Recombinant expression and purification of the NS3 subunit of a Dengue Virus Type 3 strain isolated in Jakarta

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Abstract. A dengue virus serotype 3 strain (DENV-3) was isolated in Jakarta, Indonesia. The NS3 subunit of this strain was selected for recombinant expression to determine its suitability toward later developing a subunit recombinant vaccine for dengue hemorrhagic fever. NS3 is a non-structural protein weighing approximately 72 kDa. It plays a key role in the replication cycle of the dengue virus. The successful cloning of the DENV-3 NS3 coding sequence into a pYES2/CT vector was confirmed, and Saccharomyces cerevisiae cells were transformed with this vector, followed by the expression and purification of the recombinant protein. PCR amplification and DNA sequencing confirmed the presence of the NS3 gene, and SDS-PAGE and western blot analysis produced a specific band of ~72 kDa.

Keywords: DENV-3, NS3 protein, plasmid pYES2/CT, Saccharomyces cerevisiae

1. Introduction

Dengue hemorrhagic fever (DHF) is a contagious disease caused by the dengue virus. This virus is transmitted to humans through the bite of an infected mosquito, particularly the Aedes aegypti and Aedes albopictus species [1]. DHF is characterized by four main clinical manifestations: high fever, pain and stiffness in the joints, hemorrhage, and hepatomegaly [2]. When left untreated, DHF results in blood vessel collapse and eventual blood circulation failure.

The dengue virus belongs to the family Flaviviridae, genus Flavivirus. It is a positive sense, single-stranded RNA (ssRNA+) virus [3]. The dengue virus genome contains a single long open reading frame that encodes two types of proteins: structural (capsid, C; pre-membrane, prM; and envelope, env) and non-structural (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5). There are four serotypes of dengue virus, namely DENV-1, DENV-2, DENV-3 and DENV-4 [4]. In Indonesia, the serotypes DENV-2 and DENV-3 are the most ubiquitous, with DENV-3 causing a majority of severe cases [5].

No specific antiviral treatment is yet available for treating DHF, and patient care involves symptomatic treatment only. Therefore, the development of a vaccine would be beneficial in addressing the problem of DHF [6]. A DENV vaccine could be used as a preventive control that would avert the development of DHF and reduce the transmission of dengue virus. Viral subunit recombinant vaccines are typically highly immunogenic and safe [7, 8]. The Agency for the Assessment and Application of Technology (BPPT), a research institution in Indonesia, is currently developing subunit recombinant
vaccines against DENV using a DENV serotype 3 strain isolated in Indonesia. We are currently investigating the use of NS1, NS3, NS2B-NS3, and prM for developing such a vaccine.

Here, we describe the workflow involved in preparing the NS3 subunit (isolate 141, year 2008, Jakarta) for later incorporating it into a potential DENV-3 vaccine. This workflow comprised the following steps: recombinant clone validation, protein expression, and purification of the recombinant NS3 subunit protein. NS3 has a molecular weight of approximately 72 kDa and functions in the replication of the dengue virus in host cells [9]. The NS3 protein was chosen for incorporation into a subunit recombinant vaccine because it is considered to induce T cell responses, and conserving its sequences may help protect against other serotypes of DNV [10]. NS3 is the main target of CD8+ (cytotoxic) T cells and CD4+ helper T cells and is therefore likely to be highly immunogenic [11].

2. Methodology

2.1. DENV-3 NS3 recombinant clone validation
Clones were validated to ensure correct insertion of the NS3 gene into the vector. Insertion of the NS3 gene into the pYES2/CT vector and its transformation into Escherichia coli was previously performed in 2015 by researchers at the Center for Pharmaceutical and Medical Technology (PTFM), BPPT (unpublished data). The recombinant E. coli strain was streaked onto a Luria–Bertani (LB) agar plate, and the resulting colonies were subsequently picked and cultured in LB broth (both media were supplemented with 100 μg/mL ampicillin). The agar plates were incubated at 37 °C for 16–18 h, whereas the liquid cultures were incubated on a shaker at 37 °C, 150 rpm, for 16–18 h. The pYES2/CT vector was isolated and purified using a GeneJET Plasmid Miniprep Kit. Thermo Scientific GeneJET Plasmid Miniprep Kit utilizes an exclusive silica-based membrane technology in the form of a convenient spin column. The kit recovers up to 20 μg of high copy plasmid DNA per isolation procedure. (Thermo Fisher Scientific, Waltham, MA, USA.). The presence of the NS3 DENV-3 gene was confirmed by amplification with the polymerase chain reaction (PCR), using the 2× Phusion Green Hot Start II High-Fidelity PCR Master Mix (Thermo Fisher Scientific) and forward and reverse sequencing primers specific to the multiple cloning site sequence of the pYES2/CT vector (The primers are commercially available (pYES2/CT [AddGene]). The PCR products were separated using agarose gel electrophoresis and visualized under UV using a gel documentation system. The PCR products were sent for in-house sequencing (Sequencing was done with same institution), and the DNA sequences were analyzed using the Genetyx7 software and BlastN tool available on the NCBI website. The sequence was tested for homology against DENV-3 genomes isolated in the years 1998 and 2008.

2.2. Expression of the recombinant DENV-3 NS3 subunit in Saccharomyces cerevisiae
The NS3-pYES2/CT vector was extracted from E. coli and then was transformed into the host Saccharomyces cerevisiae. Minimal medium and Yeast Extract Peptone Dextrose broth were used for the starter and induction cultures for S. cerevisiae, respectively. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and western blot analysis were used to characterize the expressed NS3 protein. The antibodies used for western blotting were the anti-6× His antibody and anti-NS3 antibody (primary) and the HRP-labeled goat anti-mouse IgG antibody (secondary).

2.3. Purification of the recombinant DENV-3 NS3 subunit from the S. cerevisiae extracts
Im mobilized metal affinity chromatography (IMAC) with HisPur Ni-NTA Superflow Agarose (Thermo Fisher Scientific) was used to purify the recombinant NS3 protein from the crude S. cerevisiae extracts, according to manufacturer’s instructions (with the exception of a range of imidazole concentrations > 250 mM being tested in the elution buffer). The column flow-through, elution fractions, and wash fractions were all retained for analysis using SDS-PAGE.
3. Results and discussion

3.1. Recombinant clone validation
Two clones designated as clones 2 and 11 produced appropriately sized amplicon bands (1946 bp) with PCR (figure 1) [9]. An empty pYES2/CT plasmid was used as the negative control, which produced an approximately 250-bp band corresponding to the multiple cloning site (MCS) of the vector. The isolated plasmids were of high purity (A260/280, 1.8–2.0; table 1).

3.2. Bioinformatic analysis
The results of the BlastN analysis of the DNA sequences of clones 2 and 11 showed high sequence similarity between the clones and the DENV-3 genome (clone 2, 96 %; clone 11, 95 %). The gene in clone 2 was the most similar to the NS3 gene in the DENV-3 strain FW06 (GenBank accession number, AY858041), whereas the gene in clone 11 was most similar to the NS3 gene in the DENV-3 strain KJ71 (GenBank accession number, AY858044). Both the strains were isolated from patients with DHF in Jakarta in 2004 [12]. Sequence comparisons with figure 1 showed that clones 2 and 11 had sequences highly homologous with these published sequences.

3.3. Western blot analysis of the recombinant NS3 subunit
Western blot analysis of the crude extracts of the recombinant NS3 subunit expressed in *S. cerevisiae* (clones 2A, 8B, and 11C) was performed in duplicates (figure 2). NS3 has a molecular weight of 72 kDa [9], and a protein band was apparent on the SDS-PAGE gel in the region of ~70 kDa for each of the clones.

![Figure 1](image-url)  
**Figure 1.** PCR amplification of NS3-recombinant pYES2/CT clones. The NS3 gene was successfully inserted into the multiple cloning site (MCS) of the pYES2/CT vector in clones 2 and 11, as indicated by the single band at ~1946 bp (lanes 1 and 2). The empty vector control (lane 3) only yielded a band corresponding to the MCS itself. M, molecular weight size marker.
Table 1. Concentration and purity of the pYES2/CT DNA plasmid.

| Clone | Concentration (ng/μl) | Purity (A260/A280) |
|-------|-----------------------|--------------------|
| 2     | 772                   | 1.853              |
| 11    | 810                   | 1.859              |

Figure 2. Results of SDS-PAGE Analysis of NS3 DENV-3 subunit recombinant protein on Saccharomyces cerevisiae pYES2/CT

Western blot analysis showed a specific band of the same size when using the anti-6x His antibody for clone 2A (figure 3a) and the anti-NS3 antibody (figure 3b).

3.4. Purification of the recombinant NS3 subunit

The effect of imidazole concentration on purification was tested using clone 2A and a range of imidazole concentrations in the elution buffer (250, 300, 350 and 400 mM), because the recommended concentration in the kit (250 mM) may not always be sufficient to elute the resin-bound protein [13]. The total protein concentrations before and after purification using the elution buffers are shown in table 2. The 250 mM imidazole elution buffer yielded the highest total protein in the elution fraction (660.3 μg/mL). However, SDS-PAGE analysis of the purified fractions showed that the 350 mM imidazole elution buffer yielded the highest concentration of the target protein NS3, as a distinct band was observed in the region of approximately 70 kDa for this fraction (lane 9, figure 4).

Different imidazole concentrations in the elution buffer were tested in duplicates. M, molecular weight size marker.
Figure 3. Western blot analysis of recombinant DNV-3 NS3 subunit expression in *S. cerevisiae*. (a) Analysis of the crude extract with an anti-6× His antibody. The 6× His tag was fused to NS3 in the expression vector, (b) Analysis with the anti-NS3 antibody. M, molecular weight size marker.

Figure 4. SDS-PAGE analysis of the purified fractions of the recombinant DENV-3 NS3 subunit.
Table 2. Effect of imidazole concentration on the total protein concentrations in the purification fractions of recombinant NS3 protein.

| Imidazole concentration (mM) | Fraction | Before elution | After elution |
|-----------------------------|---------|---------------|---------------|
|                             |         | Total protein concentration (mg/mL) | Total protein concentration (μg/mL) |
| 250                         | 1       | 660.3         |               |
|                             | 2       | 0             |               |
| 300                         | 1       | 65.8          | 208.3         |
|                             | 2       |               |               |
| 350                         | 1       | 389.18        | 128.8         |
|                             | 2       | 30            |               |
| 400                         | 1       | 58.6          | 493.4         |
|                             | 2       |               |               |

*The same initial sample was subjected to a series of elution buffers of increasing imidazole concentrations.

4. Conclusion
The NS3 gene was successfully cloned into the pYES2/CT vector. SDS-PAGE and western blot analysis of recombinant NS3 expression in *S. cerevisiae* yielded specific bands of ~72 kDa. IMAC was used to purify the recombinant NS3, and the purification was optimized using a range of imidazole concentrations in the elution buffer. Future studies will investigate the suitability of incorporating the NS3 subunit into a vaccine against the dengue virus.

References
[1] Marbawati D 2006 *Balaba* 3 21-2
[2] World Health Organization 1997 *Dengue Hemorrhagic Fever: Diagnosis, Treatment, Prevention and Control* 2nd edition (Geneva: World Health Organization)
[3] Lodish H et al. 2000 Section 3.5 purifying, detecting and characterizing proteins *Molecular Cell Biology* 4th edition (New York: W H Freeman) available at https://www.ncbi.nlm.nih.gov/books/NBK21589/
[4] Amin H Z and Sungkar S 2013 *eJournal Kedokteran Indonesia* 1 226-33
[5] Siregar F A 2004 *Epidemiologi dan pemberantasan Demam Berdarah Dengue di Indonesia* (Medan: USU Digital Library) available at http://repository.usu.ac.id/bitstream/handle/123456789/3673/fkm-fazidah3.pdf?sequence=1
[6] Wilder-Smith A, Ooi E-E, Vasudevan S G and Gubler D J 2010 *Curr. Infect. Dis. Rep.* 12 157-64
[7] Jin X, Block O T, Rose R and Schlesinger J 2009 *Antivir. Ther.* 14 739-49
[8] Ross A L, Brâve A, Scarlatti G, Manrique A and Buonaguro L 2010 *Lancet Infect. Dis.* 10 305-16
[9] Rothman A L 2011 *Nat. Rev. Immunol.* 11 532-43
[10] Costa S M et al. 2011 *Plos One* 6 e25685
[11] Natarajan S 2010 *Genet. Mol. Biol.* 33 214-9
[12] Andriyoko B, Parwati I, Tjandraawati A and Lismayanti L 2012 *Majalah Kedokteran Bandung* 44 253-60
[13] Wu H et al. 2015 *Antimicrob. Agent s Chemother.* 59 1100-9