Fish & Shellfish Immunology 35 (2013) 351–356

Contents lists available at SciVerse ScienceDirect

Fish & Shellfish Immunology

journal homepage: www.elsevier.com/locate/fsi

Development of a novel candidate subunit vaccine against Grass carp reovirus Guangdong strain (GCRV-GD108)

Yuanyuan Tian, Xing Ye*, Lili Zhang, Guocheng Deng, Yueqiang Bai

Pearl River Fisheries Research Institute, Chinese Academy of Fishery Sciences, Lab of Aquatic Animal Genetic Engineering and Molecular Breeding, CAFS, Ministry of Agriculture Key Lab of Tropic & Subtropic Fisheries Resource Utilization and Aquaculture, Guangzhou 510380, PR China

ARTICLE INFO

Article history:
Received 6 November 2012
Received in revised form
23 April 2013
Accepted 23 April 2013
Available online 9 May 2013

Keywords:
VP4
Outer capsid protein
B cell epitopes
Neutralization assay
Relative percent survival

ABSTRACT

Grass carp reovirus Guangdong 108 strain (GCRV-GD108) was recently isolated in Guangdong province, China. M6 gene of GCRV-GD108 was speculated encoding virus major outer capsid protein VP4. Blast analysis showed that the amino acid sequence of GCRV-GD108 VP4 was homologous to the structural protein VP4 of known Aquareoviruses (27.3–32.9%). Immunogenicity prediction by DNAStar software revealed there were multiple B cell epitopes on GCRV-GD108 VP4. Prokaryotic expression vector pET32a was used to express VP4 recombinant protein (rVP4) in *E. coli* BL21(DE3) strain. As expected, the molecular weight of recombinant VP4 was about 87 kDa showed by SDS-PAGE result. Neutralization assay demonstrated that the rabbit polyclonal antibody of rVP4 could prevent virus infection efficiently. After 14 days immunization with the rVP4, grass carps were challenged with GCRV-GD108, the results showed that different doses of rVP4 (1 μg/g, 3 μg/g and 5 μg/g) all provided protection against virus infection (47–82%). The relative percent survival reached 82% in the group immunized with 3 μg/g of rVP4. ELISA revealed rVP4 induced high antibody titer in immunized fish. IgM expression levels in head kidney of grass carp were detected by RT-PCR, and the results showed that IgM expressed at a significantly higher level in immunization groups than in blank control, indicating the rVP4 can induce strong immune response. In conclusion, rVP4 is a candidate vaccine against GCRV-GD108.

1. Introduction

Aquareovirus (AQRV) is a genus of the family *Reoviridae* that infect aquatic animals. AQRV genome is composed of 11 dsRNA segments. Seven genetic groups (A to G) were established in AQRV according to the different electrophoretype of genome. In 1983, Grass carp hemorrhage virus was first reported in China [1], and was named as Grass carp reovirus (GCRV) in 1991 by the International Committee on Taxonomy of Viruses (ICTV) [2]. GCRV belongs to Group C of AQRV and is the most virulent strain in AQRVs [3].

The 11 dsRNA segments of GCRV genome encode 12 proteins including 7 capsid proteins and 5 non-structural proteins. The segmented viral genome results in high complexity and variability between different strains of GCRV, and more than 10 strains have been reported so far. A highly virulent strain was isolated recently from cultured grass carp with hemorrhage disease in Guangdong province, named 'Grass carp reovirus Guangdong strain' (GCRV-GD108). The genome of GCRV-GD108 is composed of 11 dsRNA segments as GCRV, but only encodes 11 proteins. In addition, its genome shows very different molecular properties comparing to other reported GCRV strains. Phylogenetic analysis showed that GCRV-GD108 may be a new species of genus *Aquareovirus* and is closer to *Orthoreoviruse* than any known species of *Aquareovirus* [4]. RT-PCR was used to detect hemorrhagic tissue of grass carps cultured in Guangdong, Fujian, Hunan and other provinces in China and the results indicated that GCRV-GD108 was a representative strain in southern China [5].

Grass carp (*Ctenopharyngodon idellus*) is an important freshwater aquaculture species widely cultured in Asian countries, such as China. But grass carp is vulnerable to GCRV infection especially during the fingerling stage (4 to 5 month old), resulting in more than 80% mortality rate [6]. Because of the importance of grass carp in Chinese aquaculture, GCRV vaccine development has been a hot research area. Inactivated tissue vaccine is effective in preventing viral hemorrhagic disease in grass carps, but it has limitations such as finite sources and regional variability. With the development of fish cell culture technique, grass carp kidney cell line was established successfully and subsequently inactivated virus vaccine and attenuated virus vaccine were prepared. Compared with inactivated and attenuated virus vaccine, genetically engineered vaccine

* Corresponding author.
E-mail address: gzyexing@163.com (X. Ye).

1050-4648/$ – see front matter © 2013 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.fsi.2013.04.022

has advantages of low cost, no pathogenicity, being able to be formed multivalent vaccine, etc. To prepare a genetically engineered vaccine, selection of the appropriate antigen or virulence genes is the first key step.

Among the 7 capsid proteins of AQRVs, VP1, VP2, VP3, VP5 and VP6, corresponding to λ2, λ3, λ1, μ2 and σ2 of mammalian orthoreovirus (MRV) respectively, compose the viral core and inner capsid. They play critical roles in viral transcription and replication. The outer layer of AQRVs capsid is composed of VP4 and VP7, corresponding to MRV μ1 and σ3. They play roles in viral entry into host cells [7]. As a component of the outer layer of viral capsid, VP4 is encoded by segment 6 of AQRVs, including GCRV, AGCRV, GSRV, CSRV and TRV, as well as GCRV-GD108, and VP4 is μ1 homolog of ORV [4]. (Note: As pointed out by Kim et al. [8], the numbers for VP4 and VP5 of GCRV have been swapped in recent papers, i.e., μ1 homolog VP4 was previously named VP5 and μ2 homolog VP5 was previously named VP4 in GCRV [9,10]). In this paper, when segment 6 encoded protein of GCRV was mentioned, the previously named VP5 was used to represent μ1 homolog, while segment 5 encoded protein of GCRV named VP4 represent μ2 homolog in order to keep accordance with the references.

Zhang et al. expressed recombinant VP5 of GCRV in *E. coli* and demonstrated its high immunogenicity by ELISA [11]. He et al. demonstrated that rabbit polyclonal antibody against GCRV rVP5 could provide protection against virus infection by neutralization experiments [12]. Shao et al. prepared recombinant GCRV VP5 and VP7 protein and antibodies against them. Neutralization experiments results showed both antibodies were able to neutralize GCRV, and the neutralizing activity of VP7 antibody was 3 times higher than VP5 antibody, and the mixture of VP5 and VP7 antibodies could enhance their neutralizing capacity [13]. In the current study, recombinant VP4 protein of GCRV-GD108 was expressed in prokaryotic expression system and its immunogenicity and protective effect were studied.

2. Materials and methods

2.1. Virus and bacteria

GCRV-GD108 was isolated and propagated in grass carp snout fibroblast cell line using an established method as described previously [4]. *E. coli* DH5α and BL21 (DE3) strains (Takara, Dalian, China) were cultured in Luria–Bertani (LB) broth at 37 °C.

2.2. Fish

Grass carp weighing 25–30 g were obtained from Seedling Production Base of Pearl River Fisheries Institute (Guangzhou, China) and acclimatized in the laboratory for two weeks before experimental manipulation. Fish were fed daily. Water temperature was maintained at 28 °C. Before experiments, fish were randomly sampled for the examination of virus from liver, kidney, and spleen. No virus was detected from any examined tissues of the sampled fish by RT-PCR according to methods described previously [5].

2.3. Cloning of M6 and structure analysis

M6 gene was cloned from the genome of the GCRV-GD108 and inserted into a pMD-18T vector (Takara, Dalian, China) and then transformed into *E. coil* DH5α for sequence and storage. Detailed methods were described previously [4].

Sequence analysis was performed using the Clustal W software program [14] and MEGA 5.0 [15]. Phylogenetic analysis was carried out using the Maximum likelihood method of the mega program. Percentage of the replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) was shown next to the branches [16]. Online services, http://blast.ncbi.nlm.nih.gov, http://pfam.janelia.org/, http://expasy.org/tools/# proteome and conserved domain database (CDD) were used for alignment, conserved domain and structure analysis. DNAstar software (DNASTAR, USA) was used to analyze amino acid sequence of VP4. Protein hydrophilicity, surface probability and antigenic index were predicted according to Kyte–Doolittle, Emini and Jameson–Wolf, respectively [17–19]. The most possible B cell epitopes were determined by high index value (Hydrophilicity >0, Antigenic index >0, Surface probability >1).

2.4. Plasmid construction and expression

According to the sequence of ORF of GCRV-GD108 M6 gene (GenBank accession number: ADT79743) and vector pET32a, a pair of primers with *Bam*HI and *Not*I cleavage site were designed to amplify the ORF of VP4. Sequences of the primers were: Forward: 5′ -CCCGGATCCGGAAACGTCCAGACGAACA -3′ underlined nucleotides were *Bam*HI site, reverse: 5′- CCCGCGGCCGCA-GACGGAGGAGGCCAGTATC -3′ underlined nucleotides, were *Not*I site. PCR products were digested with *Bam*HI and *Not*I and inserted into plasmid pET32a (Novagen, USA), then transformed into *E. coli* BL21 (DE3). The recombinant strain was grown in LB medium containing 50 μg/ml ampicillin, and shaking cultured at 37 °C overnight. After induction with 0.5 mM final concentration isopropyl-β-D-Thiogalactopyranoside (IPTG) (Weijia, Guangzhou, China) for 4 h, bacterial cells were collected by centrifugation followed by resuspension with PBS containing 0.2% TritonX-100. Cells were lysed by sonication and then centrifuged at 10, 000 rpm for 30 min at 4 °C. Supernatant and pellet were both collected and analyzed by 10% SDS-PAGE stained by coomassie-blue [20].

2.5. Purification of the recombinant protein

The inclusion bodies were resuspended in denaturing buffer (0.1 M Tris–Cl (pH 8.0), 10 mM DTT, 8 M urea) and dialyzed twice against for 24 h binding buffer before purification. Proteins were purified with His·bind kits (Novagen, USA) pre-charged with Ni^{2+}. The purification was performed according to the manufacturer's protocol. To remove imidazole, the purified proteins were dialyzed twice against PBS for 24 h and then kept at 4 °C. The protein concentration was detected by measuring the absorption at 280 nm using BSA as a standard, and the purity was monitored by SDS-PAGE.

2.6. Preparation of polyclonal antibody and enzyme-linked immunosorbent assay (ELISA)

Before immunization, 3–4 ml blood of New Zealand white rabbit was collected as negative serum control. Each rabbit was injected at multiple points subcutaneously with 0.2 mg of rVP4 emulsified in Freund's complete adjuvant (FCA), and boosted immunization was performed with 0.2 mg rVP4 at day 14, 28, 35 and 42. The blood was collected at day 49 to prepare antiserum. Titer of antiserum was determined by ELISA. Briefly, 100 μl stock solutions containing 1 μg rVP4 were added to each well of 96-well plate. After incubation overnight at 4 °C, plates were washed and blocked with 5% skimmed milk powder blocking buffer. Plates were washed and 4-fold dilutions of test sera were added to the plates. After incubation at 37 °C for 1 h, plates were washed and incubated with peroxidase-conjugated sheep anti-rabbit antibodies (Weijia, China) at 37 °C for 1 h. Plates were washed again, 0.1 ml TMB (Weijia, China) was added and color developed for 30 min at room temperature. The reaction was stopped by adding 100 μl 2 M sulfuric