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Stability of virus-like particles of red-spotted grouper nervous necrosis virus in the aqueous state, and the vaccine potential of lyophilized particles

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ABSTRACT

Virus-like particles (VLPs) are multi protein complexes mimicking the structural properties of the native virus. The development of freeze-dried formulations of such complex protein structures remains a challenge. Red-spotted grouper nervous necrosis virus (RGNNV) causes mass mortality in fish culture, and RGNNV VLPs have been suggested to be promising vaccine candidates. In the present study, the stability of RGNNV VLPs in the liquid state was investigated over a 4-week period, along with the influence of freeze-drying on VLP stability. RGNNV VLPs were completely degraded after one week at 37 °C followed by 3 weeks at ambient temperature, and they were partially degraded after 4 weeks at 4 °C. Therefore, the inherent stability of RGNNV VLP in an aqueous milieu is insufficient for long-term storage. When RGNNV VLPs were freeze-dried in the presence or absence of sugar stabilizers, sorbitol was found to improve VLP stability whereas mannitol reduced it. VLP preparations freeze-dried with sorbitol or without stabilizer were as immunogenic as control (non-freeze dried) VLPs, whereas VLPs freeze-dried in mannitol were less immunogenic. These results indicate that freeze-dried RGNNV VLPs have potential as vaccines.

1. Introduction

Virus-like particles (VLPs) are multiprotein structures that mimic the organization and conformation of authentic native viruses but lack the viral genome [1]. VLPs have received much attention as next-generation vaccine platforms. Traditional virus vaccines use the virus itself, after inactivation, as vaccine antigen. However, the inactivation process can lead to a reduction in immunogenicity because of structural alterations [2]. Since VLPs do not contain the genetic material of the native virus but possess immunogenic properties similar to the native virus [3] they require no inactivation process and are recognized to be safer. At the same time, they are much larger and more complex than most protein-based biopharmaceuticals: their molecular weights are in the megadalton range, unlike therapeutic monoclonal antibodies (150 kDa) [4,5]. Moreover, the structural and immunogenic properties of VLPs are influenced by factors such as ionic strength, temperature, pH and choice of host cell [3]. Therefore, identifying the unique properties of a VLP preparation is critical for controlling its quality as a vaccine.

Nervous necrosis viruses (NNV) are non-enveloped RNA viruses and the causative agents of viral encephalopathy and retinopathy (VER) of fish [6]. NNVs are one of the most serious threats to the larval and juvenile stages of fish. They occur in at least 70 types of cultured or wild marine fish as well as fresh water fish [6]. Outbreaks have caused serious economic losses in aquaculture with a mortality rate reaching 100% [7]. Red-spotted grouper NNV (RGNNV) is recognized as one of the most important species because VER outbreaks are mainly caused by the RGNNV [8]. The control of infections by NNV has to be particularly strict because they are transmitted horizontally as well as vertically [9,10]. Formalin-inactivated, recombinant capsid protein and VLP vaccines have been proposed [11–13]. The capsid proteins of RGNNV produced by recombinant genetic techniques self-assemble into VLPs of 25 nm diameter, and in previous observations immunization of fish with NNV VLPs provided marked protective immunity against RGNNV [14].

Both efficacy and cost-effectiveness are significant considerations in fish vaccination. A vaccine is not useful if the cost of vaccination exceeds the economic loss caused by the pathogen. A degree of cost-saving in vaccine production can be achieved by genetic recombination technology because it facilitates large scale production and generates

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high yields. However, protein antigens are generally unstable in the liquid state, and the need for a cold chain is a drawback in terms of practical use of a vaccine [15].

Generally, the use of cold chains is unaffordable for users as well as manufacturers of fish vaccines. Most VLP-based vaccines have been developed for human applications, with the use of cold chains and stabilizers in mind. Meanwhile little attention has been paid to the inherent instability of VLPs in solution, and there have been few attempts to investigate the influence of freeze-drying on VLP antigens. Recovering such multi protein complexes after freeze-drying without losses of vaccine potency remains a major challenge [16].

Freeze-drying can provide the best way to increase the stability of protein-based biopharmaceuticals and eliminate the need for cold chains. In the present study, the stability of RGNNV VLPs in the liquid state over a four-week period was investigated, and the structural properties and immunogenicity of freeze-dried preparations were examined for the first time.

2. Material and methods

2.1. Ethics

Five-week-old female BALB/c mice were obtained from Orientbio Inc (Seongnam, South Korea) and acclimatized for 1 week prior to VLP immunization. Animal experiments were conducted in accordance with National Institutes of Health guidelines for the care and use of laboratory animals (NIH Publications No. 8023) and with the guidelines for animal experiments of Chung-Ang University.

2.2. Preparation of RGNNV VLPs

RGNNV capsid protein was produced and purified as described previously [17]. *Saccharomyces cerevisiae* producing RGNNV capsid protein was cultured in flasks containing YPDG medium (150 ml, composition: 3% glucose, 3% galactose, 1% yeast extract, 2% peptone) at 30 °C, with shaking at 230 rpm, cultured for 3 days and harvested by centrifuging at 4090 × *g* for 10 min. The cells were disrupted by vortexing with glass beads (Biospec Products, USA), and debris was removed by centrifuging at 16,200 × *g* for 15 min. The capsid protein in the supernatant was purified by heparin chromatography using 2 ml POROS50 HE resin (Applied Biosystems, USA). Elution fractions containing capsid protein were dialyzed against storage buffer (10 mM Tris, 0.5 M NaCl, 50 mM L-glutamine, 50 mM L-arginine, 0.5% glycerol, 0.05% Tween 80, pH 7.6).

2.3. SDS-PAGE analysis

Samples were diluted in storage buffer, mixed with Laemmli 2X PAGE buffer and heated at 87 °C for 10 min. Proteins were fractionated on 12% polyacrylamide gels and visualized by silver staining.

2.4. Size-exclusion chromatography

Size-exclusion chromatography (SEC) was performed as described previously [18]. RGNNV VLP samples (80 μg of RGNNV capsid protein) were loaded on Superose-6 resin (1.5 × 32 cm, GE Healthcare) and equilibrated with Tris-based running buffer (10 mM Tris, 0.5 M NaCl, 0.01% Tween 80, pH 7.6). The loaded protein was eluted at a flow rate of 0.3 ml/min and elution profiles of the proteins were acquired using Autochrome 2000 software (Young Lin Instrument Co., South Korea). Twenty-seven fractions (1 ml each) were collected, and capsid protein was detected by indirect enzyme-linked immunosorbent assay.

2.5. Indirect enzyme-linked immunosorbent assay (ELISA) for detecting RGNNV capsid protein

Fractions from SEC were diluted 1:100 in phosphate-buffered saline (PBS), and 96-well microplates (Greiner Bio One, Austria) were coated overnight with the dilutions at 4 °C. The plates were blocked with 5% skim milk in PBST (PBS containing 0.05% Tween 20) at room temperature (RT, 20–25 °C) for 2 h. Capsid protein was detected using mouse anti-RGNNV capsid protein polyclonal antibody (1:10,000 dilution; prepared in 0.5% skim milk in PBST) at 37 °C for 40 min, followed by horseradish peroxidase (HRP)-conjugated anti-mouse IgG antibody (Bethyl, USA) at 37 °C for 40 min. Color was developed with *o*-phenylenediamine (Sigma, USA), and measured at 492 nm.

2.6. Freeze-drying of RGNNV VLPs and recovery from freeze-drying

RGNNV capsid protein from all freeze-drying conditions was prepared at 0.1 mg/ml by dilution with storage buffer. Non-stabilizer conditions refer to use of storage buffer alone. Sorbitol/Mannitol plus conditions contain 10% (w/v) sorbitol and mannitol, respectively. The pH of all suspensions was adjusted to 7.6 prior to freeze-drying. Samples were frozen at −80 °C and lyophilized in a freeze-dryer (Martin Christ, Germany). For recovery, the lyophilized samples were resuspended in 1 ml distilled water, and contents of capsid protein and proportions of VLPs were analyzed by SDS-PAGE and SEC, respectively.

2.7. Transmission electron microscopy (TEM) analysis of RGNNV VLPs

RGNNV VLPs were absorbed onto carbon-coated grids (TED PELLA INC., USA) and negatively stained with 2% uranyl acetate. TEM was performed on an energy-filtering microscope (LIBRA 120, Carl Zeiss, Germany) at a final magnification of 50,000 X.

2.8. Immunization of mice with RGNNV VLPs

Sixty female BALB/c mice were divided into five groups (PBS, control, non-stabilizer, +sorbitol and +mannitol). Mice received three subcutaneous injections of 2 μg of RGNNV VLPs at two-week intervals. The PBS group received 100 μl of PBS per dose. Blood samples were collected ten days after the third immunization. Sera were stored at −20 °C for further study.

2.9. Determination of IgG titers of anti-RGNNV capsid protein

Anti-RGNNV IgG titers were determined as described previously [19]. 96-well microplates (Greiner Bio One) were coated with 100 ng/well of purified RGNNV VLPs and blocked with 5% skim milk in PBST. The plates were incubated with serial dilutions of mouse sera at 37 °C for 1 h, followed by HRP-conjugated anti-mouse IgG (Bethyl) at 37 °C for 40 min. Color was developed as described above. Endpoint titers were established at OD 2× the OD of control sera (mice immunized with PBS).

2.10. Determination of anti-RGNNV neutralizing antibody titers

Anti-RGNNV neutralizing antibody titers were determined as described previously [14] with modifications. E−11 cells were seeded at 3×10^4 cells per well in 96-well tissue culture plates (BD Bioscience, USA) and cultured for three days at 25 °C in L-15 medium (Gibco, USA) containing 5% fetal bovine serum (GenDEPOT, USA), 1% penicillin-streptomycin (Invitrogen, USA) and 50 μg/ml of normocine (InvivoGen, USA). Sera were serially diluted from 1:150 to 1: 328,050 with L-15 medium, then mixed 1:1 with RGNNVs prepared in Hanks' Balanced Salt solution (2×10^3 $TCID_{50}$). In the present study, RGNNV strain SgNag05 (kindly donated by Professor Toyohiko Nishizawa, Chonnam National University) was used. The mixtures were incubated at RT for