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Development of a Recombinant Protein Vaccine Based on Cell-Free Protein Synthesis for Sevenband Grouper *Epinephelus septemfasciatus* Against Viral Nervous Necrosis

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Sevenband grouper, *Epinephelus septemfasciatus*, is becoming an important aquaculture species in Korea. However, viral nervous necrosis disease is a large problem causing mass mortality in sevenband grouper aquaculture. Recombinant protein vaccines are one of the best methods to reduce these economic losses. However, the cell-based expression method mainly produces inclusion bodies and requires additional procedures. In this study, we expressed a recombinant viral coat protein of sevenband grouper nervous necrosis virus (NNV) using a cell-free protein synthesis system. The purified recombinant NNV coat protein (rNNV-CP) was injected into sevenband grouper at different doses followed by a NNV challenge. Non-immunized fish in the first trial (20 μg/fish) began to die 5 days post-challenge and reached 70% cumulative mortality. In contrast, immunized fish also starting dying 5 days post-challenge but lower cumulative mortality (10%) was observed. Cumulative morality in the second trial with different doses (20, 4, and 0.8 μg/fish) was 10%, 40%, and 50%, respectively. These results suggest that rNNV-CP can effectively immunize sevenband grouper depending on the dose administered. This study provides a new approach to develop a recombinant vaccine against NNV infection for sevenband grouper.

Keywords: Viral nervous necrosis (VNN), nervous necrosis virus (NNV), cell-free protein synthesis (CFPS), recombinant protein, sevenband grouper

Introduction

Viral nervous necrosis (VNN) is a significant fish disease that causes severe economic losses in marine aquaculture worldwide. It has been reported in a variety of marine aquaculture species, including rock bream (*Oplegnathus fasciatus*), olive flounder (*Paralichthys olivaceus*), barramundi (*Lates calcarifer*), turbot (*Scophthalmus maximus*), and sevenband grouper (*Epinephelus septemfasciatus*) since the first report in bigeye trevally (*Caranx sexfasciatus*) in the 1980s [14, 22, 29]. VNN-affected fish develop vacuolization in the central nervous system with abnormal swimming behavior and record high mortality rates (up to 100%) in larvae and juvenile stages [23].

Nervous necrosis virus (NNV), the causative agent of VNN, is a small non-enveloped icosahedral virus containing bi-segmented single-strand positive RNA as the genetic material. RNA1 (approximately 3.1 kb in length) encodes an RNA-dependent RNA polymerase for viral replication, whereas RNA2 (1.4 kb) encrypts a viral capsid protein. NNV belongs to the family *Nodaviridae* and the genus *Betanodavirus*. Betanodaviruses are divided into four genogroups based on the RNA2 sequence: barfin flounder nervous necrosis virus (BFNNV), red-spotted grouper nervous necrosis virus (RGNNV), striped jack nervous necrosis virus (SJNNV), and tiger puffer nervous necrosis virus (TPNNV) [24].

Because of the global spread of NNV among various fish species, vaccines for this virus are urgently required. Several NNV vaccines have been developed, such as a formalin-inactivated vaccine, a recombinant protein vaccine, a DNA vaccine, a double-stranded RNA Poly(I:C), live

vaccine, and virus-like particle vaccine [5, 18, 26, 28, 33, 34, 36]. A recombinant protein vaccine using an *Escherichia coli* cell-based system is a particularly popular tool to develop vaccines. However, only a few vaccines are commercially available, as cell-based protein synthesis is tedious and labor intensive.

Cell-free protein synthesis (CFPS) systems derived from crude cell extracts have been established as a research tool for protein expression [3]. CFPS has several advantages compared with a traditional cell-based method, including easy modification of reaction conditions depending on the target protein [12]. Moreover, the cell-free format reduces the number of protein synthesis steps by avoiding the additional gene-cloning step, enabling faster development [11, 37]. The absence of a cell wall makes active monitoring easy and sampling rapid, and improves direct manipulation of the protein synthesis process [11, 37]. CFPS has emerged as a powerful technology to satisfy the growing demand for simple and efficient intact protein production [3]. CFPS is useful for producing vaccines and drug delivery and high-throughput production for protein libraries in structural genomics [7–9, 11]. Bundy *et al*. [2] reported that the MS2 bacteriophage coat protein expressed by CFPS can be produced at a 14 times higher yield than that of other systems. In addition, infectious poliovirus has been produced by mixing the RNA with a cell-free extract of human cells without parental genomes or progeny viruses [35].

Since the first report of VNN in Korea in 1989, a VNN outbreak has been detected every year from various cultured marine fish, such as sevenband grouper, rock bream (*Oplegnathus fasciatus*), red drum (*Sciaenops ocellatus*), and olive flounder (*Paralichthys olivaceus*) [4, 27, 31, 32]. Sevenband grouper is becoming a popular aquaculture species in Korea. However, VNN outbreaks have been detected in sevenband grouper aqua-farms during the summer, resulting in huge economic losses [13].

In this study, we developed a recombinant protein vaccine using the CFPS with an NNV Korean isolate (SGYeosu08). We surveyed the effect of the vaccine on sevenband grouper with a NNV challenge. This study provides a new approach to develop a recombinant protein vaccine against NNV infection for sevenband grouper.

Materials and Methods

Fish and Virus

Sevenband grouper was purchased from an aqua-farm with no history of VNN. Prior to the experiments, 10 fish were sampled randomly from the stock, and brain samples were examined for betanodavirus by reverse transcription polymerase chain reaction (RT-PCR) analysis according to a previous report [25]. The NNV (SGYeosu08) used in this study was isolated in 2008 from Yeosu, Korea and propagated in the striped snakehead (SSN-1) cell line. SSN-1 cells were grown at 25°C in Leibovitz L-15 medium (Sigma Aldrich, St. Louis, MO, USA) with 10% fetal bovine serum (Gibco, Grand Island, NY, USA), 150 U/ml penicillin G, and 100 μg/ml streptomycin. NNV was inoculated on a confluent SSN-1 cell monolayer and incubated at 25°C for viral replication. Viral samples were aliquoted in small volumes and stored at −80°C until use.

NNV Coat-Protein Gene Cloning

Viral RNA was extracted from cultured viral samples using the miRNeasy Mini Kit (Qiagen, Madison, WI, USA), and cDNA was synthesized using M-MLV reverse transcriptase (Bioneer, Deajeon, Korea) following the manufacturer's protocols. The cDNA was amplified in 20 μl of a PCR mixture containing 5 μl of 10× Ex Taq buffer, 4 μl of 2.5 mM dNTP mixture (each), 0.5 μl of Ex Taq (5 U/μl), and 20 pmol of the PCR primer set. The PCR conditions were pre-denaturation at 95°C for 5 min, 30 cycles of denaturation for 1 min at 95°C, 1 min annealing at 58°C, and 1 min extension at 72°C, followed by a 5 min final extension at 72°C. The amplified products were purified using a gel extraction kit and cloned into the pCR2.1 vector using a Topo cloning kit (Invitrogen, Carlsbad, CA, USA). The primers were re-designed to harbor specific restriction enzyme sites to clone the full open reading frame of the NNV coat protein gene into the pET23(a) vector. The stop codon nucleotide sequence was substituted in the reverse primer to express the N-terminal histidine tag of the expressed protein. The amplified NNV coat protein gene with the new primer set was purified and subcloned using the method described above. All primers were designed based on the viral genome sequence reported previously [15].

Cell-Free Protein Synthesis

The recombinant NNV coat protein (rNNV-CP) was synthesized using the cell-free protein expression method and the ExiProgen protein synthesis system (Bioneer), according to the manufacturer's instructions. Briefly, the pET vector containing the NNV coat protein gene was incubated with an *E. coli* extract that included T7 RNA polymerase, ribosomes, and tRNA required for protein expression at 30°C. The target protein was expressed through transcription of the DNA sequence into the mRNA sequence followed by translation of the mRNA into a protein using amino acids and an energy source. The proteins expressed were purified by Ni-affinity column chromatography, and the purified proteins were dissolved in the supplied storage buffer containing 50 mM Tris-Cl (pH 7.6), 100 mM NaCl, 1 mM DTT, 0.1 mM EDTA, 0.05% NaN_3, and 50% glycerol.

rNNV-CP Vaccination Experiment

Forty fish (mean body weight, 10 g) were divided into three

tanks (groups A, n = 15; B, n = 15; and C, n = 10) and reared at 25°C. Fish in group A were injected intramuscularly with NNV at a dose of $10^{2.8}$ $TCID_{50}$/fish. Fish in group B were administered 20 μg rNNV-CP/fish, and fish in group C were injected intramuscularly with L15 medium as a control. Fish in groups B and C were challenged with NNV at a dose of $10^{2.8}$ $TCID_{50}$/100 μl/fish after 14 days of rNNV-CP or L15 medium injections. The challenged fish were reared and observed daily for 2 more weeks.

Dose-Dependent Vaccination Experiment and NNV Titrations of Challenged Fish

Forty fish (mean body weight, 20 g) were reared in four tanks (n = 10/tank). Thirty fish (n = 10/dose) were administered rNNV-CP intramuscularly at doses of 20, 4, and 0.8 μg/fish, respectively, and the remaining 10 fish were injected with L15 medium as a control. All fish were challenged with NNV at a dose of $10^{3.8}$ $TCID_{50}$/fish 2 weeks later. The challenged fish were observed daily for 2 more weeks. Brain tissues of all challenged fish (dead or alive) were collected for titration of NNV. The tissue samples were homogenized with nine volumes of L15 medium and centrifuged at 5,000 ×g for 20 min, and the supernatant was used for virus titration. Viral infectivity was calculated according to the Reed and Muench method [30].

Results

rNNV-CP Synthesis Using CFPS

The sodium dodecyl sulfate-polyacrylamide gel electrophoresis results for the rNNV-CP are shown in Fig. 1. The molecular mass of expressed rNNV-CP was approximately 41.5 kDa, which was the same size as the deduced amino acids from RNA2. The expressed protein was purified successfully using affinity reactions between Ni-NTA magnetic nanoparticles and the six-histidine tag attached at the C-terminus of the protein (Fig. 1). rNNV-CP was obtained as a soluble fraction at a concentration of about 0.2 mg/ml.

rNNV-CP Vaccination Experiment

The mortality of fish challenged with NNV at a dose of $10^{2.8}$ $TCID_{50}$/fish is shown in Fig. 2. NNV infection caused mortality in the sevenband grouper. Most of the dead fish showed abnormal swimming behavior and dis-equilibrium. Fish in group A began to die 4 days after the NNV challenge and showed 60% cumulative mortality. The immunized (group B) and non-immunized fish (group C) were challenged with NNV ($10^{2.8}$ $TCID_{50}$/fish) 11 days after the rNNV-CP injection. Fish injected with L-15 medium (group C) began to die on day 16 (day 5 post-challenge) and showed 70% cumulative mortality. In contrast, the immunized fish

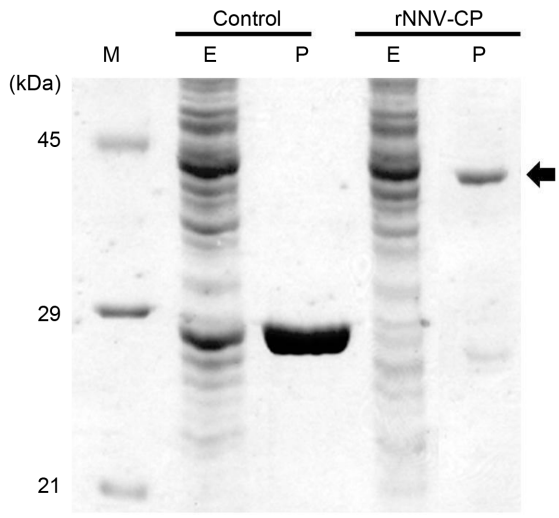

Fig. 1. rNNV-CP was expressed and purified using cell-free protein synthesis.

Each expressed and purified sample was subjected to 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (arrow indicates rNNV-CP). Control, positive control (AcGFP); rNNV-CP, recombinant NNV coat protein. Lane M: protein size marker; lane E: unpurified expressed sample; and lane P: purified sample.

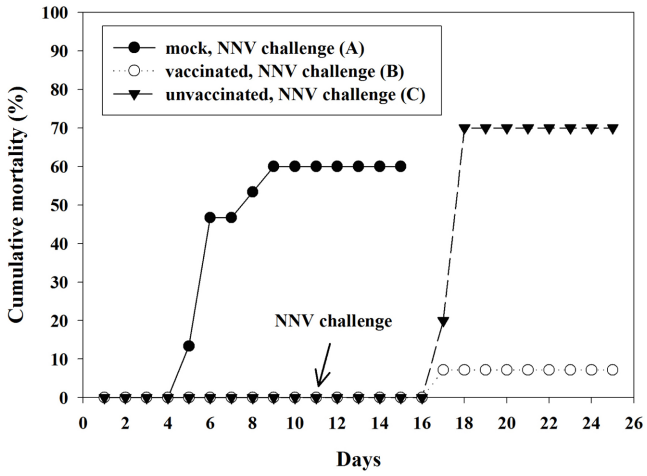

Fig. 2. Mortalities in nervous necrosis virus (NNV)-infected sevenband grouper.

(**A**) Naïve fish infected with NNV at a dose of $10^{2.8}$ $TCID_{50}$/fish. (**B**) Fish injected with the purified recombinant NNV coat protein (rNNV-CP). (**C**) Fish injected with L15 medium (control). The arrow indicates the NNV challenge day at a dose of $10^{2.8}$ $TCID_{50}$/fish.

(group B) also started to die on day 16 but showed only 10% cumulative mortality, which was significantly lower than that in the controls (groups A and C cumulative mortality, 10%).

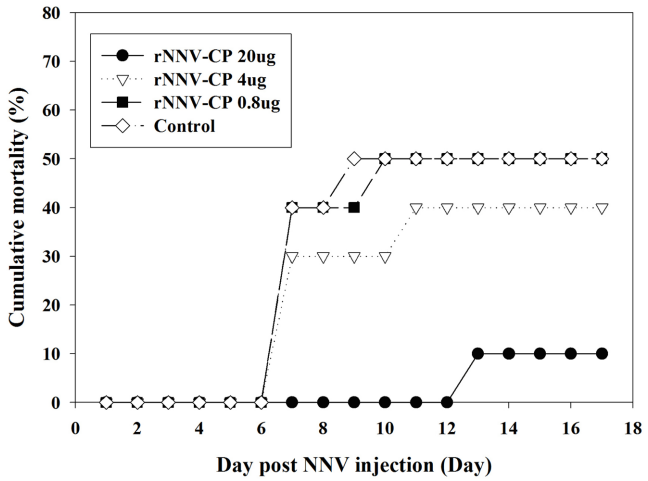

Fig. 3. Mortalities in nervous necrosis virus (NNV)-infected sevenband grouper followed by immunization with various doses of the purified recombinant NNV coat protein (rNNV-CP).

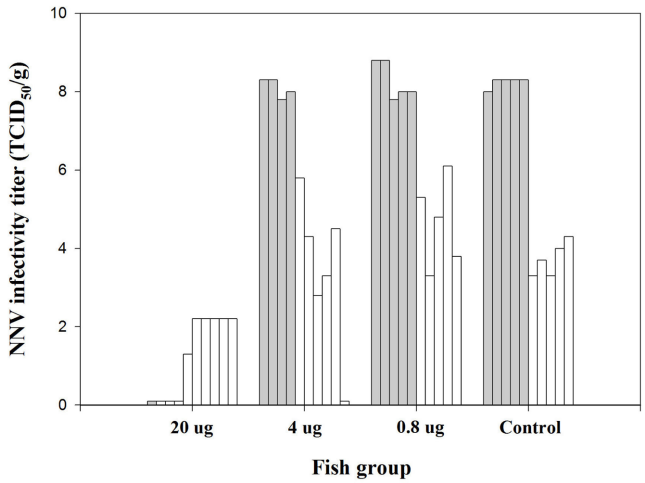

Fig. 4. Comparison of nervous necrosis virus (NNV) infectivity titers in brain of dead and surviving sevenband grouper in the dose-dependent vaccination experiment.
Grey bars indicate dead fish and white bars indicate surviving fish.

Dose-Dependent Vaccination Experiment

Mortalities following the NNV challenge at different rNNV-CP doses are shown in Fig. 3. Purified rNNV-CP was administered intramuscularly at doses of 20, 4, and 0.8 μg/fish, and L15 medium was used as a control. All fish were challenged with NNV 2 weeks later at a dose of $10^{3.8}$ $TCID_{50}$/fish. The non-immunized group began to die on day 6 and showed 50% cumulative mortality. The groups injected with 0.8 and 4 μg rNNV-CP showed 50% and 40% cumulative mortalities, respectively, whereas the group injected with 20 μg rNNV-CP showed 10% mortality. These results demonstrate rNNV-CP dose-dependent mortality from the NNV infection.

NNV Titrations of Challenged Fish

Forty fish samples from the dose-dependent experiments (n = 10/group) were used to determine the viral infectivity titers (Fig. 4). The titers of all dead fish were $>10^{7.8}$ $TCID_{50}$/g, whereas those of surviving fish in the groups administered L15 medium and 0.8 and 4 μg rNNV-CP had a range of titers from $\leq 10^{1.8}$ (under the detection limit) to $10^{6.05}$ $TCID_{50}$/g. Interestingly, all fish in the 20 μg immunized group had very low titers ($\leq 10^{2.3}$ $TCID_{50}$/g), and NNV was not detectable from one dead fish in this group, suggesting that the fish did not die from the NNV infection. This result demonstrates that administering rNNV-CP at a dose of 20 μg/fish was effective for protecting sevenband grouper from NNV infection.

Discussion

Vaccines are available commercially for more than 17 species of fish and have been developed mainly against bacterial diseases, until now [1]. Although a large number of viral disease vaccines have been licensed, only a few are used in the aquaculture industry. Recombinant protein vaccines have a number of advantages, including low production cost and safety [6]. *E. coli* is the most desirable host for recombinant protein expression; however, insoluble and/or nonfunctional proteins can be produced in this system. Recombinant proteins are expressed as either soluble or insoluble protein when overexpressed in *E. coli*. Soluble proteins are properly folded and can function biologically, whereas insoluble proteins aggregate into inclusion bodies, making them easy to purify, but they are improperly folded and biologically inactive. Therefore, insoluble forms must be solubilized and refolded by several additional processes, which are often expensive and time consuming. Lorenzen *et al*. [21] renatured a VHSV glycoprotein that was overexpressed in *E. coli* to immunize rainbow trout. Most previous studies on a recombinant vaccine against NNV used inclusion bodies [10, 33, 36] or spent time purifying the appropriately folded protein [17, 18]. Thus, it is necessary to develop a rapid method to produce soluble recombinant proteins.

CFPS has advanced as a recombinant protein synthesis tool in the past decade [3]. *E. coli*-based CFPS platforms

have been developed to greatly improve the manufacturability of viral recombinant proteins [3]. For example, Bundy *et al*. [2] synthesized the bacteriophage MS2 coat protein and truncated human hepatitis B core antigen using the CFPS system. Furthermore, viral structural proteins have been successfully expressed and CFPS has been used for drug delivery and gene therapy agents [2].

Viral coat protein spontaneously encapsulates the RNA transcripts when it is folded [20]. Poly(I:C) and RNA transcripts induce an antiviral status in fish [16]. Moreover, previous research demonstrated that not only native NNV but also virus-like particles of NNV induce interferon response [17, 19]. Similar to our study, Lai *et al*. [17] produced recombinant coat protein of orange-spotted grouper NNV, but based on the conventional *E. coli* expression system, and revealed that this protein induced the interferon pathway. However, it is difficult to produce recombinant coat protein that is not including any transcript at the present time. It could be another great suggestion to address indirect effects of transcripts encapsulated in recombinant coat protein.

NNV is a very small virus containing bi-segmented RNAs (called RNA1 and RNA2) as genetic material. RNA1 encodes a RNA-dependent RNA polymerase, whereas RNA2 encrypts a coat protein. Because only RNA2 encodes a viral coat protein, this gene was used for producing a recombinant protein similar to other studies. The expressed proteins were purified by Ni-affinity column chromatography, which is a widely used tool to purify expressed target protein harboring 6× histidine tag. Then, since we assumed the storage buffer is also one of the constituents of the CFPS system that we used, we were not concerned about storage buffer affects. In addition, it is necessary to compare with virus challenging experiment that used medium as a negative control. Thus, we injected medium into fish for the unvaccination group.

Tanaka *et al*. [33] reported a similar mortality rate (10%) from experiments using insoluble recombinant proteins produced by *E. coli*. However, they injected 60 mg of the recombinant protein/fish twice, which was three times higher than that used in the present study. The insoluble protein is an unfolded form of the viral coat protein, whereas the soluble form is similar to the intact NNV coat protein. Thus, the soluble form is more effective to immunize fish against NNV than the insoluble fraction. Lai *et al*. [17] suggested that the three- dimensional structure of the protruding surface of the recombinant protein is important for stimulating the host immune system. They immunized orange-spotted grouper (*Epinephelus coioides)* with a purified orange-spotted grouper NNV recombinant coat protein and detected NNV-specific antibodies [17]. Those authors injected the purified recombinant protein at a dose of 1.5 μg/g body weight, which was sufficient to stimulate a high specific antibody titer. We used 0.77 μg/g body weight (20 μg/mean 26 g body weight in the second trial) and successfully protected fish from death due to NNV infection (Fig. 3). This result suggests that rNNV-CP had a more powerful vaccination effect at a low concentration. Besides this, it is easy to scale up to industrial capacity because the rNNV-CP manufacturing process is based on CFPS. The CFPS reaction components and conditions are easily manipulated and improved, making the protein synthesis amenable to automation and miniaturization [12]. Moreover, improvements in translation efficiency yield exceed 1 mg protein/ml of reaction medium [12]. Thus, protein production using CFPS is cost-effective compared with that of conventional *E. coli* expression systems.

In conclusion, rNNV-CP was successfully expressed and purified using CFPS. Sevenband grouper was effectively immunized against NNV infection using rNNV-CP. Administering different doses (0.8, 4, and 20 μg/fish) resulted in different mortality rates. Administering rNNV-CP at a dose of 20 μg/fish successfully protected all fish from death against NNV challenge. This is the first study to produce recombinant protein of a fish virus based on CFPS and apply it as a vaccine for a fish viral disease. This study offers a new innovative approach to develop recombinant vaccines.

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