Fish & Shellfish Immunology 34 (2013) 348–355

Contents lists available at SciVerse ScienceDirect

Fish & Shellfish Immunology

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

Oral vaccination of BacFish-*vp6* against grass carp reovirus evoking antibody response in grass carp

Renyu Xue [a,1], Lin Liu [a,1], Guangli Cao [a], Shiying Xu [b,c], Jinghui Li [b], Yong Zou [b], Hui Chen [b], Chengliang Gong [a,*]

[a] School of Biology & Basic Medical Sciences, Soochow University, Suzhou 215123, China
[b] Jiangsu Center for Control and Prevention of Aquatic Animal Infectious Disease, Nanjing 210036, China
[c] College of Life Sciences, Nanjing Normal University, Nanjing 210046, China

ARTICLE INFO

Article history:
Received 5 March 2012
Received in revised form
12 July 2012
Accepted 21 November 2012
Available online 29 November 2012

Keywords:
Grass carp reovirus
vp6 gene
Bombyx mori
Baculovirus expression system
Oral vaccine

ABSTRACT

To obtain a vaccine and evaluate its immune efficacy as an oral vaccine against Grass Carp Reovirus (GCRV), a donor vector pFastBac-FA-VP6-ph-VP6 containing two GCRV *vp*6 genes, one driven by the *Megalobrama amblycephala* β-actin promoter and another one controlled by baculovirus polyhedrin promoter was constructed to generate the recombinant baculovirus BacFish-*vp6*. From the hemolymph of 5th instar silkworm inoculated with BacFish-*vp6*, a 53 kDa recombinant VP6 protein could be detected. And the infected pupae collected at 120 h post-inoculation with BacFish-*vp6* were used to make freeze-dried powder as an oral vaccine. When the grass carps were orally administrated with feed containing 1%, 5% and 10% of the freeze-dried powder, their specific antibody against VP6 could be detected. Further studies showed that *vp6* has been transcripted in the grass carp kidney (CIK) cells infected with BacFish-*vp6* and the different tissues of orally vaccinated fish. All the results suggested the powder of the silkworm pupae infected with BacFish-*vp6* could possibly be used as an orally administered vaccine.

1. Introduction

Grass carp reovirus (GCRV), the most pathogenic aquareovirus, as a large family of double-stranded RNA (dsRNA) viruses, was first identified from a breakout of hemorrhagic disease affecting a vast majority (~85%) of fingerling and yearling grass carp from southern China [1,2]. However, current vaccines include inactivated vaccine and attenuated vaccine, whose features were the high costs and difficulty in administering it in aquatic environment [3]. To improve the production of grass carp and reduce the economic losses, more efficient and economic preventative strategy of GCRV infection are urgently desired in China. GCRV VP7 had be expressed in *Escherichia coli* and suggested that rVP7 could be used as a potential subunit vaccine against GCRV infection [3,4]. VP5 protein was reported that it was involved in viral infection, and could be suitable for developing subunit vaccine for the control of GCRV infection [5]. VP6 is one of the 7 structural protein components of GCRV mature particle, encoded by GRCV s8 gene and involved in forming the viral core. It is recognized as the counterpart of protein σ2 of mammal reovirus (MRV) and occupied similar positions, exhibiting as the nodules on the surface of inner capsid, which has been co-expressed with enhanced green fluorescence protein in the insect cells by Bac-to-Bac insect expression system [6].

In recent years, baculovirus was also adopted for gene delivery into mammalian cells *in vitro* and *in vivo* [7–10]. However, investigations of the potential of recombinant baculoviruses as immunizing reagents to mediate protective immunity against viral infections are very limited [11–13]. Moreover, baculovirus-specific neutralizing serum antibodies are not induced in animals [14–16]. In addition, it is known that silkworm pupae are edible in Korea, China and Vietnam. Furthermore, silkworm baculovirus expression system (BES) is potential for not only low-cost but also high capacity production (up to 20% of total cell protein) [17]. These features make the silkworm as an ideal system of expression and delivery package for producing oral vaccine. Expressed UreB and HspA of *Helicobacter pylori* in silkworm pupae showed that the recombinant proteins had good immuno-protective efficacy after oral immunization of mouse [18].

* Corresponding author. Tel./fax: +86 512 65880183.
E-mail address: gongcl@suda.edu.cn (C. Gong).
[1] The authors contributed equally to this work.

1050-4648/$ – see front matter © 2012 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.fsi.2012.11.024

Oral vaccination route has the advantage that the vaccine is cheap and easy to be administered without causing any stress to the fish. So oral vaccination with antigen included in the feed would be the ideal method of vaccine delivery to fish. In this study, we constructed a recombinant *Bombyx mori* nucleopolyhedrovirus (BmNPV) in which one GCRV *vp6* gene was driven by a β-actin promoter of *Megalobrama amblycephala* and another GCRV *vp6* gene was driven by polyhedrin promoter of BmNPV to deliver and express *vp6* gene. Then the potential that the recombinant VP6 used as a vaccine against GCRV infection was evaluated. The results showed that specific antibody could be induced in grass carp by orally administrated with the freeze-dried powder made from the silkworm pupae infected with the recombinant BacFish-*vp6* (PIB), suggesting the powder could possibly be used as an orally administered vaccine.

2. Materials and methods

2.1. Cloning of vp6 gene

Grass carp reovirus (GCRV) was kindly provided by Animal & Plant Inspection and Quarantine Technology Centre of Shenzhen. Viral genomic RNA were extracted with QIAGEN Viral RNA Mini Kit (Qiagen, Germany) and converted to cDNA with RNA PCR KitVer.3.0 (Qiagen, Germany) according to the manufacturer's instructions. The *vp6* gene was amplified by using a primer pair of GCRV-EI-6(5′-GGC GAA TTC ATG GCA CAG CGT CAG TTT TTC GG-3′, the underline indicates *Eco*R I site) and GCRV-HD-6(5′-TCG AAG CTT AGA CGA ACA TCG CCT GCG C-3′; the underline shows *Hin*d III site) and ligated into the pMD19T simple vector (Sangong, Shanghai, China) to obtain plasmid pMD19T-*vp6*. The recombinant plasmid was then sequenced.

2.2. Construction of a donor vector pFastBac-FA-VP6-ph-VP6

The promoter of β-actin gene was amplified from the genomic DNA of *M. amblycephala* with the primers FA-SM (5′-TCT CCC GGG CTC TTA CAG GAA ACG GGT C-3′, the underline shows *Sma* I site) and FA-XH (5′-CTA CTC GAG ATT GGA GCT CAA AGT GAG G-3′, the underline shows *Xho* I site) according to the deposited sequences in GenBank (GenBank ID: AY170122), and cloned into the pMD19T simple vector to generate plasmid pMD19T-β-actin. The recombinant plasmid was sequenced after being identified with restriction enzymes.

The *vp6* gene excised from pMD19T-*vp6* by digestion with *Eco*R I/*Hin*d III was ligated into the pFastBac^{TM}Dual (Invitrogen, USA) to generate pFastBac-ph-VP6. The promoter of β-actin gene cut from pMD19T-β-actin by digestion with *Sma* I/*Xho* I was cloned into the *Sma* I/*Xho* I sites of pFastBac-ph-VP6 to obtain pFastBac-FA-ph-VP6. The another *vp6* gene was amplified from plasmid pMD19T-*vp6* with primers GCRV-XH-6 (5′-TAT CTC GAG ATG GCA CAG CGT CAG TTT TTC GG-3′, the underline shows *Xho* I site) and GCRV-KN-6 (5′-GCT GGT ACC TAG ACG AAC ATC GCC TGC GC-3′; the underline indicates *Kpn* I site) and inserted into pFastBac-ph-VP6 after the recovered PCR product was digested with *Xho* I and *Kpn* I to generate the final vector of pFastBac-FA-VP6-ph-VP6, in which one *vp6* gene was under the control of BmNPV polyhedrin promoter (Pph) and another one was driven by β-actin promoter of *M. amblycephala* (Fig. 1).

2.3. Preparation of VP6 polyclonal antibody

A *vp6* gene was inserted into *Eco*R I/*Hin*d III sites in pET-28a(+) (Novagen, Darmstadt, Germany) to construct pET28a(+)-VP6, and the fusion protein was expressed in *E. coli* strain BL21. Then the harvested bacteria were subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) after boiling with the same volume of loading buffer (100 mM Tris-Cl, pH 6.8, 200 mM DTT, 4% SDS, 0.2% bromophenol blue, and 20% glycerol) for 5 min. The concentrations of the stacking and separating gels were 5% and 10%, respectively. The gel was stained with Coomassie Brilliant Blue R-250 after electrophoresis. The proteins in the gel were transferred to a piece of PVDF membrane. The membrane was firstly blocked in PBST (137 mM NaCl, 2.68 mM KCl, 8.06 mM Na_2HPO_4, 1.47 mM KH_2PO_4 and 0.05% Tween 20) plus 3% Bovine Serum Albumin (BSA) for 2 h at room temperature and washed three times (each for 10 min) with PBST, and subsequently incubated with PBST containing 3% BSA and diluted mouse anti-His6 (TianGen, Beijing, China) (1:1000) for 2 h at room temperature, then it was washed three times (each for 10 min) with PBST. After incubation for 1 h at room temperature in the same PBST with HRP-conjugated goat anti-mouse IgG (1:2000) (Biosynthesis Biotechnology, Beijing, China) and being washed with PBS (137 mM NaCl, 2.68 mM KCl, 8.06 mM Na_2HPO_4 and 1.47 mM KH_2PO_4), the protein band was visualized with DAB (3, 3-Diamonobenzidine tetrahydrchloride) chromogenic substrate (2 mg DAB and 20 μl of 30% H_2O_2 in 10 ml of PBS).

The recombinant protein purified using Ni-NTA agarose (Qiagen, Shanghai, China) was used to immunize ICR mice (Soochow University, Suzhou, China) by subcutaneous and celiac injection [5,19,20] to induce the antibody which was identified by Western blotting with the prepared mouse anti-VP6 (1:100) and HRP-conjugated goat anti-mouse IgG(1:2000).

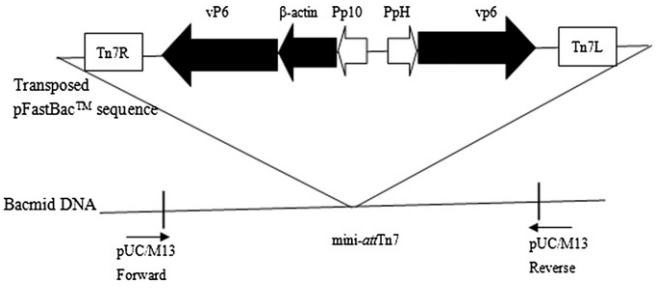

Fig. 1. Schematic representation of recombinant baculovirus and analysis recombinant Bacmid DNA by PCR.
A *vp6* gene was controlled by baculovirus polyhedrin promoter and another *vp6* gene driven by the β-actin promoter of *M. amblycephala* was cloned at downstream of the baculovirus *p10* promoter.

2.4. Construction and proliferation of recombinant Bacmid baculoviruses

The pFastBac-FA-VP6-ph-VP6 vector was transformed into *E. coli* DH10Bac/BmNPV (a gift from Prof. WB Wang of Jiangsu University) to generate recombinant Bacmid-*vp6* using the Bac-To-Bac baculovirus expression system (Invitrogen, USA) following the manufacturer's instructions. The recombinant Bacmid-*vp6* is too large (larger than 135 kb in size) to perform a restriction analysis. Therefore PCR analysis was carried out to identify the recombinant Bacmid-*vp6* with the M13 forward primer (5′-CCC AGT CAC GAC GTT GTA AAA CG-3′) and M13 reverse primer (5′-AGC GGA TAA CAA TTT CAC ACA GG-3′). These primers were flanking the mini-*att*Tn7 within the *lac*Z α-complementation region where *vp6* was harbored (Fig. 1). Then the confirmed Bacmid-*vp6* DNA was transfected into BmN cells using FuGENE HD Transfection Reagent (Roche Diagnostics, USA) to generate the recombinant baculovirus BacFish-*vp6*. Once the cells appeared infected, the virus from the cell culture were harvested as the P1 viral stock, and continuously