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Protective immunity in gibel carp, *Carassius gibelio* of the truncated proteins of cyprinid herpesvirus 2 expressed in *Pichia pastoris*

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ABSTRACT

Cyprinid herpesvirus 2 (CyHV-2) infection is a newly emerged infectious disease of farmed gibel carp (*Carassius gibelio*) in China and causes huge economic losses to the aquaculture industry. In this study, the three membrane proteins encoded by genes ORF25, ORF25C, and ORF25D of CyHV-2 were truncated and expressed in yeast, *Pichia pastoris*. Screening of the recombinant yeasts was done by detecting the truncated proteins using Western blot. Through immunogold labeling, it was shown that proteins binding the colloidal gold were presented on the surface of cells. In the experiment of inhibition of virus binding by the recombinant truncated proteins, the $TCID_{50}$ of the tORF25 group ($10^{4.1}$/ml) was lower than that of tORF25C ($10^{4.6}$/ml) or tORF25D groups (10^{5}/ml). These results suggested that the proteins may be involved in attachment of the virus to the cell surface. Healthy gibel carp were immunized with 20 μg of tORF25, tORF25C, and tORF25D proteins, and the control group received PBS. Interleukin 11 (IL-11) expression in the spleens of the immunized fish peaked at day 4 and the complement component C3 (C3) genes were significantly up-regulated at day 7 post-immunization. Specific antibodies were measured in the three immunized groups and the titer detected in the tORF25 group reached 327, that was significantly higher than the tORF25C (247) or tORF25D (228) groups. When the immunized fish were challenged with live CyHV-2 by intraperitoneal injection the relative percent survival (RPS) of the tORF25, tORF25C, and tORF25D immunized groups was 75%, 63%, and 54%, respectively. The feasibility of the *P. pastoris* yeast expression system for the production of the recombinant truncated proteins and their apparent bioactivity suggests that tORF25, tORF25C, and tORF25D are potential candidate vaccines against Cyprinid herpesvirus 2 infection in gibel carp.

1. Introduction

Gibel carp, *Carassius gibelio*, belonging to the family *Cyprinidae* and the genus *Carassius*, represents one of the most important farmed freshwater fish species in China with a total annual production of about 2.59 million tons [1]. However, the diseases of gibel carp caused by both viral and bacterial infections have become of great concern. The cyprinid herpesvirus 2 (CyHV-2) is one of the most infectious viral pathogens of gibel carp [1] and causes herpesviral haematopoietic necrosis (HVHN) disease that is characterized by skin hemorrhaging, bleeding and pale gills, enlarged spleen and kidney, and internal organ hemorrhaging [2]. This mortality can reach 90–100% [2].

Cyprinid herpesvirus 2 (CyHV-2) is a member of *Cyprinivirus* that also includes carp pox (CyHV-1) and the koi herpesvirus (CyHV-3) [3]. Historically, the host species for CyHV-1 and CyHV-3 were considered to be common carp and koi carp (a variety of *Cyprinus carpio*), respectively, while CyHV-2 was thought to be a pathogen of goldfish or gibel carp [2,4]. CyHV-2 was first reported in 1992 and caused significant economic damage to the goldfish (*Carassius auratus*) aquaculture industry in Japan [5]. The disease was recognized as a major pathogen of goldfish not only in Japan but also in the USA [6,7], Taiwan [8], Australia [9], New Zealand [10], and the UK [11]. Doszpoly et al. were the first to report the occurrence of CyHV-2 in gibel carp instead of the usual host goldfish [12]. Mass mortality caused by CyHV-2 infection has been observed in farmed gibel carp in China since 2010 that appears to be spreading

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in the Yancheng area of Jiangsu province [13]. Now this disease has also been detected in Hubei, Jiangxi, Hebei, and Hunan provinces in China and effects fish in a much larger area [2].

An effective prevention and treatment program for HVHN disease in gibel carp is desparately needed. Vaccination has been shown to be an effective approach to prevent impacts to cultured fish from virus infections [14]. To date, many vaccines for fish have been developed and are widely used for preventing and reducing the impact of diseases in aquaculture [15–17]. A large number of structural genes from many viruses have been expressed in yeast expression systems [18–20]. This has led to the licensing of vaccines against hepatitis B virus and papillomaviruses in humans [21]. One of the most widely used yeast expression systems is based on the methylotrophic *Pichia pastoris* strain, that has been successfully used to produce more than 500 proteins both for basic laboratory research and industrial purposes [22,23].

The whole genome sequence of CyHV-2 (GenBank No. NC_019495) was analyzed and the 36 membrane proteins were predicted in 2013 [24]. The CyHV-3 envelope glycoproteins of ORF25 family had been demonstrated that they were possibly suitable as components of subunit vaccine against CyHV-3 infection [25]. In our previous work, the full length ORF25 family genes (ORF25, ORF25C, and ORF25D) of CyHV-2 were cloned and inserted into the plasmid pPICZα B, and then they were expressed in *P. pastoris*. But it was hardly detected the anticipated objective strap sizes from expression products by Western blot. This result may be caused by the low expression level or early termination of expression for total length genes. To obtain high yields and immunogenics of three recombinant proteins, the regions contained most of the antigenic determinants, and outside of the cell membrane were chosen and expressed in this study. The *in vitro* characteristics of the recombinant truncated proteins were investigated by Western blot, immunogold labeling, and inhibition of virus binding. Additionally, the ability of the recombinant truncated proteins to change the expression of immune-related genes, induce specific antibodies in gibel carp, and protect fish against CyHV-2 challenge was investigated. The results supported that it was feasible to develop and produce a yeast-derived vaccine against CyHV-2 for use in gibel carp.

2. Materials and methods

2.1. Gibel carp

Apparently healthy gibel carp (mean weight, 158 ± 18 g) were obtained from a local farm, and were acclimatized to laboratory conditions at the Yangtze River Fisheries Research Institute. The fish were kept at 25 °C in an aerated freshwater tanks and fed a commercial carp diet daily for two weeks before the experiment was initiated.

2.2. Virus and cells

The CyHV-2 was originally isolated and identified from diseased gibel carp [2]. Gibel carp brain (GiCB) cell line was used to propagate CyHV-2 *in vitro* [26]. Cells were grown at 28 °C in M199 medium (Sigma, USA) supplemented with 100 IU/ml penicillin G (Sigma, USA), 100 mg/ml streptomycin (Sigma, USA), 2 mmol/L L-glutamine (Sigma, USA) and 10% fetal bovine serum (FBS; Sigma, USA).

2.3. Yeast strain, media and culture conditions

The *P. pastoris* KM71 strain pPICZa B vector, *E. coli* strain DH5a, and the antibiotic zeocin were purchased from Invitrogen (CA, USA). The *E. coli* DH5a was cultured in low salt lauria bertani (LLB) broth that contained 1% (w/v) peptone (Oxoid, England), 0.5% (w/v) yeast extract (Oxoid, England), and 0.5% (w/v) NaCl (Sinopharm Chemical Reagent Co., Ltd, China), and was used as a cloning host for vector storage and amplification. *P. pastoris* transformants were screened on yeast extract peptone dextrose medium with sorbitol (YPDS) agar plates including 1% yeast extract, 2% peptone, 2% dextrose (Sinopharm Chemical Reagent Co., Ltd, China), 1M sorbitol (Amresco, USA), 2% agar (Promega, USA), and 100 μg/ml zeocin. Additionally, zeocin was added to yeast extract peptone dextrose medium (YPD) plates, which were comprised of the same components as the YPDS agar plates except that sorbitol was omitted so that three concentrations of zeocin could be obtained including 500, 800, and 1000 μg/ml for the isolation of multi-copy insert tansformants. A single tansformant with the plasmid was inoculated in the buffered minimal glycerol-complex medium (BMGY) that contained 1% yeast extract, 2% peptone, 100 μM potassium phosphate (pH 6.0, Sinopharm Chemical Reagent Co., Ltd, China), 1.34% yeast nitrogen broth (Amresco, USA), 0.4 μg/ml biotin (Amresco, USA), and 1% glycerol (Sinopharm Chemical Reagent Co., Ltd, China). The buffered minimal methanol-complex medium (BMMY) was used to induce protein expression and was the same as the BMGY medium except that glycerol was replaced by methanol (Sinopharm Chemical Reagent Co., Ltd, China).

2.4. Construction of the expression plasmids and transformation of P. pastoris

The truncated ORF25, ORF25C, and ORF25D (tORF25, tORF25C, and tORF25D) genes of CyHV-2 were amplified by PCR using specific primer pairs (Table 1). *Eco*R I and *Sac* II sites were added to the forward and reverse primers, respectively. The reactions were carried out following the procedures: 95 °C for 10 min, 30 cycles of 94 °C for 20 s, 56 °C for 30 s, 72 °C for 30s, and 72 °C for 10 min. The purified PCR products were cloned in frame downstream of the α-factor, yielding the recombinant plasmids pPICZα tORF25, pPICZα tORF25C, and pPICZα tORF25D (Fig. 1). The recombinant plasmids were confirmed by DNA sequencing.

2.5. Screening and expression of recombinant truncated proteins in P. pastoris

2.5.1. Transformation of P. pastoris and screening of multiple inserts

The preparation of competent cells and the transformation of *P. pastoris* strain KM71 were performed as previously described [27]. In this study, the recombinant plasmid was linearized with *Sac* I and integrated into competent cells of *P. pastoris* strain KM71 by electroporation using an electroporator (Eppendorf, electroporator 2510). The transformants were plated on YPDS agar plates that contained 100 μg/ml zeocin and incubated at 30 °C for 3–10 days. Colonies with inserts were further selected on YPDS plates that contained 500, 800, and 1000 μg/ml zeocin and incubated at 30 °C for another 3–10 days.

2.5.2. Expression and purification of recombinant truncated proteins in P. pastoris

A single KM71 transformant with the plasmid was cultured in 20 ml BMGY at 30 °C overnight until the optical density reached 2–6 at 600 nm. The yeast were harvested and resuspended in 5 ml of BMMY. Methanol was added every 24 h to a final concentration of 0.5% (v/v) to induce protein expression. The yeast cells were discarded after centrifugation at 4000 g for 30 min. The supernatant was loaded onto 5 ml HisTrap FF column (GE Healthcare, England) and washed with the lysis buffer supplemented by 40 mM imidazole (Amresco, USA), pH 7.4. Recombinant protein was eluted