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Process Optimization for Influenza NA-VLP Vaccine Development using Baculovirus/Insect Cell System

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ABSTRACT

Recombinant protein based virus-like particles (VLPs) vaccine produced in insect cell using baculovirus expression vector was one of the methods developed to overcome the limitations of influenza vaccine egg-based production. This study focused on bioprocess development of influenza NA-VLP vaccine from the baculovirus/insect cells system. The NA-VLP production and purification processes were optimized. Infection of recombinant NA baculovirus at MOI 1 into $1x10^6$ cells/ml insect cells in active growing phase for 4 days resulted in the highest NA-VLP level. The NA-VLP could be mostly purified by two conventional methods i.e. ultrafiltration and size exclusion chromatography. Firstly, NA-VLP was concentrated and purified from a major contaminant protein, albumin, by the cross flow ultrafiltration using 500 kDa membrane. More than 97% of albumin removal was achieved in this process. The largest NA-VLP could be obtained after size exclusion chromatography. Albumin was also further removed from this step. It was found that recombinant NA baculovirus could not be efficiently removed by these two steps purification process. One more simple purification by anion exchange (AEX) chromatography using flow-through mode is recommended.

Keywords: Baculovirus/insect cell system, Influenza virus, NA-VLP

INTRODUCTION

Two types of influenza vaccines recommended by World Health Organization (WHO) are live-attenuated influenza vaccine and inactivated influenza vaccine (Cox, 2012). Embryonated chicken eggs are currently used for influenza virus production (Ren et al., 2015). However, egg-based production manufacturing system requires six to eight months for strain isolation to final dose formulation and validation and has limitation when insufficient egg supply and egg-substrate was infected with avian influenza virus during the outbreak (Ravin et al., 2012; Thompson et al., 2013; Ren et al., 2015; Kang et al., 2009). Thus, new methods for influenza virus vaccine production are in need.

Recombinant protein based virus-like particles (VLPs) vaccine produced in cell culture manufacturing system (i.e. mammalian cells, plant cells and insect cell) using a variety of vectors and gene delivery techniques was one of the methods developed to overcome the limitations of egg-based production (Ravin et al., 2012; Thompson et al., 2013). VLPs contain no genetic material thus they are unable to replicate and infect. Therefore, they are safe for human and environment (Thompson et al., 2013; Kang et al., 2009). Previously, influenza VLPs were developed as empty particles consist of influenza proteins such as hemagglutinin (HA), neuraminidase (NA), matrix protein M1 and matrix protein M2 (Ren et al., 2015; Thompson et al., 2013; Kang et al., 2009). It was later found that the influenza VLPs containing only an NA was also able to bud out of cells and form particles with morphology similar to influenza virions (Lai et al., 2010).

Animal Cell Culture research group at KMUTT had developed the influenza virus-like particle strain A/Thailand/1(Kan1)/2004 (H5N1) based on NA (NA-VLP) produced by baculovirus expression vector/insect cell system (BEVS). Insect cells produces recombinant NA protein when infected with the baculovirus expressing *NA*. In this system, baculovirus infection caused cell lysis. Baculovirus progeny is produced and bud out of the infected cells together with the NA-VLP. Therefore, cell debris and baculovirus must be removed from the NA-VLP (Khanefard, 2014; Vicente et al., 2011; Kawaoka et al., 1989). In addition, all protein contents in culture medium such as albumin

which is a major protein from fetal bovine serum supplemented in the culture medium must be removed.

In this study, NA-VLP production using different multiplicity of infection (MOI) of baculovirus infected into insect cells and time of harvest (TOH) were optimized. Process optimization for NA-VLP purification from baculovirus infected cell culture was also carried out.

MATERIALS AND METHODS

Materials

Spodoptera frugiperda (Sf9) cell line was purchased from Invitrogen Crop., USA. The recombinant NA baculovirus was constructed by Animal Cell Culture laboratory in King Mongkut's University of Technology Thonburi.

NA-VLP production

Sf9 insect cells were grown in TNM-FH medium supplemented with 10% FBS in shake flask at 27°C until reached mid-log phase. Cells at $1x10^6$ cells/ml were infected by recombinant NA baculovirus (rBV) at MOIs 1 and 3. Culture was sampled at 0, 1, 2, 3, 4, 5, 6 and 7 day(s) post-infection. Cells in all samples were counted in hemocytometers. Samples were centrifuged at 5,500 rpm for 10 minutes to remove infected cells. The supernatant was examined by Western blot analysis using anti-NA (H1N1) monoclonal antibody (Sino Biological, China) as a primary antibody. Intensity of recombinant NA protein monomer specific band on the Western blot was estimated by Image J program.

NA-VLP Purification

Culture of Sf9 insect cells infected with recombinant NA baculovirus was harvested by centrifugation at 5,500 rpm for 10 minutes. The supernatant was collected and proceeded to buffer exchanged by ultrafiltration using cross flow filtration membrane, 500 kDa hollow fiber member (surface area 73 cm^2, GE Healthcare, USA) with the transmembrane pressure controlled at 80 ml/min of feed flow rate. A low-salt Tris buffer (20 mM Tris-HCl containing 0.5 M NaCl, pH 7.5) was used for buffer exchange. The culture supernatant

was firstly diluted with 400 ml of 20 mM Tris-HCl containing 0.5 M NaCl, pH 7.5 and ultrafiltration was carried out until a 100 ml of sample remained in reservoir. Buffer exchange was repeated two times. After that, sample in reservoir was concentrated about 5 times. The membrane was washed with 5 ml of 20 mM Tris-HCl containing 0.5 M NaCl, pH 7.5 three times for product recovery. Total protein content, baculovirus titer and the formation of virus-like particle by transmission electron microscope of samples collected from retentate, permeate and washing fraction were determined.

Size exclusion chromatography. The sample from ultrafiltration was further concentrated using 100 kDa NANOSEP (Pall Life Sciences, Puerto Rico). Retentate was collected for purification by size exclusion chromatography. The concentrated sample at 500 µl was loaded into a phosphate buffered saline (PBS, pH 7.4) equilibrated Superose^{TM} 6 Increase 10/300 GL column (GE Healthcare). One column volume of PBS at flow rate of 0.5 ml/min was used for elution. Fractions were collected and analyzed by Native PAGE and SDS-PAGE. Western blot analysis using either anti-NA antibody or anti-GP64 antibody to detect NA-VLP and baculovirus, respectively was also carried out.

Virus titration

The recombinant baculovirus titer was determined by end-point dilution method (O'Reilly et al., 1992). The sample was diluted tenfold dilutions with TNM-FH containing 10% FBS. After that, 10 µl of each dilution was added into a well of a 96-well plate containing 1×10^4 cells/well of Sf9 cells. The plate was sealed with parafilm and putted in the box containing wet cotton and incubated at 27 °C for 7 days. The viral titers was determined as $TCID_{50}$/ml and/or converted to pfu/ml.

Transmission electron microscopy (TEM)

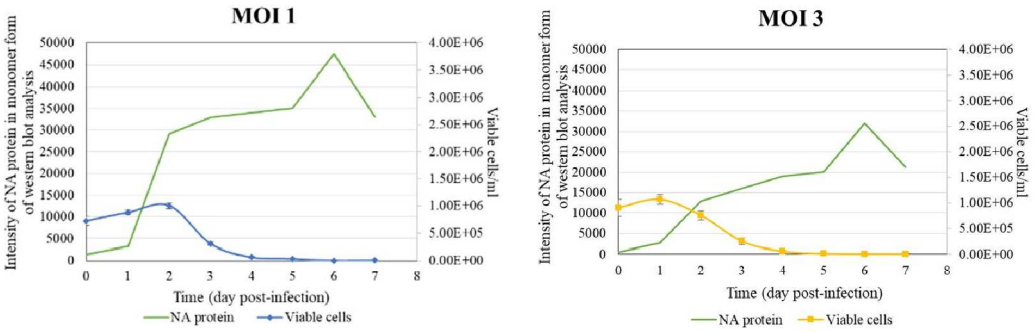
Sample was dropped onto a discharged 400 mesh formvar-carbon coated copper grid. The grid was stained with 2% phosphotungstic acid. Influenza VLPs was visualized on a transmission electron microscope (HITACHI, HT7700) at an accelerating voltage of 100 kV.

RESULTS AND DISCUSSIONS

Optimization of NA-VLP production

The NA-VLP production was optimized by varying multiplicity of infection (MOI) at MOI 1, 3 and 5 to infect into insect cells at $1x10^6$ cells/ml. Optimal time of harvest (TOH) was also observed. Western blot analysis revealed the recombinant NA protein, NA-VLP monomer at molecular weight ~51 kDa. The highest recombinant NA protein, determined by the intensities of specific band on the Western blot, was obtained when Sf9 cells were infected with recombinant baculovirus at MOI 1 on day 6 and MOI 3 on day 6 post-infection (Figure 1 A). However, almost all infected insect cells died after 4 days post-infection and many cellular proteins may be released from death cells. Thus, it is recommended to harvest the NA-VLP product earlier. It was found that production levels of NA-VLP obtained from day 4 and 5 using both MOIs were non-significantly different (Figure 1 B). MOI 1 was chosen as optimal MOI. This will safe cost for both baculovirus usage for NA-VLP production and virus removal for NA-VLP purification.

(A)



(B)

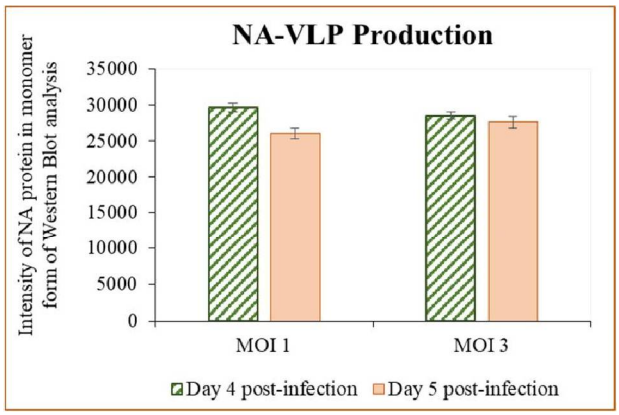


Figure 1. Insect cells growth and recombinant NA protein production when recombinant baculovirus at MOI 1 and 3 (A). Comparison of recombinant NA protein production level (on the same blot) from both samples harvested on day 4 and 5.

NA-VLP Purification

Ultrafiltration

Our previous studies (data not shown) have shown that transmembrane pressure at 0.3 bar and feed flow rate at 80 ml/min were optimal conditions for ultrafiltration process since some impurities such as albumin were removed.

A 100 ml of sample from Sf9 infected with recombinant baculovirus at MOI 1 was buffer exchanged into 20 mM Tris-HCl buffer with 0.5 M NaCl, pH 7.5, and further five times concentrated by ultrafiltration using 500 kDa molecular weight cutoff. SDS-PAGE shows that some proteins were filtered through the membrane, into permeate fraction (Figure 2 A lane 5). The albumin, major protein in fetal bovine serum, was removed by ultrafiltration process by 97.55% (Table 1). It was found that recombinant NA protein in monomer form and polymers remained in the retentate after buffer exchange and concentration (Figure 2 B lane 4). They were found assembled into the NA-VLP as demonstrated by electron micrograph (Figure 3 A-B). Due to their large rod shape particles, recombinant NA baculovirus were retained on the membrane (Figure 3 C) which must be removed in the purification process.

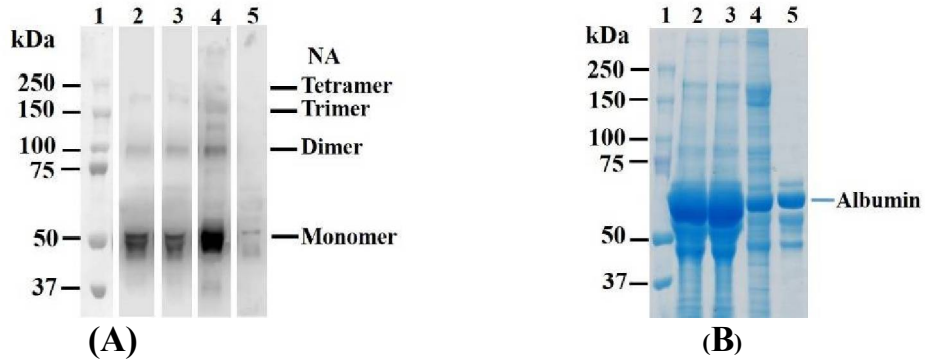


Figure 2. Recombinant NA protein monomer and its polymers after filtration using 500 kDa hollow fiber membrane (area 73 cm^2). SDS-PAGE staining with Coomassie blue (A) and Western blot analysis using anti-NA (H1N1) antibody (B). Lane 1: Protein marker, Lane 2: Culture supernatant from Sf9 cells infected with recombinant baculovirus at MOI 1, Lane 3: Sample from lane 2 after pre-filtration using 0.45 µm syringe filter, Lane 4: Sample obtained in retentate after buffer exchange and concentration, Lane 5: Sample obtained in permeate after buffer exchange.

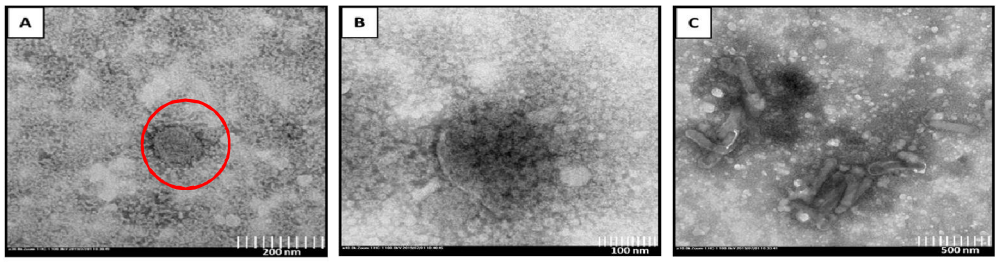


Figure 3. Electron micrograph of NA-VLP from retentate sample after ultrafiltration using 500 kDa hollow fiber membrane. A and B shows NA-VLP. C shows recombinant NA baculovirus.

Size exclusion chromatography

The retentate obtained from ultrafiltration was further concentrated using 100 kDa NANOSEP and subjected to a Superose^{TM} 6 Increase 10/300 GL column, size exclusion chromatography. The chromatogram shows proteins in samples were separated by sizes since many peaks were observed (Figure 4 A). The first 3 peaks (fraction17-36) contained recombinant NA protein and its polymers in different sizes (Figure 4 B). Albumin was separated as seen in fraction 40-41 (Figure 4 C). Therefore, size exclusion chromatography could be used for albumin removal in which < 0.04 mg was detected (Table 1).

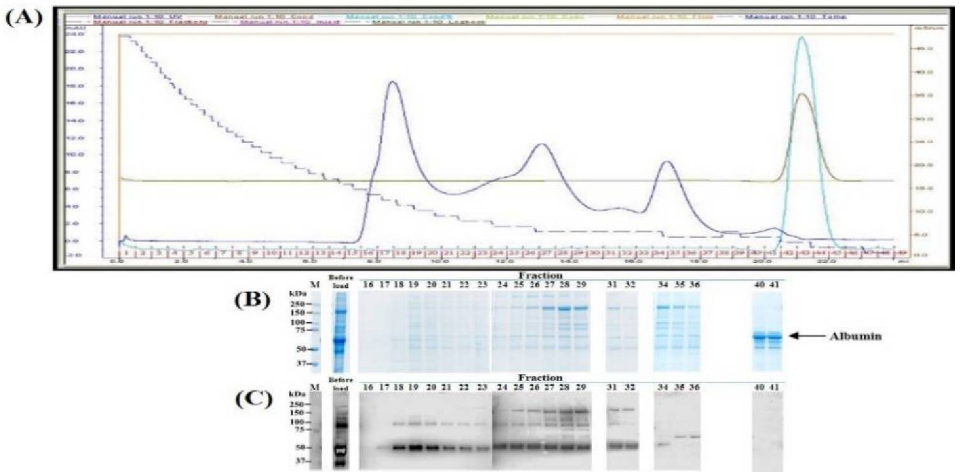


Figure 4. Purification of NA-VLP after ultrafiltration by a size exclusion chromatography (SEC) using Superose^{TM}6 Increase 10/300 GL column (A). SDS-PAGE staining with Coomassie blue stain (B) and Western blot analysis using anti NA antibody (C).

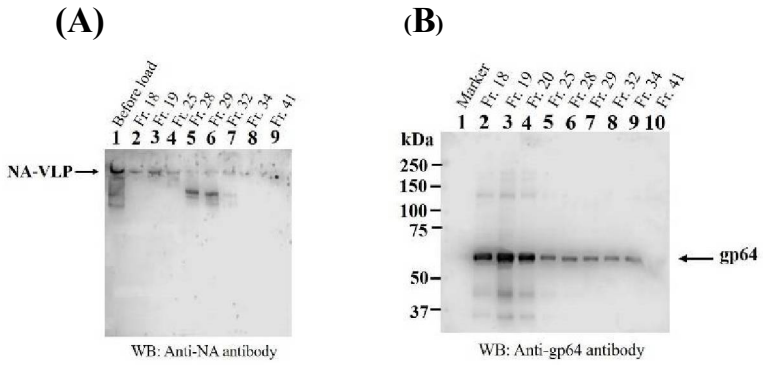


Figure 5. Western blot analysis of samples from size exclusion chromatography separated by Native PAGE (A) and SDS-PAGE (B) using; anti-NA H1N1 monoclonal antibody for detection of NA-VLP and anti- gp64 monoclonal antibody for detection of recombinant NA baculovirus, respectively.

Figure 5 A shows NA-VLP were mostly found in the first two peaks (fraction 18–19 and fraction 28-29. The NA-VLP in the first peak had the biggest size since they could not enter the PAGE pore sizes. As for the recombinant baculoviruses removal, due to their large sizes, they were found in the first peak together with NA-VLP. Titer of virus in pooled fractions of fraction 18-22 was 9.08×10^{8} pfu (Table 1). Further purification by anion exchange (AEX) chromatography (flow-through mode) using Hitrap® Capto Q™ column (GE Healthcare Life Sciences) for baculovirus and nucleic acids removal will be carried out.

Table 1. Baculovirus titer and albumin contents from sample collected each step in purification process.

Step	Process	Total baculovirus (pfu)	Total albumin content (mg)
1	Culture supernatant (100 ml)	8.29×10^{10}	629.50
2	Ultrafiltration (20 ml)	3.94×10^{11}	15.44
3	Size exclusion chromatography (37.5 ml)	9.08×10^{8}	< 0.04

CONCLUSIONS

The NA- VLP could be produced by baculovirus expression vector/insect cells system using recombinant NA baculovirus at MOI 1 to infect $1x10^6$ cells/ml insect cells in active growing phase. The NA-VLP product should be harvested not later day 4 post-infection to avoid other contaminant proteins released from cell death. The NA-VLP could be concentrated and partial purified by the cross flow ultrafiltration using 500 kDa molecular weight cutoff. More than 97% of albumin removal was achieved in this process. Size exclusion chromatography could be used to separate NA-VLP according to their sizes. The largest NA-VLP was obtained in the first peak. It was found that recombinant NA baculovirus was not efficiently removed by these two steps purification process. Thus, further purification by anion exchange (AEX) chromatography using flow-through mode is recommended.

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