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Optimization of the Purification of Nucleocapsid Protein of Newcastle Disease Virus by Ultracentrifugation Method

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Protein purification is a process to remove impurities contains in a complex mixture and may involves a single step or by a combination of several steps. In this study, nucleocapsid (NP) protein of Newcastle disease virus (NDV) was purified using various purification steps from recombinant *Escherichia coli.* The purification steps involved ammonium sulphate precipitation, dialysis and sucrose gradient ultracentrifugation. In this study, the optimized condition for obtaining the highest NP protein yield in the final step of purification (ultracentrifugation) was determined. Two independent parameters which are centrifugal force and running time were manipulated and investigated. The optimum processing condition was determined by employing Response Surface Method (RSM). Based on the almost fully elliptical plot showed in 2D and 3D response graphs, the optimum condition for obtaining the highest NP protein yield is near to the centre points of the response surface (condition of 159000xg or 31,000rpm and 5 hours). Although the optimum time to purify the highest NP protein yield is five hours centrifugation, the NP protein could also be purified after three hours centrifugation albeit with slight reduction in the yield. Less centrifugation time is important as pre- and post-centrifugation procedures for sucrose gradient ultracentrifugation is time consuming and laborious.

Key words: Face Centre Composite Design (FCCD), Nucleocapsid (NP) protein, Purification, Response Surface Method (RSM), Ultracentrifugation

The purification of protein from various recombinant organisms is one of the most crucial steps of downstream processes in the production of protein for therapeutic and diagnostic kit use[1]. In protein purification, it is important to adopt procedures that avoid denaturing proteins, especially the protein of interest. The selected method of the purification also influences purity of protein. Protein may be purified by a single step such as affinity chromatography, or by a combination of several steps such as salt fraction, ion exchange, gel filtration, and etc[2]. In this study, nucleocapsid (NP) protein of Newcastle disease virus (NDV) was purified using various purification steps from recombinant *Escherichia coli*. The purification steps involved ammonium sulphate precipitation, dialysis and sucrose gradient ultracentrifugation. These steps of purification were adopted in this study as the purified NP protein will be used for protein-protein interaction study that requires highly purified NP protein.

NP protein is the major protein in NDV and it is highly immunogenic. It can be used in immunodiagnostic kit for NDV detection in infected chicken serum and also used in NDV propagation study as NP protein plays an important role and interacts with several NDV proteins. During virus assembly, the NP protein is most likely interacts

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with the matrix (M) protein of NDV[3] and mediates the interaction with viral lipid membrane which results in the budding and release of virus progenies from the cell plasma membrane[4]. The NP protein also interacts with phosphoprotein (P) of NDV and acts as an active form of NP protein that is used to assemble nascent RNA chain during genome replication[5]. To have better understanding of these protein-protein interactions, this study purified the NP protein to be used in the interaction studies. As protein purification is laborious and time consuming, this study determined the best conditions to purify the NP protein particularly the ultracentrifugation method.

MATERIALS AND METHODS

Purification of NP protein

Recombinant *E. coli* harboring NP gene of NDV for this study was obtained from Project Development Lab, Faculty of Engineering, International Islamic University of Malaysia. Approximately 250 ml recombinant *E. coli* cultures were grown at 37°C in LB broth until the culture reached optical density (at A_{600}) around 0.6 to 0.8. After induction with rhamnonse (0.1%) for 5 h, the cells were harvested by centrifugation. The pellets were lysed by sonication, cell extracts were recovered by centrifugation and followed by ammonium sulphate precipitation (10%). The precipitates were pelleted by centrifugation and dialysed (50 mM Tris, 100 mM NaCl, pH 7.8) extensively. The dialysed pellet were carefully layered onto 10-50% sucrose gradient and centrifuged (Optima L-90K; Beckman, USA) at various centrifugal force and running time at 4°C (Table 1). After the centrifugation, fractions were collected and analysed by SDS-PAGE to determine which fractions contained the desired protein. The purified NP protein was determined by Bradford assay.

Optimization experiment and statistical analysis

To determine the best condition for obtaining NP protein in ultracentrifugation step, two independent parameters which are centrifugal force and running time were manipulated. A series of experiments was designed by using through Face-Centred Central Composite Design (FCCD) (Table 1). The FCCD used in this study was provided in Design-Expert (Stat-Ease, Inc., version 6.0.8) software. Statistical analysis was carried out to determine which parameters in the design of an optimization experiment have the greatest influence on its behavior and performance and to obtain accurate values for those parameters. The statistical analysis involved analysis of variance (ANOVA), F-test and regression analysis.

RESULTS AND DISCUSSION

Purification of NP protein involved three purification steps after the harvested cells were lysed. For each step, the resulting sample was analyzed for the presence of NP protein by SDS-PAGE. After the first step of purification (ammonium sulphate precipitation, lane 2, Fig. 1), it can be observed that many contaminants were removed leaving the target protein in the precipitated pellet compared to the lysate (lane 1, Fig. 1). Dialysis further removed the contaminants and NP protein band can be seen clearly on the gel (lane 3, Figure 1). After sucrose gradient ultracentrifugation, NP protein was purified and very few contaminating faint bands were observed (lane 4, Figure 1). The purified NP protein size is 53 kDa which is similar to the previous isolated NP protein[1].

In determining the optimized condition for obtaining NP protein in ultracentrifugation step, centrifugal force and running time were manipulated and investigated. According to the FCCD, a total 11 experiments or runs were generated (Table 1). Experimental results from Table

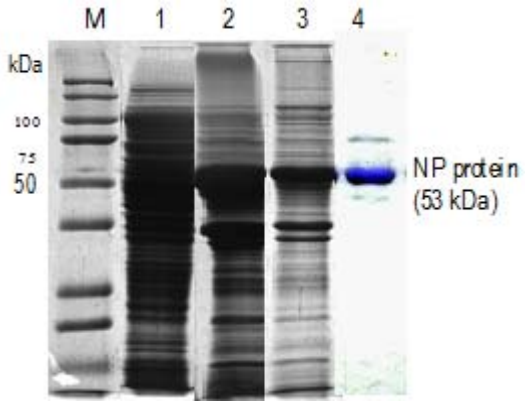

Fig. 1. SDS-PAGE of production of NP protein after (1) sonication, (2) ammonium sulphate precipitation, (3) dialysis, (4) sucrose gradient centrifugation. M, marker protein

1 were fitted to a quadratic polynomial model, thus multiple regression and regression coefficient were obtained and illustrated in the equation below in terms of coded factors:

$Y = 312.95-8.47A + 15.23B - 18.30A2 - 50.81 B2 + 28.14AB$

Where Y is the amount of NP protein; A, speeding and B, running time. However, the final equation in terms of actual factors was illutrated by the equation below:

$Y = 25.81371 + 3.46399E^{-003}A + 91.01414B - 1.83008E^{-007}A^2 - 12.70145B^2 + 1.40700E^{-003}AB$

This equation was used to determine the predited values of optimization experiments (Table 1). Table 1 showed the best condition of ultracentrifugation which results in 313.50 µg of NP protein is in runs 2, 6 and 9. The predicted values for these runs are also the highest (312.95 µg) compared to rest of the runs. The difference between the actual and the predicted values lead to the determination of the model significance via ANOVA. ANOVA gives the validity of the model and describe either the model used adequacy fits the variation observed in the response (NP protein yield) at the designed level or not[6]. There are many factors that were used to evaluate adequacy of the fitted model, the most important factors are Fisher variance also known as *F*-value. The summary of the ANOVA analysis of this study is illutrated in Table 2. The Model *F*-value of 10.16 implies the model is significant. There is only a 1.18% chance that a "Model F-Value" this large could occur due to noise. Values of "Prob > F" less than 0.05 indicated the model was significant. Based on Table 2, the quadratic term running time, B^2 ($p>F$ = 0.0048) and interactive term of speeding and running time, AB ($p>F$ =0.0202) significantly affected the yield of purified NP protein ($p<0.05$). R^2 obtained (0.9104) indicated that 91% of the two independent variables supported the response.

The determination coefficient, R^2 can be determined the goodness of fit of the regression equation that obtained from the model. The value of R^2 is in the range between zero to one, the higher value of R^2 is the better degree of correlation the actual and predicted value[7]. Based on the result, R^2 obtained from this study was strongly high which equal to 0.9104. it indicated that 91% of the two independent variables supported the response.

Table 1. Optimization experiments of sucrose gradient ultracentrifugation

Run	Centrifugal force (xg) or (rpm)	Running time (h)	Actual Value of NP protein (µg)	Predicted value of NP protein (µg)
1	288000xg (41000rpm)	7	264.29	278.74
2	159000xg (31000rpm)	5	313.5	312.95
3	288000xg (41000rpm)	5	296.63	286.17
4	72400xg (21000rpm)	7	236.25	239.41
5	159000xg (41000rpm)	3	195.98	265.22
6	159000xg (3100 rpm)	5	313.5	312.95
7	72400xg (21000rpm)	5	291	303.12
8	72400xg (21000rpm)	3	280.5	265.22
9	159000xg (31000rpm)	5	313.5	312.95
10	159000xg (31000rpm)	3	227.64	246.91
11	159000xg (31000rpm)	7	294.98	277.37

Table 2. Analysis of variance (ANOVA) of optimization experiments

Source	Sum of Squares	Mean square	F-value	Prob > F
Model	14296.26	2859.25	10.16	0.0118*
A	430.95	430.95	1.53	0.2708
B	1392.33	1392.33	4.95	0.0767
A^2	848.46	848.46	3.02	0.1429
B^2	6539.11	6539.11	23.25	0.0048*
AB	3167.44	3167.44	11.26	0.0202*
Residual	1406.49	281.30		
Lack of Fit	1406.49	468.83		
Pure Error	0.00	0.00		
R-Squared	0.9104			
Adjusted R-Squared	0.8209			

A = speeding, B = running time * $p<0.05$ is significant different

By using ANOVA, it generates the 3D response surface and 2D contour plots. According to Tanyildizi *et al* (2005)[8], the 3D response surface and 2D contour plots could be used to determine the optimum values of the variables. Based on the almost fully elliptical plot showed in 2D and 3D response graphs (Figure 2 and 3) obtained from the present study, the optimum condition was near to the centre points of the response surface (condition of 159000xg or 31,000rpm and 5 hours). Although the optimum time to purify the highest NP protein yield is five hours centrifugation; based on the optimization experiments, the NP protein could also be purified after three hours centrifugation albeit with slight reduction in the yield (Table 1, run 8).

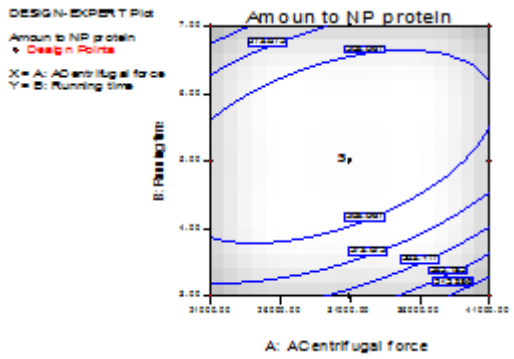

Fig. 2. 2D response surface shows the effect of speeding (centrifugal force) and running time on purification of NP protein

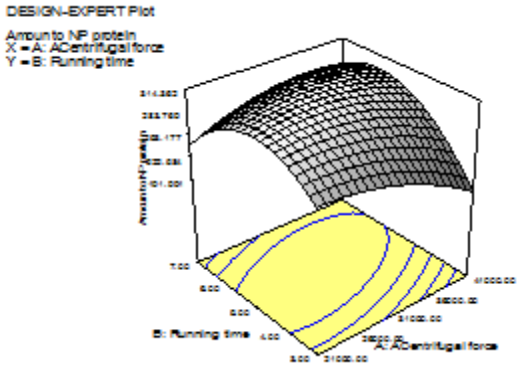

Fig. 3. 3D response surface shows the effect of speeding (centrifugal force) and running time on purification of NP protein

CONCLUSIONS

In this study, the optimized condition of ultracentrifugation to purify NP protein was successfully identified (condition of 159000xg or 31,000rpm and 5 hours). The purified NP protein obtained in this study can be used for protein-protein interaction study as well as diagnostic kit.

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