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Full Length Research Paper

Prokaryotic expression and purification of grass carp reovirus capsid protein VP7 and its vaccine potential

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The 11 dsRNA fragmental genome of grass carp reovirus (GCRV) is enclosed in five inner core proteins and two outer capsid proteins. The Glutathione S-transferase (GST) fusion protein expression vector pGEX-4T-3 was employed to clone and expression of GCRV outer capsid gene vp7, which was amplified by reverse transcription-polymerase chain reaction (RT - PCR) from infected Grass carp. The recombinant GST-fusion protein rVP7 was induced by 1 mM IPTG in Dh5 and confirmed by SDS-PAGE and Western blot assays using both anti-GST-tag and anti-VP7 monoclonal antisera. An expected 52-kDa rVP7 was highly expressed, and was mainly exhibited in the formation of the inclusion body. After purification, rVP7 was intraperitoneally injected to the experimental mice to produce anti-rVP7 polyclonal serum. *In vitro* microneutralization assay indicated that polyclonal antibody against rVP7 could neutralize GCRV, and suggested that rVP7 had the potential to be used as subunit vaccine against GCRV infection. The present study paved the way for further characterization of the immunogenicity of viral outer capsid protein VP7 in grass carp *Ctenopharyngodon idellus* and could be based to develop antibody or antigen detection assays for GCRV pathogen.

Key words: Grass carp reovirus, VP7 protein, prokaryotic expression, western blot, microneutralization.

INTRODUCTION

Grass carp reovirus (GCRV), as a prototype member of aquareovirus, is a pathogen causing pandemic hemorrhage disease of the grass carp *Ctenopharyngodon idellus* and identified as the most causative pathogenic aquareovirus with high mortality (Qiu et al., 2001; Cheng et al., 2008). For the reason that grass carp *C. idellus* is the first cultured fish species, efficient and economic preventative strategy of GCRV infection is urgently needed in China.

Due to the lack of study on the immunogenicity and function of GCRV proteins, information on the structural proteins of GCRV was mainly deduced from its mammalian counterpart mammalian orthoreoviruses (MRV). Among the structural proteins of GCRV, VP7 and VP5 are the outer capsid proteins of the virus. VP7 is encoded by the S10 gene fragment and composed of a total of 276 amino acid residues (Benavente et al., 2007). The 3D structure and genome sequences demonstrated that there is a higher level of sequence homology in structural proteins between GCRV and MRV (Cheng et al., 2010), but *vp7* gene of GCRV and MRV shares the most divergent sequences and lowest amino acids identity (Jaafar et al., 2008; Cheng et al., 2008; Attoui et al., 2002).

In the past several decades, progress has been made in the research on the grass carp hemorrhagic disease in China. The reverse transcription-polymerase chain reaction (RT-PCR) techniques have been developed for pathogen diagnosis (Li et al., 1999; Seng et al., 2004; Zhang et al., 2008, 2010) . The most important advances about GCRV includes the biochemical and ultra-structural characteristics of GCRV and molecular strategies on diagnosis of GCRV (Ma et al., 2008; Guo et al., 2010). Vaccination has proven to be a very useful strategy in controlling pathogens in aquaculture, particularly bacterial pathogens (Heng et al., 2011). Furthermore, polyclonal antibodies against 1 and 63 were reported able to

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neutralize Mammalian reovirus *in vitro* (Chandran et al., 1999). Current vaccines include inactivated vaccine and attenuated vaccine, whose features were the high costs and difficulty in administering it in aquatic environment. Subunit and oral vaccine remians urgently desired against GCRV for the fish cultivation industry (Rangel et al., 1999; Heng et al., 2011). Additionally, Zhang et al. (2008) had expressed His-tagged VP7 protein in *Escherichia coli* and develop the protein-based detection assay for GCRV, but the vaccine potential of VP7 remians unknown. In this paper, we investigated the vaccine potential of GST-fused rVP7 through *in vitro* microneutralization assay.

MATERIALS AND METHODS

Virus isolation and propagation

GCRV infected grass carp fingerlings (3 to 4 months of age) were obtained from a fish farm in Jiangsu, China. GCRV isolates from infected fingerling grass carp with typical hemorrhagic symptoms (dead and moribund fish fingerlings) were inoculated in the *C. idellus* kidney cell (CIK). CIK cells were maintained as stock cultures in DMEM (Dulbecco's Minimal Essential Media) and re-plated 2 days before infection assays.

Viral RNA extraction and RT-PCR

GCRV purification and RNA extraction followed the reported method (Seng et al., 2002). RT- PCR assay was carried out by using total RNA from tissue infected with GCRV as template. To obtain virus RNA from infected tissue, genomic dsRNA was extracted from purified GCRV using a Trizol method (Invitrogen). For preparing RNA from virus- infected cell culture, 0.5 to 1.0 ml of virus-infected cell supernatant was pelleted by centrifugation at 8000 rpm for 15 min to pellet the virus- cell suspension for further total RNA extraction. Total RNA was obtained according to the manufacturer's instructions and was resuspended in DEPC-treated water and stored at −80°C until use.

Vp7 gene was amplified with the N-terminal primer 5'-TCCCCCGGGGGATGGGGAACGTTCAAACCTCCGT-3' and the C-terminal primer 5'-ACGCGTCGACGTC TTAATCGGATGGCTCCAC-3'. The PCR amplification was then carried out as follows: 5 min at 94°C for pre-denaturation, followed by 34 cycles each consisting of denaturation at 94°C for 30 s, annealing at 50°C for 30 s and extension at 70°C for 70 s, with the final extension at 72°C for 10 min by using a DNA thermal cycler. Form each reaction amplicons were analyzed in 1.0% (w/w) agarose gels in TAE buffer for 30 min and followed by UV-light transillumination and image capture.

Constructions of expression vector

Cloning sites *BamH1* and *Sal1* were selected according to the multiple cloning sites of the Glutathione S-transferase (GST) fusion protein expression vector pGEX-4T-3 (GE Healthcare) and VP7 gene sequence. Recombinant plasmid was confirmed by restriction enzyme digestion and sequencing (Co., Ltd. Shanghai Sangon). The correct recombinant plasmid was named as pGEX-4T- vp7 containing the full length VP7 cDNA was identified according to the size of inserted segment by PCR and was purified using a plasmid DNA purification miniprep kit (Promega).

Cultivation and induction condition of the recombinant protein

E. coli strain DH5 was cultured in Luria-Bertani medium (LB) containing 100 µg/ml ampicillin and grown overnight at 37°C and 180 rmp. The pre-inocula were then transferred to 500 ml LB medium containing ampicillin of the same concentration at a ratio of 1:10. Expression of GST fusion protein was induced with 0.1 mM isopropy1- -d-thiogalactopyranoside (IPTG) for 4 h at 37°C.

Expression and purification the recombinant protein

The GST-rVP7 contained in inclusion bodies was extracted as described previously (Zhang et al., 2008; Pathak, 2008) with some modifications. Briefly, after ultrasonic treatment, the sediment was collected at 4°C by centrifugation at 12000 rpm for 15 min. The samples (post-induction samples, host bacteria transformed with empty expression vector) were suspended with equal volume of 2 × SDS lysis buffer (Takara). The suspensions were incubated for 5 min at 100°C with stirring. The resulting cell lysate was centrifuged at 12000 rpm for 10 min. Then the extracted *E. coli* proteins were resolved by SDS-PAGE on vertical slab gels (5% stacking and 12% resolving gel). Protein bands were stained with 2.5% Coomassie Blue R- 250 for visualisation with control wide molecular range markers (Takara) as molecular estimates. Protein sample of GCRV virions were also run here for comparison purposes. The recombinant protein was purified with the PAGE gel extraction kit (Co., Ltd. Shanghai Sangon). The purified recombinant protein was subjected for further SDS-PAGE analysis.

Western blotting analysis

For western blotting, the gel was soaked in transfer buffer (20 mM tris-HCl, 196 mM glycine, 40%(v/v) methanol, pH 8.0). The resolved proteins were electro-transferred on to the nitrocellulose membrane according to methods described previously (Yin et al., 2004; Kayali et al., 2008). The membrane was then blocked in phosphate-buffered-saline (PBS) supplemented with 5% milk powder and diluted rat monoclonal IgG (1:2000) or diluted polyclonal antibody (1:300). Color development was performed by incubating with alkaline phosphatase-conjugated anti-mouse IgG (Sigma), followed by adding substrates: bromocholoroindolyl phosphate (BCIP) and nitroblue tetrazolium (NBT), or with alkaline horseradish peroxidase- conjugated anti-Rabbit IgG (Ding National Bio Co., Ltd), and followed by adding diaminobenzidine (DAB) (Co., Ltd. Shanghai Sangon) substrate.

Antiserum preparation

Four female and four male 4- week-old Balb/c mice were purchased from the Animal Holding Unit (AHU) of the Second Military Medical University (Shanghai, China) . The mice were injected and followed the way as (Wu et al., 2008; Ma et al., 2009) intra-peritoneally (i.p.) with an emulsion of purified protein and Freund's complete adjuvant (FCA) (1:1 in volume). Two weeks later, a mixture of (1:1 in volume) of purified protein and Freund's incomplete adjuvant was administered. Finally, two subsequent i.p. and intravenous (i.v.) injections of purified protein were administered at 2 weeks interval. The mice were then bled 3 days after the last injection. Serum was collected at 4°C for overnight and stored until further use.

Microneutralization assay

Neutralization effect was measured using GCRV viruses to infect CIK cells as described previously (Yang et al., 2007; Cohen et al., 2007). Briefly, after series of two-fold diluted heat-inactivated serum

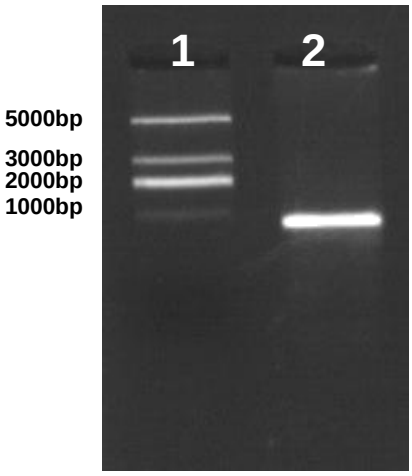


Figure 1. 1% agarose gel eletrophoresis of VP7 gene amplified from GCRV-infected cells through RT-PCR. Lane 1: 1 kb DNA Marker (1000 to 4000 bp); Lane 2: RT-PCR products of GCRV vp7 gene with the corresponding molecular weight of about 900bp.

samples (1:20 to 1:2560) were loaded in 96-well tissue culture plates, about 50 TCID50 GCRV were added, and the plates with virus/serum dilution mixtures were incubated at 27°C for 48 h to allow neutralization to take place. Mock-infected cells served as positive control here, while infected cells with pre-immunized control serum served as negative control. The assay was performed in triplicate and the viral cytopathic effect (CPE) was observed every 6 h. To visualize the results, the plate was stained with crystal violet-formaldehyde stain (0.013% crystal violet, 2.5% ethanol, and 10% formaldehyde in 0.01 M PBS). The crystal violet solution was then removed to a designated waste container. Finally, the cell monolayer in each well was rinsed with approximately 200 µl, 1×PBS for image capture.

TCID50 (50% tissue culture infective dose) assay

In order to confirm the neutralization ability of the polyclonal antibody, virus titration was assayed using the supernatant of above *in vitro* microneutralization assay as described previously (Fang et al., 2008). The virus stock was serially diluted with DMEM and then used for infection of CIK. Cell cultures were infected for 1 h, and fed with 200 µl fresh medium. 48 h post infection, 96 well plates were observed under light microscope for typical CPE. The TCID50 value was calculated using Reed Muench method.

RESULTS

Extracted RNA used for GCRV segment amplification

To obtain the coding sequence of VP7 protein from total RNA extracted from virus-infected cells, RT-PCR was performed by using specific primer pairs for VP7 gene. Figure 1 showed that fragment up to 0.9 kb in length could be detected by RT-PCR from the total RNA sample.

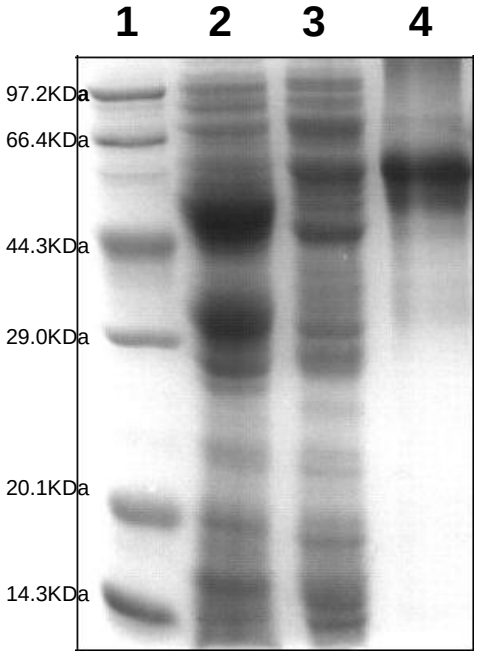


Figure 2. SDS-PAGE (12%) analysis of the GST-fusion protein rVP7 expressed in *E. coli*. Proteins gel was fixed and stained with Coomassie Brilliant Blue R- 250. Lane 1: Molecular weight maker; Lane 2: total cell extract of uninduced bacterial; Lane 3: total cell extract of bacterial after 4h induction with 1 mM IPTG at 37°C; Lane 4: Purified rVP7.

Expression and purification of recombinant protein

When the Vp7 gene was cloned into the prokaryotic expression vector pGEX-4T-3, it was confirmed by restriction enzyme digestion and sequence analysis (data not show). To obtain the over-expressed recombinant protein, bacteria containing pGEX-4T-vp7 was induced by IPTG at 4 h. A protein of 52 kDa in molecular weight was induced by IPTG, which was consistent with the expected size of rVP7 (Figure 2, Lane 3) . We found that the rVP7 protein mainly exists in the form of inclusion bodies rather than in the supernatant. Then we used a protein purification kit (Co., Ltd. Shanghai Sangon) to purify rVP7 from PAGE gel. Lane 4 of Figure 2 demonstrated the purified rVP7 for polyclonal antibody production.

Western blot analysis with rVP7 and polyclonal anti-rVP7

Polyclonal antibody, anti-rVP7, was produce in mice by using the purified rVP7 as immunogen. To test the specificity of the antiserum, extracts of the rVP7- expressing bacteria was immunoblotted with anti-rVP7 serum (Figure 3a), as well as the monoclonal anti-GST antiserum as control (Figure 3b). It appeared both anti-GST and anti-rVP7 could specifically recognize rVP7 (Lane 3 of Figures

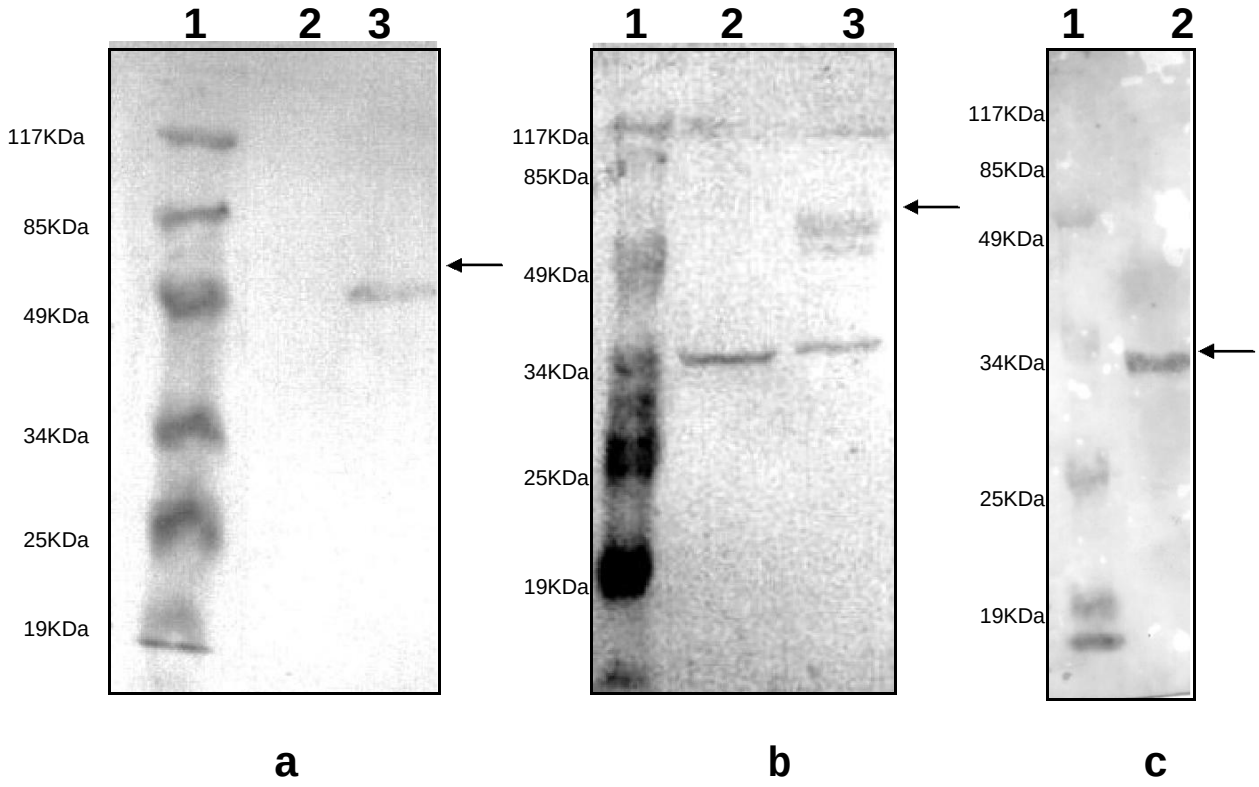


Figure 3. Western blotting analysis of the rVP7 and viral VP7. Proteins from 12% PAGE gels were transferred to nitrocellulose. (a) membrane was probed with anti-GST monoclonal antiserum and detected by NBT-BCIP substrate, Lane 1: Molecular weight maker; Lane 2: total cell extract of uninduced bacterial; Lane 3: total cell extract of bacterial after 4 h induction with 1mM IPTG at 37 ; (b) membrane was probed with anti-rVP7 polyclonal antiserum and detected by DAB substrate. Lane 1: Molecular weight maker; Lane 2: total cell extract of uninduced bacterial; Lane 3: total cell extract of bacterial after 4 h induction with 1 mM IPTG at 37 ; (c) membrane was probed with anti-rVP7 polyclonal antiserum and detected by DAB substrate. Lane 1: Molecular weight maker ; Lane 2: cellular extract of GCRV infected cells.

3a and b), in contrast to the uninduced bacteria (Lane 2 of Figures 3a and b). The anti-rVP7 could detect VP7 from the GCRV-infected cells (Figure 3c), which confirmed the viral origin of rVP7.

Microneutralization assay and virus titration

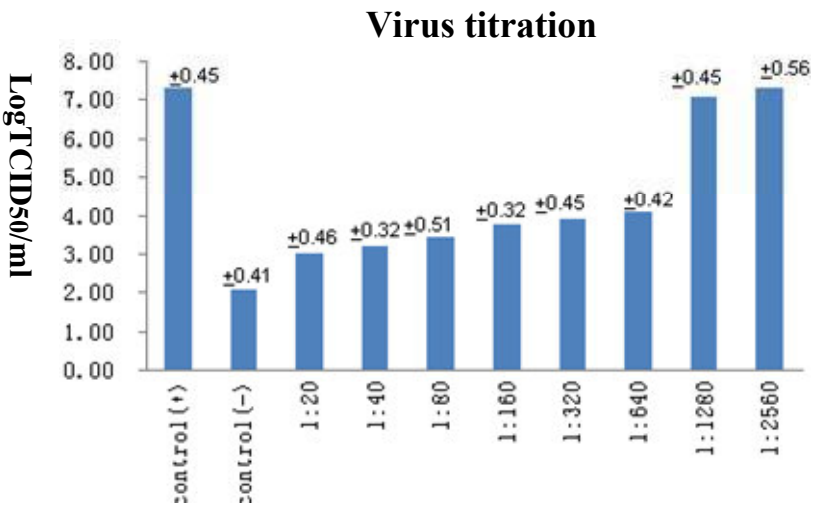
As described previously, the anti-VP7 polyclonal antibody was able to recognize the viral VP7 protein. An *in vitro* microneutralization assay was performed to measure the neutralizing ability of this polyclonal antibody. Figure 4a indicated that the anti-rVP7 antibody had strong neutralizing capability (1:640) for GCRV. For more accuracy, the supernatant of the infected cells in 96 well plates was collected for viral titration assay. The viral production reached 10^7 TCID50/ml for the cells infected with GCRV when mixed with anti-VP7 sera of 1:1280 and 2560 dilution, while low viral progeny of 10^2-10^3 TCID50/ml was observed for the wells with anti-VP7 sera of GCRV. The genetically-engineered oral vaccines are ranging from a dilution of 1:20 to 1:640 (Figure 4b).

DISCUSSION

Up to date, aquaculture in the world suffers from the frequent outbreak of epidemic disease. The grass carp reovirus is identified and regarded as the most patho-genic aquareovirus (Zhang et al., 2010). GCRV particle is composed of 7 proteins, VP1-VP7. Among the 7 structural proteins, the VP1, VP2, VP3, VP4 and VP6 are involved in forming the viral inner core, while the remaining VP5 and VP7 proteins comprise the outer capsid shell of the virus (Cheng et al., 2008). Among all the above proteins, only the outer capsid protein VP5 is seen to be reported as a subunit vaccine (He et al., 2011). Current inactivated and attenuated vaccines have been limited because their high costs and inconvenience (Heng et al., 2011). Based on its neutralization ability, the passive administration of neutralizing outer capsid VP7 polyclonal antibodies could provide an immediate treat-ment strategy for emergency prophylaxis and treatment considered the most promising effective and safe drugs

Serum dilution

a

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OFFENDING COMMAND: .buildcmap

STACK:

-dictionary-
/WinCharSetFFFF-V2TT9BF4ACCAt
/CMap
-dictionary-
/WinCharSetFFFF-V2TT9BF4ACCAt
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