Production of Scale Drop Disease Virus Self-assembled Major Capsid Protein Nanoparticles for Fish Vaccine Development

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Abstract

Scale drop disease virus (SDDV) causes scale drop syndrome in Asian sea bass. Vaccination is considered an effective method for the control of this disease. SDDV vaccines that have been tested are inactivated vaccine and subunit vaccine. The chemical or physical treatment used to inactivate the virus and denaturing agent used for subunit vaccine purification have been known to modify their antigenicity. This usually results in a short immune response, weaker cell-mediated and mucosal immune responses, and possibly less effective in preventing viral entry. Therefore, new form of an antigen called self-assembled protein nanoparticles (SAPNs) to improve antigen stability and immunogenicity was proposed. These SAPNs were obtained from the oligomerization of recombinant SDDV major capsid proteins (MCP) produced by the baculovirus expression vector system. A genetically engineered baculovirus vector was constructed and used for expression of His-tag fused SDDV MCP gene in insect cells as confirmed by reverse transcription PCR (RT-PCR). A specific protein band at approximately 53 kDa, corresponding to the recombinant His-tag MCP protein, was detected in the baculovirus-infected insect cells by Western blot analysis. This recombinant MCP protein was partially purified by ultracentrifugation using double layers of 25 and 70 % sucrose. The electron micrograph of the purified sample revealed many particles with sizes ranging from 50-100 nm. These recombinant MCP proteins seem to be able to self-assemble to form nanoparticles in well-ordered arrays that mimic the repetitiveness, geometry, and shape of the SDDV virus. It is anticipated that a stable and better immunogenicity SDDV vaccine could be developed.

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1. Introduction

Asian sea bass (*Lates calcarifer*) is one of the aquaculture products. In 2011, Thailand produced 13,697 tons of Asian sea bass from aquaculture that generate income of 1,720.75 million baht. There are many factors affecting Asian sea bass production, such as water quality, fish starter quality, and diseases (Office of agricultural economics, 2014). According to FAO report, pathogens that caused diseases in Asian sea bass are such as viruses (Nodavirus and Iridovirus), bacteria (*Streptococcus iniae* and *Tenacibaculum maritimum*), and parasites (*Neobenedenia* spp.), etc. Viral diseases are more difficult to control than others due to a lack of anti-viral agents and proper treatment (Dhar *et al.*, 2014).

Since 1992, scale drop syndrome in Asian sea bass has been reported in Malaysia, Singapore, and Indonesia. Infected fish showed darkened bodies, scale loss, tail and fin erosion, pallor of gills, and sometimes exophthalmia. Besides, severely infected fishes stop schooling, sometimes showed spiral swimming with 40-50 % mortality (de Groof *et al.*, 2015). Originally, scale drop syndrome was believed to be caused by bacteria, *Tenacibaculum maritimum*. Since antibiotic treatment was not effective, the cause of this disease was speculated to be viruses. de Groof *et al.* (2015) identified virus particles from diseased fish and named scale drop disease virus (SDDV). SDDV displays icosahedral symmetry and is characterized by a central DNA-protein complex, an outer proteinaceous capsid formed by major capsid protein (MCP), and an intermediate lipid membrane associated with polypeptides that cover genetic materials. In Thailand, SDDV-infected *Lates calcarifer* case was reported in a farming area of Eastern province in 2016. The case was confirmed by PCR technique using primers specific to *MCP* gene and DNA sequencing (Senapin *et al.*, 2018). And recently, Charoenwai *et al.* (2020) also found the SDDV case in the farming area at Laemsing, Chanthaburi, Thailand. These cases indicate the SDDV is spreading in Thailand.

Vaccines for SDDV protection had previously been developed. Formalin-inactivated and BEI-inactivated SDDV whole virus vaccines showed some protections against SDDV infection in Asian sea bass but weaker than the *E. coli* produced recombinant major capsid protein (rMCP) (de Groof *et al.*, 2015). Because both inactivated viruses and subunit vaccines tend to produce a weaker immune system response than live viruses, immunologic adjuvants and high amounts of antigen were required to provide an effective immune response against the inactivated pathogen.

Self-assembled protein nanoparticles (SAPNs) are oligo-polymerized protein monomers. Thus, they could be designed to accommodate many repetitive protein antigens.

SAPNs can be used as a vaccine by itself or as antigen- presenting particles and surface-displayed antigens, etc. It provides a strong immune response and long- term protection similar to virus-like particles (VLPs). Hervé *et al.* (2017) previously produced SAPNs to elicit an immune response against Respiratory Syndrome Virus (RSV). These SAPNs were made from the nucleoprotein (N) of the virus nucleocapsids, a major target of antigen-specific cytotoxic T-cell response. The self-assembly of N protein protomers led to the formation of supramolecular nano-rings of 15 nm diameter. These SAPNs were found to reduce viral load in the lungs of immunized mice after virus challenge (Hervé *et al.*, 2017). SAPNs could be produced by many expression systems, i. e. bacterial system (Lokesh *et al.*, 2002), yeasts system (Saraswat *et al.,* 2016), plant systems (Calhoun *et al.,* 2007), and baculovirus expression vector system (BEVS) (Lin *et al.*, 2001). The BEVS is an established tool producing a range of recombinant proteins and multi- protein complexes, including virus- like particles in insect cells.

Since viral capsids are protein assemblies with a discrete size, space, and a specific aggregation number, we aimed to produce the recombinant SDDV major capsid protein using the baculovirus expression vector system. It is anticipated that a high antigenicity and stability SAPNs could be formed by the recombinant SDDV major capsid protein (SDDV-MCP) produced in the baculovirus- infected insect cells.

2. Materials and Methods

2.1 Generation of baculovirus expressing vector

SDDV major capsid protein gene (*SDDV-MCP*) in pGEM was provided by the Center of Excellence for Shrimp Molecular Biology and Biotechnology (Centex Shrimp), Mahidol University, Bangkok, Thailand. The *SDDV-MCP* gene was amplified by PCR using forward primer 5′-GCGGATCCATGTCATCTATT GCAGG-3′ and reverse primer 5′-CGCTCGAGT TACAAGATCG GAAATC- 3′ with underlined sequences for *Bam*HI and *Xho*I, respectively. The PCR amplified MCP gene was inserted into *Bam*HI and *Xho*I sites of a donor plasmid, pFastBac™ HTB (Invitrogen, USA). The recombinant plasmid was transformed, using the heat- shock transformation method, into DH10Bac *E. coli* (Bac-to-Bac®, Invitrogen, USA) containing a baculovirus genome (bacmid). The recombinant bacmid was generated after the MCP gene transposition into its Tn7 attachment site within the *lacZ* in *E. coli*. White colonies were selected and MCP gene insertion in the bacmid was confirmed by PCR using primers specific to the *LacZ* (data not shown). Recombinant bacmid was extracted from selected DH10Bac *E. coli* using an alkaline lysis method followed by isopropanol precipitation. *Spodoptera frugiperda* insect cells (Sf9) were grown as monolayer culture at 27 ºC in TNM- FH medium supplemented with 10 % fetal bovine serum (Hyclone, USA). The cell was transfected with recombinant bacmid using Cellfectin® II (Invitrogen, USA) to generate recombinant

baculovirus stock. The recombinant baculovirus was titrated by the endpoint dilution method (O'Reilly *et al.*, 1994).

2.2 SDDV MCP gene expression in insect cells

The *SDDV MCP* gene expression analysis was carried out by reverse transcription-PCR (RT-PCR). Briefly, the recombinant baculovirus-infected insect cells were harvested and extracted by the addition of TRIZOL reagent (Invitrogen, USA), and their total RNA was obtained. Total RNA extract was next treated with DNase I (Thermo Scientific, USA) according to manufacture protocol. The first-strand cDNA synthesis reaction is catalyzed by reverse transcriptase according to manufacture protocol (Thermo Scientific, USA) using oligo (dT) primers. The synthesized cDNA was then used as the template for PCR using primers specific to the MCP gene at 5′ and 3′, as described in 2.1, for detection of the MCP transcription.

2.3 Recombinant SDDV MCP production

Sf9 insect cells were grown as a cell suspension in an Erlenmeyer flask and shake at 120 rpm at 27 °C. When cell density reached 1×10^6 cells/ mL, cells were infected with recombinant baculovirus at the multiplicity of infection (MOI) of 5. Infected cells were harvested at day 3 post-infection by centrifugation at 800×g for 5 minutes and washed twice with phosphate buffer saline (PBS), pH 7.4. The cell pellet was kept at -80 °C freezer for further experiment.

2.4 Recombinant SDDV MCP analysis by SDS-PAGE and Western blot

The infected cell pellet was resuspended with TN buffer (50 mM Tris, 150 mM NaCl, pH 7.8) and lysed using a 1 mL syringe with 25G hypodermic needle. The cell lysate was centrifuged at 16,000xg for 10 minutes to remove cell debris. The supernatant was boiled for 5 minutes with SDS/ PAGE loading buffer. The boiled sample was subjected to 10 % polyacrylamide gel electrophoresis (PAGE), and proteins were visualized by InstantBlue™ Coomassie Protein staining.

Proteins from SDP-PAGE were also transferred from the polyacrylamide gel to a nitrocellulose membrane (Bio-Rad) for Western blot analysis. The membrane was incubated with 5% skim milk in PBST (phosphate buffer saline with 0.1 % Tween-20) for 1 hour at room temperature and then incubated with antibody against 6×His-tag (eBioscience™, Thermo Fisher Scientific, USA) at 1:1,000 dilution in PBST, overnight. After 3 times washing with PBST, the membrane was incubated with horseradish peroxidase-conjugated goat anti-mouse IgG (Cell Signaling Technology, USA) at 1:5,000 dilution in PBST for 2 hours at room temperature. After washing, the membrane was incubated with chemiluminescent substrate (Westar Supernova, Cyanagen, Italy) for 5 minutes in dark condition at room temperature and place in a blot scanner for protein detection or incubated with 3,3',5,5'-tetramethylbenzidine (TMB, Sigma, USA) substrate for 20 minutes at room temperature.

2.5 Recombinant SDDV MCP purification

Infected cells, total cell number at 6×10^7 cells, were lysed as described in 2.4. Two concentrations of sucrose solutions, 25 % (w/v) and 70 % (w/v), were prepared in TN buffer. Half mL of 70% sucrose was first loaded into a 4.4 mL polyallomer centrifuge tube (Thermo Scientific, USA), followed by 1 mL of 25 % sucrose and 2.5 mL of cell lysate at the top. Centrifugation was performed using TH-660 rotor at 150,000×g, 4 ºC for 90 min. Interphase between sucrose layers was collected for transmission electron microscope visualization.

2.6 Recombinant MCP protein analysis by transmission electron microscope

A formvar/ carbon film, 400 mesh, copper grid (Electron microscopy sciences, USA) was placed on top of a drop of protein sample solution for 5 minutes and partially air-dried. The excess sample was removed by filter paper. The sample adsorbed grid was washed twice by placing on the top of water drops followed by 2 % phosphotungstic acid (Fluka, Australia) in deionized water for 1 minute. The access dye was removed, and the grid was air-dried before observed under a transmission electron microscope at 100 kV (HT7700, Hitachi).

3. Results and Discussion

3.1 Generation of baculovirus expressing vector

The baculovirus was genetically engineered to express *SDDV MCP* gene by gene insertion into the baculovirus genome, bacmid. This resulted in recombinant bacmid carrying the *SDDV MCP* gene under the control of the baculovirus strong promoter called polyhedrin. This recombinant bacmid was then introduced into insect cells with Cellfctin® II to facilitate the DNA transduction. Baculovirus gene expression, including *MCP* gene, protein synthesis, and virus particle formation, were then taking place in the transduced insect cells followed by budding out of new recombinant baculovirus progenies. These budded recombinant baculoviruses can be directly infected into neighboring insect cells and hence becoming the expression vector for expression of the *MCP* gene. To determine whether or not this recombinant baculovirus could express the *SDDV MCP* gene in infected insect cells, the MCP mRNA was detected by reverse transcription PCR. Total RNA was extracted from the infected insect cells treated with DNase to remove the remaining DNA that could result in false positive. In this assay, all mRNA including the one transcribed from the *MCP* gene will be reversed to cDNAs. The cDNAs were next used as the template for PCR with MCP gene-specific primers. A PCR band at a similar size to the PCR amplified MCP gene was shown in Figure 1, indicating the presence of MCP cDNA, a representative of MCP mRNA. Thus, the recombinant baculovirus vector successfully delivered and expressed the *MCP* gene in infected Sf9 cells. The recombinant MCP protein was therefore expected to be produced.

3.2 Recombinant MCP protein production

After the *MCP* gene expression had

been confirmed in the recombinant baculovirus-infected insect cells, the recombinant MCP protein synthesis was determined. Infected cells were harvested 3 days post-infection. Cell lysis was carried out, and intracellular proteins in the supernatant were collected after centrifugation. This supernatant was subjected to SDS-PAGE and Western blot analysis. Due to the presence of histidine-tag, provided at the upstream of the MCP gene cloning site of the pFastBac™ HTB, the recombinant MCP protein was expected to be fused with the His-tag at its N-terminal. The calculated molecular weight of the recombinant MCP protein according to their amino acid sequences was approximately 50 kDa. With the extra fusion parts, 6xHis and rTEV protease cleavage site, total molecular weight of the recombinant MCP protein was expected to be approximately 53 kDa. Figure 2 shows a single band at approximately 53 kDa detected by the anti-His monoclonal antibody, suggesting that this protein had His-tag fused. Since the detected protein had a size of calculated molecular weight MCP protein with His-tag fusion and the successful expression of the *MCP* gene, it could be concluded that recombinant MCP protein was produced by the infected Sf9 cells.

3.2 SAPNs formation

As results are shown in 3.1, the recombinant MCP protein was produced intracellularly in the recombinant baculovirus-infected cells. An attempt to purify this protein was made by using ultracentrifugation with sucrose gradients. The intracellular protein fraction was loaded on top of two layers of sucrose solutions, 25 % (w/v) sucrose and 0.5 mL of 70 % (w/v) sucrose in TN buffer. After centrifugation, 3 interphases and 4 layers were observed (Figure 3A). Since only two layers of sucrose gradients were loaded, two more interphases found after ultracentrifugation might be new sucrose gradients generated after top loading with the protein samples.

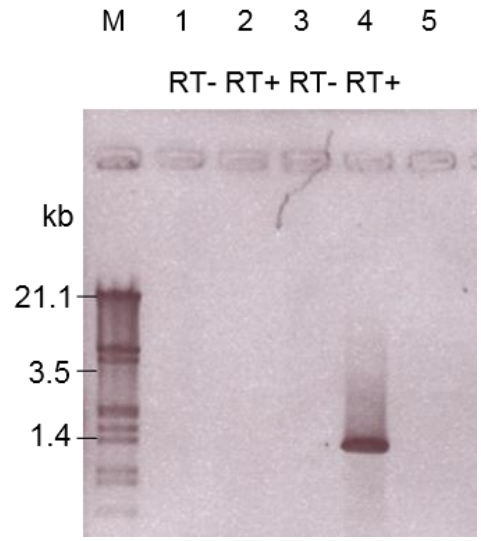

Figure 1 MCP gene expression analysis by reverse transcription PCR using cDNA converted from mRNA obtained from Non-infected Sf9 cells (Lane 1 and 2, without and with reverse transcriptase enzyme, respectively) and recombinant baculovirus infected Sf9 cells (Lane 3 and 4, without and with reverse transcriptase enzyme, respectively). PCR without DNA template was included as a negative control (Lane 5).

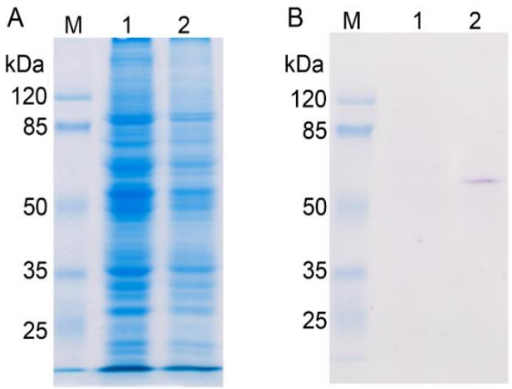

Figure 2 Protein analysis of Sf9 insect cells by SDS- PAGE (A) and Western blot analysis using anti- His monoclonal antibody (B). Non-infect cells (Lane 1) and recombinant baculovirus infected Sf9 cells (Lane 2). Marker (Lane M).

Six samples as labeled in Figure 3A were collected and subjected to SDS- PAGE (Figure 3B) and Western blot analysis (Figure 3C). Western blot results show that a high amount of recombinant MCP protein, shown as more intense bands, were found in the lower layers with higher sucrose concentration, from no. 4 to 5. Some proteins, including the recombinant MCP protein, even passed through the 70 % sucrose (Figure 3C Lane 5 and 6) suggested that they were large- size proteins. These results suggested that the recombinant MCP protein may be in aggregate forms. To verify this, the protein sample in layer no. 5 was visualized by a transmission electron microscope. Electron micrograph revealed particle sizes range from 50 nm to 100 nm (Figure 4), and the majority were small size particles (~50 nm). It seems that the recombinant MCP protein could self- assemble into particles at nano- size, which is a highly suitable size range for optimal interactions with various cells of the immune system (Bachmann and Jennings, 2010; Oyewumi *et al.*, 2010). The high density and structurally ordered antigenic array presented by these MCP SAPNs could provide multiple binding events coincide between the nanoparticle and the immune cell receptors (Lopez-Sagaseta *et al.*, 2016)

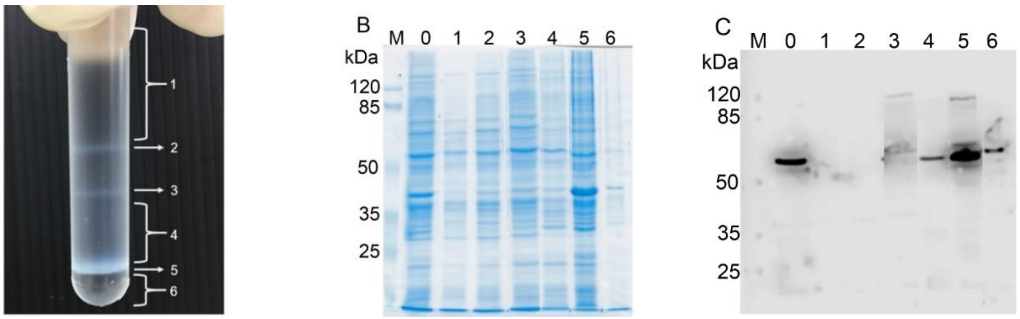

Figure 3 Ultracentrifugation of infected Sf9 cell lysates using double layers (25 and 70 % sucrose layers) (A) Six samples were analyzed by SDS-PAGE (B) and Western blot analysis using anti- His monoclonal antibody (C) Infected Sf9 cell lysate before centrifugation (Lane 0). Proteins in layer no. 1-6 (Lane 1-6, respectively).

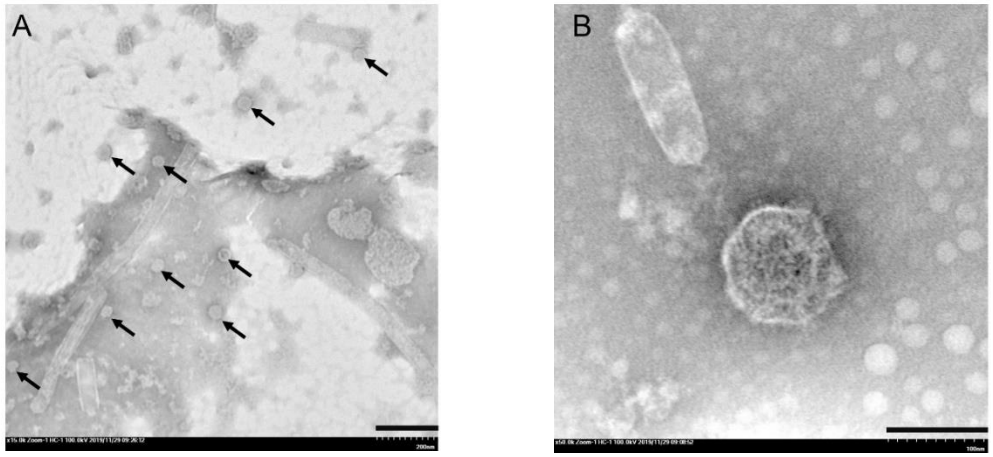

Figure 4 Electron micrographs of recombinant MCP protein obtained from layer no. 5. The protein was negative stained with 2 % phosphotungstic acid and observed under transmission electron microscope at 15,000x (A) and 50,000x (B) magnification respectively. Black arrows indicate assemble form of recombinant MCP protein. The scale bar shown 200 nm and 100 nm for (A) and (B), respectively.

Since many viruses can form hollow protein shells (capsids), protecting their genome from the external environment, it was possible that the recombinant MCP protein could form self-assembled MCP nanoparticles. Milo (2013) suggested that assemble usually takes place in the crowded environment of cells, where ~200 mg/mL of irrelevant cellular proteins are present. The baculovirus expression system should be able to drive the cells to produce recombinant MCP protein at the level that meets this condition. Environmental conditions also play roles in capsid protein assemble or disassemble. For example, ionic stengths vary particle sizes and assemble time since they provide electrostatic repulsion force between the capsid subunit that hinder the assembly of large particle. In this study, 150 mM NaCl was used in the buffer. Most particle sizes observed under transmitted electron microscope were averaged around 50 nm in diameter. This might implicate that this salt concentration promoted 50 nm capsid protein assembly (Lutomski *et al*., 2017).

Protein nanoparticle vaccines are safe due to the lack of genetic materials. They can be easily engulfed by antigen-presenting cells because they have well- defined shapes and sizes resembling a virus particle with a repetitive epitope on their surfaces. This feature constitutes a multivalence, which is able to cross- link B cell receptors and results in the maturation of naïve B cells (Chackerian, 2007). In addition, nanoparticles vaccines have also been reported to induce memory cytotoxic T cell responses against malaria via cross-presentation (McCoy *et al*., 2013). Most importantly, nanoparticle vaccines could be self-adjuvants (Kaba *et al*., 2009; Karch *et al*., 2017).

Therefore, self-assemble MCP nanoparticles is a promising SDDV nanovaccine candidate that offers an excellent solution to the long-standing issue of safety versus immunogenicity.

4. Conclusions

A new type of SDDV vaccine is an alternative to the inactivated vaccine. The subunit vaccine, SAPNs, was produced in insect cells using a genetically engineered baculovirus as a vector to carry the *SDDV MCP* gene to be expressed. After baculovirus infection, insect cells expressed the MCP gene and produced the recombinant MCP protein. These recombinant MCP proteins could assemble and form MCP nanoparticles, as revealed by the transmission electron micrograph. The MCP SAPNs will be further studied on their ability to induce better immune responses than the previously developed SDDV subunit vaccines.

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