Purification of NS2B-NS3 dengue virus serotype 3 protein as raw material for dengue virus vaccine

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Abstract. Dengue virus serotype 3 has been recognized as the predominant serotype in many occurrences of Dengue Hemorrhagic Fever (DHF) in Indonesia. Performing DHF prevention could be done with vaccination. BPPT institution was developing a vaccine, which is made from NS2B-NS3 recombinant protein. This protein is one of the non-structural proteins that arrange the genome of DENV and it has a molecular weight of 83 kDa. This research aims to isolate and purify NS2B-NS3 protein DENV-3 from transformant cell of Saccharomyces cerevisiae. Purification of NS2B-NS3 protein is done with HisPur Ni-NTA Magnetic Beads method. Optimization of purification is done by increasing the concentration of imidazole as protein binder in the elution buffer starting from 250 mM until 500 mM. The validity of purified protein was tested qualitatively with Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) method and quantified by Bichinconinic Acid (BCA) method. The results revealed us that NS2B-NS3 protein was purified optimally at 300 mM imidazole by HisPur Ni-NTA Magnetic Beads method. SDS-PAGE analysis showed that there was a specific band with size 83 kDa in lane result of elution with 300 mM imidazole and based on the result of protein quantification obtained that it has the highest percentage of purification effectiveness is 16.38 %.

Keywords: dengue virus, NS2B-NS3 protein, purification, protein quantification.

1. Introduction
Dengue Hemorrhagic Fever (DHF) is a major public health problem worldwide [1]. This disease is the most common disease in tropical and subtropical regions [2]. Indonesia is one of the countries with endemic area of dengue hemorrhagic fever (DHF), the largest endemic area amongst the tropical countries [3], and ranks second out of 30 countries with the highest number of dengue cases [4].

Dengue Hemorrhagic Fever is caused by hemorrhagic fever virus or dengue virus (DENV) [5]. The virus itself has four types of serotypes, namely DENV-1, DENV-2, DENV-3 and DENV-4 [1]. Dengue virus serotype 3 (DENV-3) is the predominant serotype, which is the main cause of dengue fever in Indonesia [6]. DHF, which caused by DENV-3 shows the clinical conditions that are more severe than other serotypes. In addition, this serotype can cause higher mortality rate [7,8].

Research institutes in Indonesia has conducted collaboration in dengue vaccine research consortia as a preventive effort of the disease. The institutions which were involved in the consortium are the
Ministry of Health, the Eijkman Institute, Universitas Indonesia (UI), Universitas Gajah Mada (UGM), Institut Pertanian Bogor, Biofarma, Lembaga Ilmu Pengetahuan Indonesia (LIPI), dan Badan Pengkajian dan Penerapan Teknologi (BPPT). BPPT institution is given a task to develop a vaccine made from NS2B-NS3 protein from DENV serotype 3 obtained from the Jakarta area.

Non-structural protein (NS2B-NS3) is one of the proteins that make up the genome of DENV. The protein serves to assist the replication process, as well as modulate viral pathogenesis [9]. Rothman reported that NS2B-NS3 protein boosts the human immune response to dengue virus by triggering the activity of T cells, namely CD4+ cells and CD8 + cells [5]. Ross et al. [10] reported that a vaccine made from recombinant proteins have high immunogenicity and high level of security.

Early stage of the research that has been conducted by BPPT in manufacturing dengue vaccine based on NS2B-NS3 protein is the cloning process of NS2B-NS3 protein coding subunit gene using a plasmid vector pYES2/CT. Genes from NS2B-NS3 protein have been successfully inserted in a plasmid vector pYES2/CT [11]. Previous research reported that the results of cloning have been successfully validated and the recombinant NS2B-NS3 protein has been expressed in Saccharomyces cerevisiae [12].

Protein used as a raw material to be made into vaccine has to be purified first [13]. The process of purifying proteins with His-tagged affinity can be done on the expressed proteins into the cells of Saccharomyces cerevisiae [14]. Recombinant proteins which contain polihistidin tagged is purified with the method of Immobilized Metal Affinity Chromatography (IMAC) based on the interaction between transition metal ions in a matrix with a specific amino acid chain [14]. HisPur Ni-NTA Magnetic Beads is a device that can be used for protein purification with IMAC method [15].

Azzizah [16] reported that purified recombinant NS2B-NS3 protein with His-tagged marker has not been successfully done with HisPur Ni-NTA Magnetic Beads. The concentration of 200 mM imidazole in the elution buffer was not optimal to elute the protein. Imidazole serves to elute the His-tagged protein that binds to the resin, so that the protein can be purified [17]. Variations in the imidazole concentration in elution buffer that can be used to purify the protein start from 250 mM to 500 mM [15]. Therefore, optimization of the concentration of imidazole starting from a concentration of 250 mM, 300 mM, 400 mM and 500 mM in elution buffer needs to be done so that the purification process with HisPur Ni-NTA Magnetic Beads can be successfully carried out.

Results of NS2B-NS3 protein purification with HisPur Ni-NTA Magnetic Beads method need to be validated by Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) method and Bichinconinic Acid (BCA) method. SDS-PAGE method is an effective method used to analyze the purified proteins based on molecular weight [13]. BCA method is one of the methods used to measure protein concentration effectively [18]. Purified NS2B-NS3 protein that has been validated will be used as the raw material of dengue vaccine.

This study aims to isolate NS2B-NS3 protein of DENV serotypes 3 from transformants Saccharomyces cerevisiae and optimize NS2B-NS3 protein purification using Ni-NTA HisPur Magnetic Beads. The hypothesis of this study is that NS2B-NS3 protein of DENV serotypes 3 can be isolated and purified using Ni-NTA HisPur Magnetic Beads.

2. Methods

2.1. Cultivation of transformants Saccharomyces cerevisiae
Cultivation of Saccharomyces cerevisiae which expresses NS2B-NS3 protein was conducted using the quadrant streak. Yeast cells were streaked four times in four different areas on the surface of Minimal Medium (MM) agar. Medium was incubated at 30 °C for 48 hours.

2.2. NS2B-NS3 protein isolation of transformants Saccharomyces cerevisiae
A single colony of Saccharomyces cerevisiae from INVSc samples 3 and 6a1 was grown in MM liquid medium containing 2 % glucose. The medium then incubated in an incubator shaker at 30 °C at 300 rpm for 24 hours. Optical density of the sample cell suspension of S. cerevisiae INVSc 3 and 6a1 that are grown in the medium were measured at a wavelength of 600 nm (OD₆₀₀) using a spectrophotometer. One ml of S. cerevisiae cell suspension is taken, then the measured in OD₆₀₀. OD₆₀₀ value is used to calculate the volume of cell suspension that will be taken to be put in induction medium. The formula for computing the volume of culture to be taken is using equation (1):

\[
\frac{0.4 \times 50 \text{ ml}}{\text{OD}_{600 \text{ score}}} \quad (1)
\]
the concentration of purified protein is measured by BCA.

are repeated until the supernatant contains the His

by vortex every 5 minutes of incubation time. Resin is collected on the bottom of the micro tube using a magnetic stand. Supernatant containing the His

process is repeated 1 time. Micro
tube using a magnetic

by vortex for 10 seconds, then incubated for 30 min at 40°C.

buffer is added. The mixed solution is homogenized using a vortex for 10 seconds. Micro tube is placed in a magnetic stand to separate the resin in the bottom of the tube, then the supernatant is discarded, while the pellets formed are resuspended in induction medium. Induction medium used is a liquid MM medium containing 2 % galactose and 2 % raffinose. The medium was incubated for 48 hours in an incubator shaker at 300 rpm. Culture resulted in induction medium was inserted into the falcon tube to centrifuge at 12,000 rpm for 2 minutes. Formed supernatants were discarded, and then 500 µL of PBS 1x is added to the remaining pellet. The cell suspension is sonicated for 20 min, then stored at -20°C.

2.3. Verification of NS2B-NS3 protein isolate with SDS-PAGE

Results of NS2B-NS3 protein isolation from samples 3 and 6a1 of INVSc is taken as much as 30 µL, then mixed with 20 µL loading dye 6X. The sample is heated in boiling water at a temperature of about 100°C for 15 minutes. The 20 mL sample and 4 mL marker are inserted into the well. Electrophoresis gel running is performed for 90 minutes at a voltage of 150 V. Gel is removed from the mold after the running process is completed, and then the gel is soaked in staining solution gel (Coomassie Brilliant Blue). Gel is incubated at 30°C in the incubator shaker for 60 minutes at a speed of 50-60 rpm. Staining solution gel is removed, and then destaining solution gel is incorporated into the gel. The gel is soaked for 10 minutes at 30°C in an incubator shaker at a speed of 50-60 rpm. The washing process with destaining solution gel is repeated three times.

2.4. Protein concentration measurement method Bichinconinic Acid (BCA)
The protein concentration is measured by a standard curve protein Bovine Serum Albumin (BSA) at a concentration of 20-2000 µg/mL. 2mg/mL BSA stock is diluted using a solvent (PBS 1x). Total volume of solvent and BSA to be used for dilution of BSA is based on the procedure in the kit manual [Thermo Scientific]. 0.1 mL of standardized BSA protein and protein samples are inserted into the microplate. 2.0 mL of working reagent is added to each tube. The mixed solution is homogenized and incubated for 30 minutes at 37°C, then allowed to stand at room temperature. Absorbance values of BSA and protein sample are then quantified by ELISA microplate reader at a wavelength of 562 nm. BSA absorbance value standard curve is used to measure the concentration of protein samples and the percentage of purification effectiveness. Percentage of purification effectiveness is obtained from the percentage of protein concentration ratio before and after purification. Formula of the percentage of protein purification effectiveness is as described in equation (2):

\[ X = \frac{B}{A} \times 100\% \] (2)

X is the percentage of protein purification effectiveness. A is the concentration of the protein before purification, while B is the concentration of protein after purification.

2.5. NS2B-NS3 Protein purification

Proteins purification is performed using Hook-6x His Protein Spin Purification kit. 40 µL of HisPur Ni-NTA magnetic beads is incorporated into 1.5 mL micro tubes, and then 160 mL equilibration buffer is added. The mixed solution is homogenized using a vortex for 10 seconds. Micro tube is placed in a magnetic stand to separate the resin in the bottom of the tube, and then the supernatant is discarded, 400-µL of equilibration buffer is added, and then homogenized with the vortex for 10 seconds. Micro tube is placed in a magnetic stand to separate resin in the bottom of the tube, and then the supernatant is discarded, 400-µL of the results of protein isolation is taken, and then mixed with 400-µL equilibration buffers in micro tube already containing resin. The solution is homogenized by vortex for 10 seconds, then incubated for 30 min at 25°C. Resin is collected on the bottom of the tube using a magnetic stand, then the supernatant or the flow-through is stored for process validation using SDS-PAGE. Micro tube containing resin is added with 400-µL wash buffers. Resin is collected on the bottom of the micro tube using magnetic stand, then the supernatant is discarded. The washing process is repeated 1 time. Micro-tubes containing resin is mixed with 25 µL elution buffer, then homogenized using the vortex for 15 seconds. Samples are incubated for 15 minutes and homogenized by vortex every 5 minutes of incubation time. Resin is collected on the bottom of the micro tube using a magnetic stand. Supernatant containing the His-tagged protein is stored as elution 1. Elution stages are repeated until elution 2 is obtained. The elution results obtained and the resin are verified, and then the concentration of purified protein is measured by BCA.
3. Results and discussion

Visualization of the isolated proteins using SDS-PAGE method shows that cells of transformants *S. cerevisiae* sample 3 and sample 6a1 were showed a positive expressing NS2B-NS3 protein (figure 1). This positive transformation result is consistent with the result of previous research about *S. cerevisiae* transformation [16]. The protein was successfully isolated from transformants *S. cerevisiae*. SDS-PAGE visualization resulted indicate a specific band 83 kDa in size. NS2B-NS3 protein has a molecular weight of 83 kDa [10].

NS2B-NS3 recombinant protein was successfully expressed by *S. cerevisiae* as galactose contained in MM media serves to induce the expression of the protein. Transformant cells were cultivated in the induction medium Minimal Medium (MM) liquid containing 2% galactose and raffinose 2%. Galactose induces the expression by activating the promoter GAL1. Galactose induces the activity of Gal4p protein contained in GAL1 promoter. The protein also functions as a regulator of the process of transcription and translation, thus forming protein [19].

Purified protein was tested qualitatively using SDS-PAGE. Results data visualization of SDS-PAGE shows that purification of NS2B-NS3 protein of DENV-3 both from sample 3 and sample 6A1 are successfully performed with a concentration of 250 mM imidazole and 300 mM (figure 1). The thickened ribbon formed in the strip of elution results with molecular size of 83 kDa represents the fact that NS2B-NS3 protein purification is successful. Ribbon with the size of 83 kDa on the results of SDS-PAGE gel visualization shows that NS2B-NS3 protein is found in cells of *S. cerevisiae*. The protein is then purified successfully because it has a His-tagged affinity marker that serves to facilitate the purification of recombinant protein targets [20].

Purified protein was tested qualitatively with BCA method. Data of protein concentration before and after purification shows that the purified protein from sample 6a1 eluted with 300 mM imidazole has the highest concentration, which is 287.14 µg/mL, with the highest percentage of purification effectiveness, which is 17.08 %, while the purified protein of sample 3 has the highest concentration in elution results with 500 mM imidazole, namely 212.86 µg/mL, with the percentage of purification effectiveness of 14.61 %. Looking only at the quantitative test results, the optimization of purification is performed with 300 mM and 500 mM imidazole. However, based on the results of qualitative tests using SDS-PAGE, it shows that there is no ribbon in the purified protein result that is eluted with 500 mM imidazole. Therefore, optimization of purification in the next stage is done only with the use of 250 mM imidazole and 300 mM.

Results of NS2B-NS3 protein purification optimization is tested qualitatively using SDS-PAGE (figure 2 and figure 3), and then quantified to determine the concentration of purified protein and protein purification effectiveness (table 1). Visualization of NS2B-NS3 protein shows that specific
Figure 2. Results visualization of NS2B-NS3 Protein purification of samples 3 and 6a1 with Imidazole concentration of 250 mM and 300 mM

Figure 3. Results of NS2B-NS3 Protein purification optimization of samples 3 and 6a1 at concentration of 250 mM and 300 mM Imidazole

band is formed with the size of the 83 kDa on the results of protein elution of sample 3 with 300 mM imidazole (lane 6). Thus, we can conclude that NS2B-NS3 protein purification is most optimal when done with elution buffer containing 300 mM imidazole.

The data show that the concentration of purified protein in sample 3 with 300 mM imidazole has the highest concentration value, namely 342.38 µg/mL, with a percentage of 16.38% in purification.
Table 1. NS2B-NS3 Protein concentration before and after purification with 250 and 300 mM Imidazole

| Sample | Isolation result (μg/mL) | Protein Concentration | Purification Results | % Effectiveness |
|--------|--------------------------|-----------------------|----------------------|----------------|
|        |                          |                       | Imidazole (mM)       | Purified Protein (μg/mL) | Average (μg/mL) |
|        |                          |                       | 1                    | 2              | 3                  |                |
| S3     | 2090                     | 250                   | 262.86               | 264.29         | 258.57             | 261.9           | 12.53          |
|        |                          | 300                   | 278.57               | 398.57         | 350.00             | 342.38          | 16.38          |
| S6a1   | 2707.14                  | 250                   | 322.86               | 311.43         | 308.57             | 314.29          | 11.61          |
|        |                          | 300                   | 295.71               | 307.14         | 294.29             | 296.19          | 11.05          |

effectiveness. Based on the analysis, we can conclude that NS2B-NS3 protein purification eluted with 300 mM imidazole has the highest purification effectiveness.

Preliminary data of the qualitative test results of shows that purification can be performed optimally at imidazole concentration of 250 mM and 300 mM. Wu et al. [21] reported that NS2B-NS3 protein of DENV can be successfully purified from Escherichia coli cells by the method of Ni-NTA Magnetic Beads using elution buffer containing a concentration of 250 mM and 300 mM imidazole. Therefore, protein purification is performed again with the same technique at a concentration of 250 mM imidazole and 300 mM.

Based on preliminary data of qualitative test results using SDS-PAGE method, it is known that the method HisPur Ni-NTA Magnetic Beads is an appropriate method for the purification of proteins with His-tagged markers. Ni-NTA Magnetic Beads have been selected as a method of His-tagged purification because there are four sides in protein binding to a metal ion chelators are likely to result in an increase in protein binding capacity, so that the purification occurs optimally [15].

The results showed that the most optimal conditions for NS2B-NS3 protein purification can be performed by using elution buffer containing 300 mM imidazole. Purified protein is successfully obtained by using HisPur Ni-NTA Magnetic Beads method. The method is the most effective method to purify proteins based on the principles of IMAC. IMAC principle is the interaction between transition metal ions in a matrix with a specific amino acid chain [14]. This is supported also by the statement of Wu et al. who reported that NS2B-NS3 protein of DENV can be successfully purified from Escherichia coli cells by engineering HisPur Ni-NTA Magnetic Beads using elution buffer containing a concentration of 250 mM and 300 mM imidazole [20].

4. Conclusions
NS2B-NS3 protein is successfully isolated from transformants S. cerevisiae cell and optimally purified with HisPur Ni-NTA Magnetic Beads method by elution buffer containing 300 Mm imidazole.

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References
[1] Grange L, Simon-Loriere E, Sakuntabhai A, Gresh L, Paul R and Harris E 2014 Front. Immunol. 5 280
[2] Neeraja M, Lakshmi V, Lavanya V, Priyanka E N, Parida M M, Dash P K, Sharma S, Rao P V L and Reddy G 2015 J. Virol. Methods 211 22–31
[3] Sasmono R T et al. 2015 Infect. Genet. Evol. 32 165–77
[4] World Health Organization 2012 Global Strategy for Dengue Prevention and Control (Geneva: WHO Press)
[5] Rothman A L 2011 Nat. Rev. Immunol. 11 532–43
[6] Sukri N C 2003 et al. Am. J. Trop. Med. Hyg. 68 529–35
[7] Nogueira R M R 2005 Emerg. Infect. Dis. 11 1376–81
[8] Andriyoko B, Parwati I, Tjandrawati A and Lismayanti L 2012 Majalah Kedokteran Bandung 44 253–60
[9] Luo D, Vasudevan S G and Lescar J 2015 *Antiviral Res.* 118 148–58
[10] Ross A L, Brâve A, Scarlatti G, Manrique A and Buonaguro L 2010 *Lancet Infect Dis.* 10 305-16
[11] Narita V, Widyanto R M, Pambudi S and Sudiro T M 2011 *Makara Sains* 15 173–8
[12] Yang C C, Hsieh Y C, Lee S J, Wu S H, Liao, C L, Tsao C H, Chao Y S, Chern J H, Wu C P, Yueh A 2011 *Antimicrob. Agents and Chemother* 55 229–38.
[13] Marston F A O, Lowe P A, Doel M T, Schoemaker J M, White S, Angal S 1984 *Bio/Technology* 2 800.
[14] Bornhorst J A and Falke J J 2000 *Methods Enzymol.* 326 245–54.
[15] Thermo Fisher Scientific 2013 *HisPar Ni-NTA Magnetic Beads: Introduction* (Rockford: Pierce Biotechnology).
[16] Azzizah I N Validation of Clone Product Expression and Purification of NS2B-NS3 Protein Denv Serotype 3 Isolate Jakarta (Depok: Universitas Indonesia) Undergraduate Thesis
[17] Saepuloh U, Iskandriati D, Pamungkas J and Sajuthi D 2013 *Jurnal Ilmu Pertanian Indonesia* (JIPI) 18 49–54.
[18] Nollet L M L and Toldra F 2012 *Handbook of Analysis of Active Compounds in Functional Foods* (New York: CRC Press).
[19] Schneiter R 2004 *Genetics, Molecular and Cell Biology of Yeast* (Fribourg: University of Fribourg).
[20] Terpe K 2003 *Appl. Microbiol. Biotechnol.* 60 523–33.
[21] Wu H et al. 2015 *Antimicrob. Agents Chemother.* 59 1100–9.