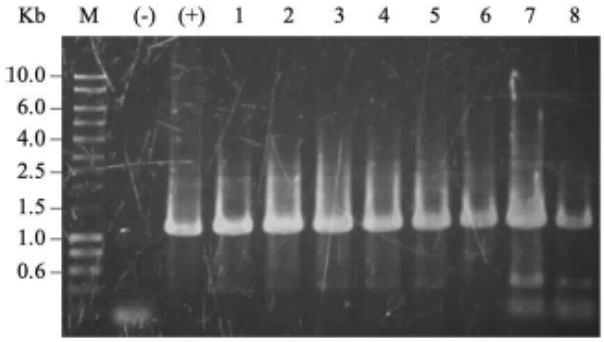

Fig. 5. Colony PCR of transformants. Lane M, DNA marker; lane (-), negative control; (+) PCR product of target gene; lane 1-8, PCR products from 8 randomly selected colonies.

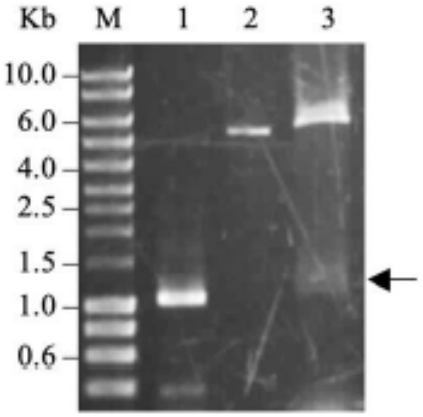

Fig. 6. Confirmation of recombinant pET::*DENV-3 ns1* by restriction enzyme digestion. Lane M, DNA marker; lane 1, PCR product of target gene; lane 2, pET22b(+)/*Bam*HI/*Sal*I; lane 3, pET::*DENV-3 ns1/ Bam*HI/*Sal*I.

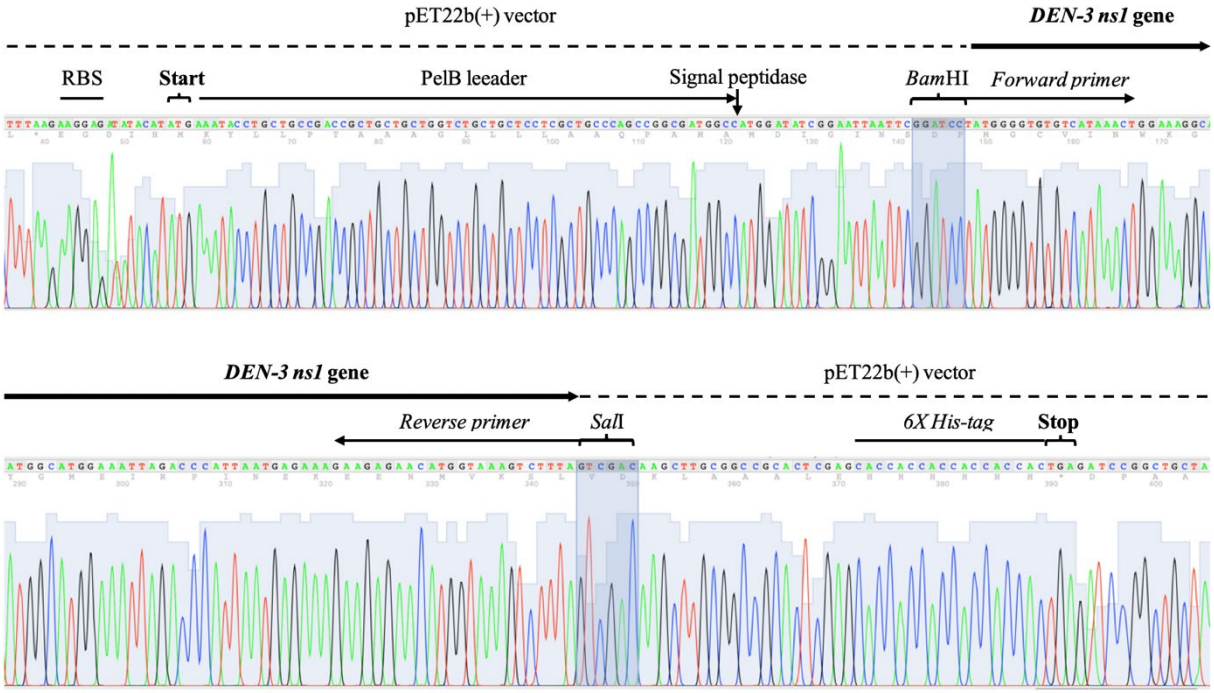

Fig. 7. Partial sequence of the recombinant construct of pET::*DEN-3 ns1*

3.2. Expression of Recombinant DENV-3 NS1 Protein

The *E. coli* BL21(DE3) cells transformed with the recombinant construct pET::*DENV-3 ns1* were grown in LB medium at 37 ºC for 3 hours and then recombinant protein was expressed by induction with IPTG for an extra period of 4 hours. In this construction, recombinant NS1 protein (rNS1) was a fusion protein with a PelB sequence at the N-terminus and a His-tag region at the C-terminus. This contributes an additional 5 kDa to its molecular weight to obtain a full rNS1 protein of approximately 44 kDa. To get the best clones for protein expression, eight positive clones were used in the expression experiments. Expression analysis by SDS-PAGE showed that a significant protein band of approximately 44 kDa appeared in five clones (Fig. 8, lanes 2, 3, 5, 7, 8). The expected protein was not expressed in the negative control sample (E. coli BL21(DE3) transformed with empty vector pET22b(+). This observed protein molecular weight of approximately 44 kDa matched the predicted theoretical protein molecular weight of rNS1. The result implied that rNS1 was successfully expressed in *E. coli*. Assessment of protein expression in the soluble and insoluble cell lysate fractions revealed that a significant amount of proteins was distributed into the insoluble fraction (Fig. 8b).

To confirm expression of rNS1, Western blot analysis was applied using an anti-His tag primary antibody. For two positive clones (5 and 7), the Western blot signal pattern revealed the presence of a significant signal band with an expected size of approximately 44 kDa, whereas no signal band was observed at the control clone (*E. coli* BL21(DE3) strain carrying empty vector pET22b(+)) (Fig. 9). The Western blot pattern also showed some smaller-sized signal bands in clones 5 and 7. This phenomenon has been observed in some previous studies [13,14]. This data confirmed that the rNS1 protein was successfully expressed in *E. coli*.

3.3. Optimization for Expression of Recombinant DENV-3 NS1 Protein

By optimizing multiple parameters, a high expression level of recombinant protein could be achieved. The most commonly used approaches for maximizing recombinant protein expression in *E. coli* include optimizing codon usage bias and screening expression conditions. Other approaches could also be considered, such as designing an optimal promoter, adjusting vector copy number, or increasing mRNA longevity. In this study, the expression level of rNS1 protein was maximized by optimizing inducer concentration and growth conditions.

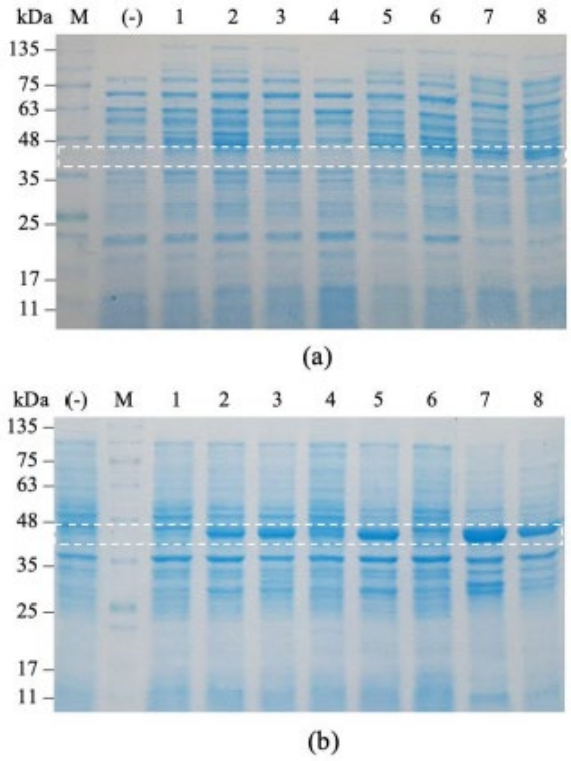

Fig. 8. Expression analysis of the recombinant DEN-3 NS1 (rNS1) protein in different clones by SDS-PAGE. Lane M, protein marker; lane (-), extract from *E. coli* BL21(DE3) carrying empty vector pET22b(+); lane 1-8, extracts from eight *E. coli* BL21(DE3) clones carrying recombinant construct of pET::*DENV-3 ns1*. (a) soluble protein fraction (supernatant) and (b) insoluble protein fraction (pellet). Predicted rNS1 protein was marked in the dotted square.

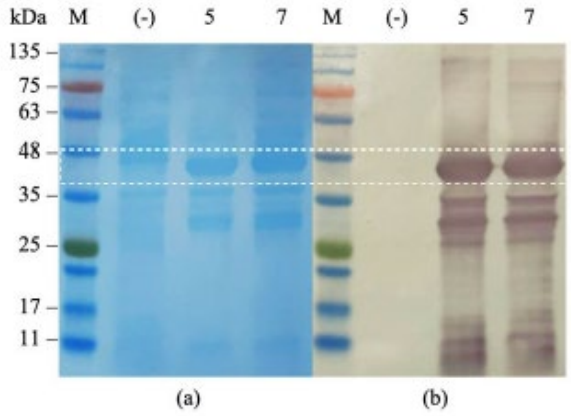

Fig. 9. Western blot analysis of rNS1 expression. (a) Ink stain pattern and (b) Western blot pattern. Lane M, protein marker; lane (-), extract from *E. coli* BL21(DE3) carrying empty vector pET22b(+); lane 5 and 7, extracts from clone 5 and 7 of *E. coli* BL21(DE3), respectively carrying recombinant construct of pET::*DENV-3 ns1*. Predicted rNS1 protein was marked in the dotted square.

3.3.1. IPTG concentration

In this study, the *DEN-3 ns1* gene was inserted into pET22b(+) at *Bam*HI/*Sal*I sites under the control of the T7 promoter. This promoter is recognized only by T7 RNA polymerase, whose gene has been fused into the chromosome of *E. coli* BL21(DE3) and is under the control of the lacUV5 promoter. Therefore, the target gene of *DENV-3 ns1* could be expressed in *E. coli* BL21(DE3) only in the presence of T7 RNA polymerase, which is induced by isopropyl-beta-D-thiogalactopyranoside (IPTG) [15].

There were several studies on the effect of IPTG concentration on the expression level of recombinant protein. The optimal IPTG concentration was different in each study. Therefore, determining the optimum IPTG concentration is an important factor for increasing the level of protein expression. In this study, an IPTG concentration range of 0 - 0.5 mM was investigated. rNS1 protein was not expressed without IPTG induction and was slightly expressed at the concentration of 0.01 mM IPTG. The expression of rNS1 protein was significantly increased at 0.05 mM IPTG and almost similar at a range of 0.05 - 0.5 mM (Fig. 10). The optimal concentration of IPTG chosen for further experiments was 0.05 mM.

3.3.2. Induction temperature and time

Several studies demonstrated that more soluble proteins were produced at lower induction temperatures [16]. However, lowering the induction temperature results in lower expression level. In this study, expression experiments were performed at three different temperatures: 10 ºC, 25 ºC, and 37 ºC. Expression of rNS1 protein was not observed at 10 ºC, but expression levels were increased at 25 ºC and 37ºC. The highest expression level was reached at 37ºC (Fig. 11). In this study, lower temperatures did not yield soluble rNS1 protein. This phenomenon was also observed in previous studies [14].

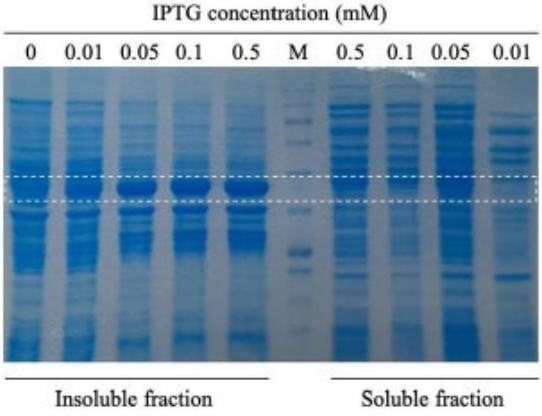

Fig. 10. Expression analysis of rNS1 protein by SDS-PAGE at different IPTG concentrations. Lane M, protein marker; expression was induced by different IPTG concentrations of 0, 0.01, 0.05, 0.1 and 0.5 mM.

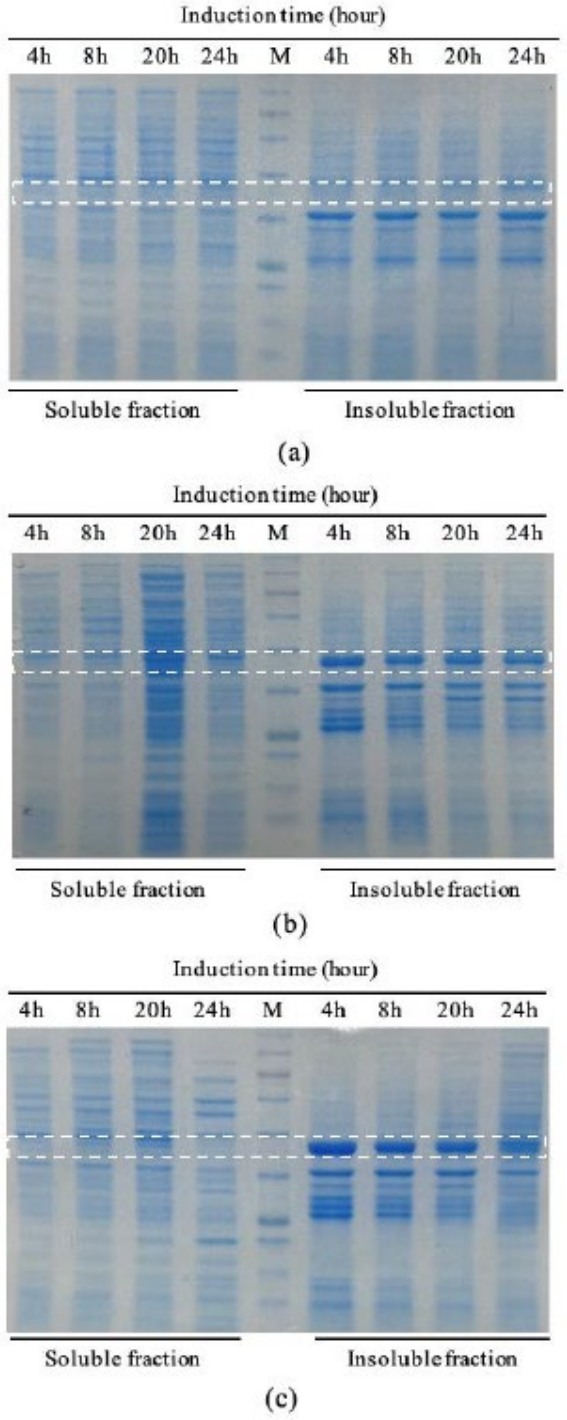

Fig. 11. Expression analysis of rNS1 protein by SDS-PAGE at different induction temperatures and induction time. (a) induction at 10 ºC; (b) induction at 25 ºC and (c) induction at 37 ºC. Lane M, protein marker.

The suitable time of protein expression was determined by evaluating the protein expression profile in an SDS-PAGE pattern. In this study, the expression level was estimated at 4 h, 8 h, 20 h, and 24 h after induction. The expression level of rNS1 protein was highest at 4h post induction and decreased with longer induction times of 8 h, 20 h, and 24 h. A low expression level of recombinant protein at 20 h and 24 h after induction was observed in other studies [14]. Therefore, it is important to control optimum expression time since overgrowth may result in cell death and protein turnover. The expression time of NS1 selected for further studies was four hours.

3.3.3. Effect of ethanol concentration

A previous study provided evidence that the expression of several recombinant proteins was enhanced by adding ethanol to the medium during the induction period [17]. In this study, the effect of ethanol on the growth and expression of rNS1 protein in *E. coli* was investigated. The result showed that the growth of *E. coli* was found to be decreased in the presence of more than 2% ethanol (Fig. 12). However, it was observed that the highest expression level was achieved at 2% ethanol (Fig. 13). A comparison between normal induced protein (0% ethanol) and protein induced in the presence of 2% ethanol indicated that ethanol addition enhanced expression of rNS1 protein. Chhetri *et al*. also demonstrated that 3% ethanol gave the maximum enhancement in protein expression [17].

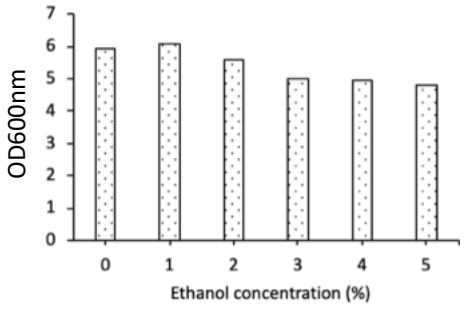

Fig 12. Effect of ethanol concentration on the growth of *E. coli*.

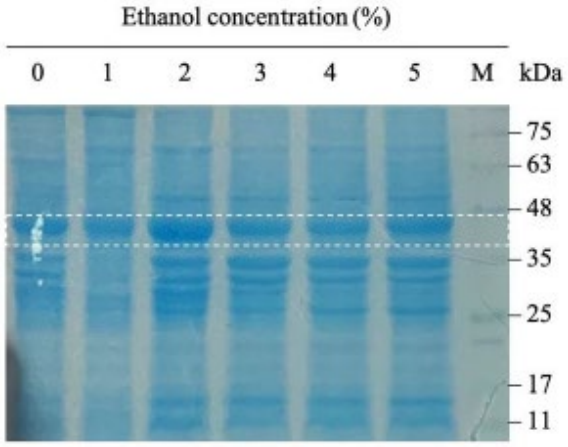

Fig. 13. Expression analysis of rNS1 protein by SDS-PAGE at different ethanol concentrations. Lane M, protein marker; lane 0 - 5, different concentrations of 0, 1, 2, 3, 4, 5%, respectively.

3.3. Purification of Recombinant DENV-3 NS1 Protein

High level expression of recombinant protein in *E. coli* often results in the formation of insoluble inclusion bodies. Inclusion bodies were simply isolated from the supernatant by centrifugation to enhance purification of target protein. In this study, rNS1 protein was also expressed as inclusion bodies. rNS1 was harvested and solubilized in a denaturing buffer containing 20 mM β-mercaptoethanol, 5 mM DTT, and 0.5% SDS. The His-tagged rNS1 protein was purified by immobilized metal ion affinity chromatography. SDS-PAGE analysis showed a single band of the expected mass of approximately 44 kDa (Fig. 14, lane 4 and 5). This data reveals that the rNS1 protein was successfully purified.

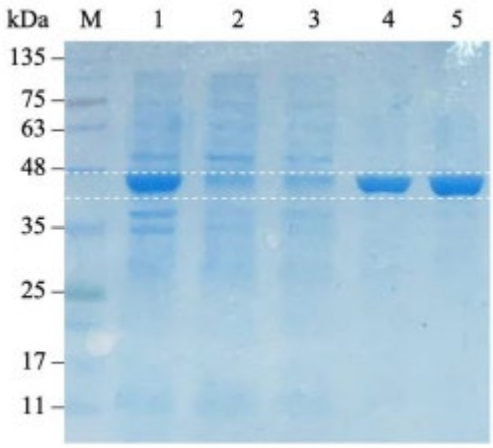

Fig. 14. Purification of rNS1. Lane M, protein marker; lane 1, crude extract; lane 2, flow through (unbound protein fraction); lane 3, wash fraction; lane 4 and 5, eluted protein fractions. Predicted rNS1 protein was marked in the dotted square.

4. Conclusion

Non-structural glycoprotein 1 (NS1) has been shown to be associated with dengue severity and is considered as diagnostic marker for early detection of dengue virus infection. The generation of pure recombinant NS1 protein in this study could provide a valuable material for the development of vaccines and diagnostic tools in future studies.

Acknowledgments

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