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Production of virus-like particles of nervous necrosis virus displaying partial VHSV's glycoprotein at surface and encapsulating DNA vaccine plasmids

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In order to use nervous necrosis virus (NNV) virus-like particles (VLPs) as a delivery tool for heterologous antigens or plasmids, we attempted to produce red-spotted grouper nervous necrosis virus (RGNNV) VLPs displaying a partial region of viral hemorrhagic septicemia virus (VHSV) glycoprotein at the surface and VLPs that are harboring DNA vaccine plasmids within the VLP. A peptide encoding 105 amino acids of VHSV glycoprotein was genetically inserted in the loop region of NNV capsid gene, and VLPs expressing the partial part of VHSV glycoprotein were successfully produced. However, in the transmission electron microscope analysis, the shape and size of the partial VHSV glycoprotein-expressing NNV VLPs were irregular and variable, respectively, indicating that the normal assembly of capsid proteins was inhibited by the relatively long foreign peptide (105 aa) on the loop region. To encapsulate by simultaneous transformation with both NNV capsid gene expressing plasmids and DNA vaccine plasmids (having an eGFP expressing cassette under the CMV promoter), NNV VLPs containing plasmids were produced. The encapsulation of plasmids in the NNV VLPs was demonstrated by PCR and cells exposed to the VLPs encapsulating DNA vaccine plasmids showed fluorescence. These results suggest that the encapsulation of plasmids in NNV VLPs can be done with a simple one-step process, excluding the process of disassembly-reassembly of VLPs, and NNV VLPs can be used as a delivery tool for DNA vaccine vectors.

Key words: Nervous necrosis virus (NNV), Virus-like particles (VLPs), Antigen surface display, Plasmids encapsulation.

Introduction

Virus-like particles (VLPs) are promising candidates for overcoming disadvantages of live vaccines and subunit vaccines in that they are safe and highly immunogenic, respectively. VLPs are usually produced by recombinant molecular technology and have structural and antigenic properties that are comparable to wild-type viral particles (Donaldson et al., 2018). Recently, vaccines based on the VLPs have been extensively investigated in mammalian viruses and several VLP-based vaccines such as Engerix-B for hepatitis B virus and Garasi Cervarix for human papillomavirus have already been commercialized in human medicine (Dai et al., 2018). Also, in fish and shellfish, researches on the utilization of VLPs as prophylactic vaccines or delivery tools for biomolecules have been conducted (Jeong et al., 2020).

Viral nervous necrosis viruses (VNNVs) are belonging to the genus *Beatnodavirus* of the family Nodaviridae, and the genome comprises two pos-

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itive-sense RNA segments, RNA1 encoding the RNA-dependent RNA polymerase and RNA2 encoding the coat protein. NNVs are known to cause massive mortality in cultured marine fish, especially in the larval stage of fish (Munday et al., 2002; Nakai et al., 2009). The induction of protective immunity against NNV by the immunization of fish with inactivated viruses or recombinant capsid proteins has been reported (Husgaro et al., 2001; Yuasa et al., 2002; Pakingking et al., 2009; Yamashita et al., 2009). However, these inactivated or subunit vaccines should be accompany with adjuvants due to poor immunogenicity. There are several papers on the production of nodaviral VLPs and the evaluation of vaccine potential (Thiery et al., 2006; Lai et al., 2014; Jeong et al., 2020).

In the present study, in order to use NNV VLPs as a delivery tool for heterologous antigens or plasmids, we attempted to produce red-spotted grouper nervous necrosis virus (RGNNV) VLPs displaying a partial region of viral hemorrhagic septicemia virus (VHSV) glycoprotein at the surface and VLPs that are harboring DNA vaccine plasmids within the VLP.

Materials and methods

Bacteria and cells

Escherichia coli BL21 (DE3) codon plus was used as a host strain to produce recombinant proteins, and cultured at 37℃ in Luria-Bertani (LB) broth and agar. E11 cells were maintained in Leibovitz medium (L15, Sigma) supplemented with 10% fetal bovine serum (FBS, Welgene) and penicillin-streptomycin (100 U/ml penicillin 100 μg/ml streptomycin, Welgene) at 25℃.

Expression of NNV capsid protein

E-11 cells were infected with RGNNV and total RNA was extracted when about 40% of cytopathic effect (CPE) was observed. Random hexamers (Promega) were used to synthesize cDNA, and the RNA2 gene encoding the capsid protein of NNV was amplified by PCR using primers in Table 1. The amplified product was cloned into a pGEM-T easy vector (Promega) and sequenced. After digestion with NdeI and HindIII, the RNA2 gene was inserted into pET28a+ vector and designated as pVLP. *E. coli* BL21 (DE3)

Table 1. Primers used in this study

Name of primer	Sequence (5' to 3')
For construction of pVLP	
NdeI-VNNRNA2	CATATGGTACGCAAAGGTGAGAAGAAATTGGC_
HindⅢ-VNNRNA2	AAGCTTGTTTTCCGAGTCAACCCTGGTG
For construction of pVLP-vG	
VNNRNA2(810)-vG(68)-F	CCATTGATTACAGCCTTGGAATCACTCAACGACCTCCGGT
VNNRNA2(810)-vG(68)-R	ACCGGAGGTCGTTGAGTGATTCCAAGGCTGTAATCAATGG
vG(384)-VNNRNA2(812)-F	AAACCATCCTGGAGGCAAAGACTGGAGATGTTGACCGTGC
vG(384)-VNNRNA2(812)-R	GCACGGTCAACATCTCCAGTCTTTGCCTCCAGGATGGTTT
For construction of pCMV-E	
NheI-EGFP-F	GCTAGCATGGTGAGCAAGGGCGAG
EcoRI-EGFP-R	GAATTCTTACTTGTACAGCTCGTCCAT
For verification of pCMV-E in VLP-eGFP	
AgeⅠ-CMV F	ACCGGTGCCAGATATACGCGTTGACATTG
EGFP-R	TGCTTGTCGGCCATGATATAG
EGFP-F	GAACCGCATCGAGCTGAA
EcoRⅠ-BGH-R	GAATTCCCATAGAGCCCACCGCAT

Underlined characters represent restriction enzyme site

was transformed with pVLP and incubated in LB broth containing kanamycin (50 μg/ml) at 37℃ until optical density at 600 nm (OD_{600}) reached between 0.5 - 0.6. The cultures were treated with 0.1 mM isopropyl-β-D- thogalactoside (IPTG) at 20℃.

Expression of partial VHSV glycoprotein on the out-warded loop region of NNV VLPs

To insert the partial sequence (68-384 position) of VHSV glycoprotein gene into the loop structure located in the protrusion domain of RGNNV RNA2 gene, both amplified genes were ligated by overlapping PCR using primers in Table 1. The amplified final fragment was cloned into a pGEM-T easy vector and sequenced. The T vector fragment digested with NdeI and HindIII was ligated to pET28a+ vector that was predigested with the same enzymes, and designated as pVLP-vG. *E. coli* BL21 (DE3) was transformed with pVLP-vG, and when the absorbance of bacteria reached between 0.5 - 0.6 at OD_{600}, the protein expression was induced by adding 0.5 mM IPTG for 3 h at 37℃, for 5 h at 27℃, or for 18 h at 20℃. After reaching an OD_{600} between 1.3-1.5, bacteria were pelleted by centrifugation. The bacterial pellets were re-suspended in PBS (pH 7.0) for sonication at 100 Hz (15 s, 4 cycles) and then centrifuged (13,000 rpm, 4℃, 15 min) to separate soluble proteins in supernatant and inclusion bodies in pellet. For the denaturation of proteins, the isolated protein samples were mixed with 5X reducing SDS-PAGE sample buffer (GeneAll), then, boiled to denature proteins. All protein samples were loaded on 10% polyacrylamide gel (ELPIS) and were visualized with Coomassie blue stain. For Western blot analysis, proteins on SDS-PAGE gel were transferred to a nitrocellulose membrane. The transferred membrane was washed with TTBS buffer (0.05% Tween 20 in TBS; 150mM NaCl, 10mM Tris-HCl, pH 7.5) and soaked in blocking buffer (3% bovine serum albumin in TBS, pH 7.5) for 2 h at room temperature (RT). After washing three times with TTBS, the blot was probed with rabbit His-probe IgG (1:2000, Santa Cruz) for 2 h at RT, and washed 3 times with TTBS, then, incubated with goat anti-rabbit alkaline phosphatase conjugated IgG (1:2000, Santa Cruz) for 2 h at RT. After additional washes, membrane was developed by BCIP-NBT (5-bromo, 4-chloro, 3-indolyl phosphate-nitro-blue tetrazolium chloride; Sigma).

Generation of VLPs containing eGFP expressing vector (VLP-eGFP)

Enhanced green fluorescent protein (eGFP) gene was amplified with a primer pair in Table 1. The PCR amplified product was inserted into a pGEM-T easy vector, then, digested with NheI and EcoRI for the ligation to pcDNA3.1+ that was predigested with the same enzymes, and named it as pCMV-E. To produce VLPs containing pCMV-E in the cavity, bacteria were simultaneously transformed with both pVLP and pCMV-E (1:1 ratio), then, selected using kanamycin and ampicillin (100 μg/ml).

Purification of VLPs and transmission electron microscopy

When OD_{600} of inducing bacterial cultures was reached between 1.3 -1.5, bacteria were pelleted by centrifugation. The bacterial pellets were re-suspended in binding buffer (0.5 M NaCl, 5 mM imidazole, 20 mM Tris-HCl, pH 6.9) for sonication at 100 Hz (30 s, 8 cycles). After centrifugation, the supernatants were filtered using a 0.45 μm syringe filter. To purify His tagging VLP from bacterial lysates, the supernatant was flown onto a nickel-based agarose (Ni-NTA) His bind resin chromatographic column (Novagen). After rinsing with washing buffer (0.5 M NaCl, 60 mM imidazole, 20 mM Tris-HCL, pH 7.9), the elution was performed by loading elution buffer (0.5 M NaCl, 0.5 M imidazole, 20 mM Tris-HCL, pH 7.9) for collecting VLPs. The amount of VLPs was measured by bicinchoninic acid assay (BCA).

To observe VLPs size and shape, the purified VLPs were put on a Formvar carbon grid, and the moist

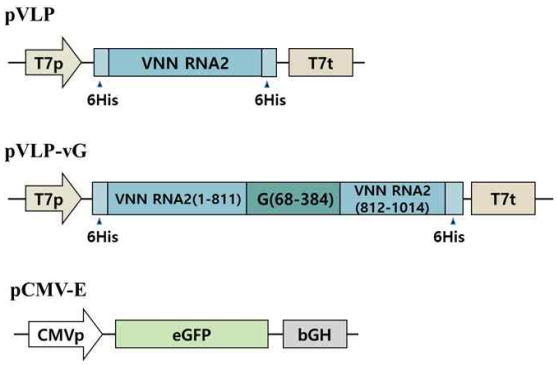

Fig. 1. Schematic representation of the expression cassette in pVLP for the expression of nervous necrosis virus capsid protein (RNA2) gene; pVLP-vG for the insertion of partial glycoprotein gene of VHSV into NNV's out-warded loop region; pCMV-E for the expression of enhanced green fluorescent protein (eGFP) under the CMV promoter. T7p, T7 promoter; T7t, T7 terminator; His, His-Tag.

of the sample was discarded by attaching a piece of paper at the edge of the grid, then, 4% uranyl-acetate was put on the grid for staining. The VLPs were observed by transmission electron microscopy (TEM).

Presence of pCMV-E in VLP-eGFP and in vitro delivery

Total DNA was isolated from purified VLP-eGFP using Exgene Clinic SV kit (GeneAll). The isolated DNA was amplified by PCR using primers in Table 1.

For in vitro delivery of pCMV-E vector using VLPs, E11 cells seeded in 35-mm dish (2 X 10^6 cells/dish) were inoculated with 10 to 80 μg of VLP-eGFP, then incubated at 25℃. After 3 days post-inoculation, cells were rinsed with phosphate-buffered saline (PBS) and supplemented with L-15 containing 10% FBS. Fluorescence of each cell was monitored by a florescent microscope.

Results

Expression of partial VHSV glycoprotein on the out-warded loop region of NNV VLPs

The expression of recombinant VLPs and VLP-vG from bacteria transformed with pVLP or pVLP-vG (Fig. 1) by inducing with 0.1 or 0.5 mM IPTG at 37℃, 27℃ and 20℃ were visualized by western blot (Fig. 2). The VLP was shown as two bands corresponding to monomer (37 kDa) and trimer (about 120 kDa) (Fig. 2A, B). VLP-vG was also shown as two bands corresponding to monomer (55 kDa) and trimer (about 150 kDa) (Fig. 2C).

Transmission electron microscopic analysis of VLPs

To analyze the size and shape of VLPs, each puri-

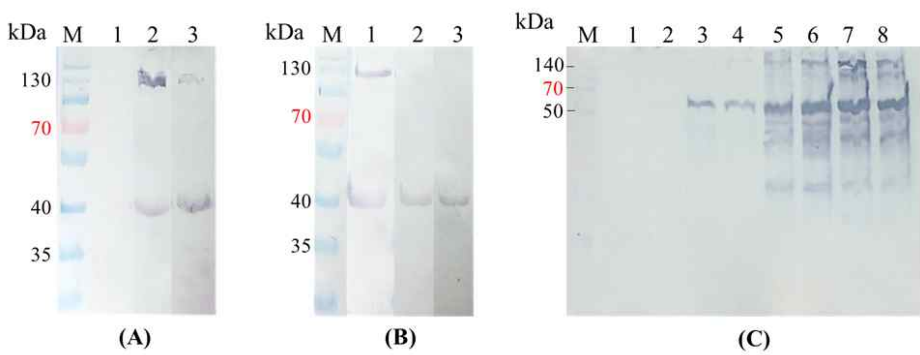

Fig. 2. Western blot analysis of recombinant NNV capsid proteins (A, B) and partial VHSV glycoprotein-inserted NNV capsid protein (C). The transformed *E. coli* with pVLP were treated with 0.1 mM IPTG at 37℃ (lane 1), 27℃ (lane 2), and 20℃ (lane 3), then, supernatant (A) and pellet (B) were separated. (C) *E. coli* transformed with pVLP-vG were treated with 0.5 mM IPTG at 37℃ (lane 1, 5), 27℃ (lane 2, 6), 20℃ (lane 3, 7), and 15℃ (lane 4, 8). supernatant (Lane 1 to 4, Supernatant; Lane 5 to 8, pellet). M indicate pre-stained protein marker (Bio-Rad).

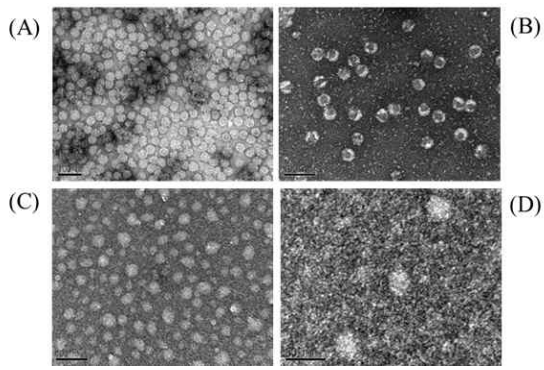

Fig. 3. Transmission electron microscopic analysis of VLPs. The purified NNV VLPs (A), VLP-eGFP (B), and VLP-vG (C) were negative stained on formvar carbon grid and were observed using a transmission electron microscope. (D) RGNNV cultured in E11 cells.

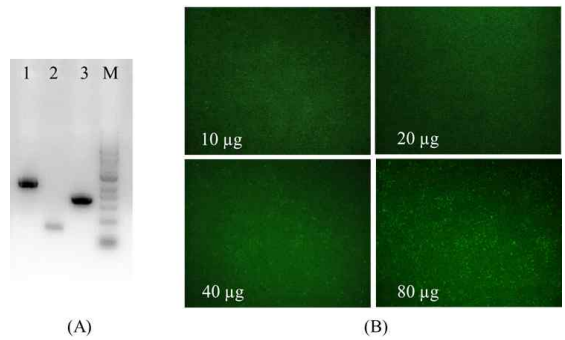

Fig. 4. (A) Analysis of pCMV-E vector in VLP-eGFP. Using total DNA isolated from purified VLP-eGFP, the PCR amplification was done using three primer pairs. Lane 1, from CMV promoter to eGFP ORF; lane 2, partial eGFP ORF; lane 3, from eGFP to bGH; M, DNA 1kb ladder. (B) E11 cells were inoculated with 10 to 80 μg of VLP-eGFP, then, eGFP fluorescence was observed using a fluorescence microscope.

fied VLP sample was observed using a negative stain TEM. The NNV VLPs and pCMV-E plasmid-containing VLPs (VLP-eGFP) were observed as regular shaped and even sized (mean diameter of about 40 nm) particles (Fig. 3A, B), while VLPs expressing partial sequence of VHSV glycoprotein (VLP-vG) were irregular in shape and variable in size (20 to 40 nm) (Fig. 3C).

Presence of pCMV-E in VLP-eGFP and in vitro delivery

To verify the presence of eGFP expression vector (pCMV) in VLPs, DNA was isolated from the purified VLP-eGFP and performed PCR amplification, by which all three regions of vector were detected (Fig. 4A).

When E11 cells were inoculated with 10 to 80 μg of VLP-eGFP, the green fluorescence was observed, and the strength was concentration-dependent (Fig. 4B).

Discussion

VLPs mimic the conformation of native viruses lacking viral genome, and are usually more immunogenic than subunit antigenic proteins (Murata et al., 2003; Roldão et al., 2010; Mohsen et al., 2017). Furthermore, the production of VLPs using *E. coli* expression system without using eukaryotic cell lines can greatly reduce the costs related to vaccine production, which is a critical factor for practical vaccines for aquaculture farms. In this study, the successful production of NNV VLPs using an *E. coli* expression system was demonstrated by electron microscopic analysis, which showed VLPs similar to native viruses in size and shape. The trimerization of NNV capsid proteins in the course of capsid assembly has been reported (Tang et al., 2002; Chen et al., 2015). In the present results, not only monomeric form but also trimeric form was detected in Western blot of NNV VLP capsid proteins despite under the reducing SDS-PAGE condition, suggesting that there may be a strong interaction among capsid proteins to form trimeric assembly.

VLPs can be used as a scaffold for displaying heterologous antigens at the surface (Crisci et al., 2012). A little information is available on the use of NNV VLPs for the surface display of foreign antigens. Xie et al. (2016) reported that a 6× histidine (His)-tag genetically inserted in the surface-exposed loop region

of orange-spotted grouper nervous necrosis virus (OGNNV) capsid gene was displayed at the surface of VLPs, and found that the loop region could carry only short peptides. In this study, we inserted a peptide encoding 105 amino acids of VHSV glycoprotein in the loop region of NNV capsid gene, and successfully produced VLPs expressing the partial part of VHSV glycoprotein. However, in the transmission electron microscope analysis, the shape and size of the partial VHSV glycoprotein-expressing NNV VLPs were irregular and variable, respectively, indicating that the normal assembly of capsid proteins was inhibited by the relatively long foreign peptide (105 aa) on the loop region. The optimum insertional peptide length that does not affect on the stability of NNV VLPs should be further analyzed.

As VLPs can be self-assembled and disassembled, macromolecules such as drugs, enzymes, and plasmids etc. can be encapsulated in the VLP cavity, enabling efficient delivery of macromolecules into target cells (Takamura et al., 2004; Donaldson et al., 2015). In nodaviruses, although Jariyapong et al. (2014, 2015) successfully produced VLPs of *Macrobrachium rosenbergii* nodavirus (MrNV) containing plasmid DNA or double-stranded RNA, they used the artificial process of disassembly-reassembly of VLPs to encapsulate the macromolecules, which are very laborious and unsuitable for aquaculture farms in an economic aspect. Therefore, in this study, we attempted to encapsulate plasmids into NNV VLPs by co-transformation of *E. coli* with NNV capsid gene expressing plasmids and DNA vaccine plasmids (having an eGFP expressing cassette under the CMV promoter). The encapsulation of plasmids in the NNV VLPs was demonstrated by PCR and cells exposed to the VLPs encapsulating DNA vaccine plasmids showed fluorescence. These results suggest that the encapsulation of plasmids in NNV VLPs can be done with a simple one-step process, excluding the process of disassembly-reassembly of VLPs, and NNV VLPs can be used as a delivery tool for DNA vaccine vectors.

In summary, the present study shows that NNV VLPs can be a delivery tool for foreign antigens by displaying at the surface and for DNA vaccine vectors by the natural encapsulation. Since this study focus only on the preparation of NNV VLPs and in vitro delivery of foreign proteins, further studies on the in vivo delivery and immunization efficacy in fish should be conducted.

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