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Protective immunity of a *Pichia pastoris* expressed recombinant iridovirus major capsid protein in the Chinese giant salamander, *Andrias davidianus*

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ABSTRACT

The major capsid protein (MCP) is the main immunogenic protein of iridoviruses, that has been widely used as an immunogen in vaccination trials. In this study, the codon-optimized giant salamander iridovirus (GSIV) MCP gene (O-MCP) was synthesized and cloned into a pPICZα B vector for secretory expression in the methylotrophic yeast *Pichia pastoris* after methanol induction. The expression of the O-MCP protein was detected by the Bradford protein assay, SDS-PAGE, Western blotting and electron microscopy. The Bradford protein assay indicated that the concentration of the O-MCP expressed was about 40 μg/ml in culture supernatants. SDS-PAGE analysis revealed that the O-MCP had a molecular weight of about 66 kDa and reacted with a His-specific MAb that was confirmed by Western blotting. Electron microscopy observations revealed that the purified O-MCP could self-assemble into virus-like particles. Healthy giant salamanders were vaccinated by intramuscular injection with the O-MCP antigen at a dose of 20 μg/individual. The numbers of erythrocytes and leukocytes in the peripheral blood of immunized Chinese giant salamanders increased significantly at day 3 and reached a peak at day 5 post-immunization. Meanwhile, the differential leukocyte counts of monocytes and neutrophils increased significantly at day 5 post-immunization compared to that of the control group. The percentage of lymphocytes was 71.33 ± 3.57% at day 21 post-immunization. The neutralization assay showed that the serum neutralizing antibody titer reached 321 at day 21 post-immunization. The GSIV challenge test revealed that the relative percent survival of Chinese giant salamanders vaccinated with O-MCP was 78%. These results indicated that the O-MCP antigen expressed by the *Pichia pastoris* system elicited significant immune response in the Chinese giant salamander against GSIV and might represent a potential yeast-derived vaccine candidate that could be used for the control of disease caused by the giant salamander iridovirus.

1. Introduction

The Chinese giant salamander (*Andrias davidianus*), that belongs to the order *Caudata* in the family *Cryptobranchidae*, is the largest existing amphibian species in the world [1,2]. In view of its high medicinal, nutritional and scientific value, the artificial breeding and culture of this animal has significantly increased and has become an important industry in China [3]. However, frequent outbreaks of infectious diseases pose a serious threat to the further development of the industry [4–7]. A Chinese giant salamander iridovirus (GSIV) was isolated from the diseased giant salamanders in Hubei Province in 2010 [3], which caused high mortality of all life stages of the Chinese giant salamander [8,9]. Currently, there are no effective methods available for the treatment and control of this disease.

Vaccination has been shown to be an effective method for the control of infectious diseases in aquatic animals [10]. To date, many vaccines for aquatic animal have been developed and are widely used for preventing and reducing the economic impact of diseases in aquaculture [11–13]. Inactivated vaccines are the most common

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type of vaccines used for aquatic animal vaccinations. However, due to the problems of antigenic competition, poor immunogenicity, high production costs and/or the lack of safety, the inactivated vaccine is limited to application [10].

Yeast expression systems have been widely applied to the expressions of structural proteins from many viruses [14–16]. This has led to the licensing of vaccines against hepatitis B virus and papillomaviruses in humans [17]. The methylotrophic *Pichia pastoris* strain is one of the most extensively used yeast expression systems, which has been successfully used to produce more than 500 proteins both for basic experimental research and industrial purposes [18,19]. However, heterologous protein expression has been plagued with problems of poor protein expression because of biased codon usage. This problem is minimized by improving the genetic code by using partly or fully rearranged synthetic codons [20,21].

The major capsid protein (MCP) accounts for 40–45% of the total polypeptides of the iridovirus, which play an important role in the induction of antiviral immune responses [22,23]. Since recombinant MCP can be used as an antigen to generate vaccines, the expression of this gene is a viable area of research [10,24]. In this study, the optimized codon sequence of the GSIV MCP was expressed in *P. pastoris* and evaluated as a potential yeast-derived vaccine for the immunization of Chinese giant salamander against GSIV infection.

2. Materials and methods

2.1. Virus, cell lines and animals

The GSIV was originally isolated and identified from diseased Chinese giant salamanders by our laboratory [8]. The epithelioma papulosum cyprini (EPC) cell line was obtained from the China Center for Type Culture Collection (CCTCC), Wuhan University and grown at 25 °C in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum. Healthy Chinese giant salamanders (weight, 50 ± 5 g) that were confirmed to be free of GSIV by PCR were obtained from the farm of Yangtze River Fisheries Research Institute. The animals were kept in plastic aquaria at 20–22 °C and fed daily with diced bighead carp meat for two weeks before the experiments were initiated.

2.2. Yeast strain and culture conditions

The *P. pastoris* KM71 strain pPICZa B vector, *Escherichia coli* strain DH5a, and the zeocin antibiotic were purchased from Invitrogen (CA, USA). The *E. coli* DH5a was cultured in low salt lauria bertani (LLB) broth 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. 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 transformants. A single transformant with the plasmid was inoculated in the buffered minimal glycerol-complex medium (BMGY) and induced protein expression in the buffered minimal methanol-complex medium (BMMY).

2.3. Codon optimization and gene synthesis

The codon usage of MCP (GenBank KF023635) from the GSIV was analyzed through Graphical Codon Usage Analyser (http://gcua.schoedl.de/), and its sequence was optimized by replacing the codons less frequently used in *P. pastoris* with those frequently used. The optimized MCP gene (O-MCP) was synthesized by GenScript (Nanjing, China).

Table 1
Primers used in this study.

Name	Primers[a] 5′ → 3′
O-MCPF	CGGAATTC CG ATGTCTAGTGTTACTGGTTCTGG
O-MCPR	TCCCCGCGG C AAGGATTGGAAAACCCATACTACC
5′AOX1	GACTGGTTCCAATTGACAAGC
3′AOX1	GCAAATGGCATTCTGACATCC′

[a] Restriction sites in the primers are underlined.

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

The synthetic DNAs that encoded the O-MCP were amplified by PCR using the primer pair of O-MCPF and O-MCPR (Table 1). *Eco*R I and *Sac* II sites were added to the forward and reverse primers, respectively. The reaction was carried out following the procedures: 95 °C for 10 min, 30 cycles of 94 °C for 45 s, 56 °C for 45 s, 72 °C for 1 min and 72 °C for 10 min. The purified PCR product was cloned between the frames of the α-factor and the 6 × His-tag. The recombinant plasmid was checked for O-MCP integration by PCR with the primer pairs of O-MCP and AOX1 (Table 1) following the procedures described above. The recombinant plasmid was confirmed by DNA sequencing.

2.5. Transformation of P. pastoris and screening of the multiple inserts

The preparation of competent cells and the transformation of *P. pastoris* strain KM71 were performed as previously described [25]. 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.

2.6. Expression and purification of O-MCP 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 by centrifugation 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 then incubated with the His-bind resin and the resin was washed and then eluted with PBS. The protein concentration in culture supernatants and purified protein samples were determined by the method of Bradford using the Bio-Rad Protein Assay kit (Bio-Rad) with bovine serum albumin (BSA) as a standard.

2.7. SDS-PAGE and Western blotting

The purified protein was mixed with an equal volume of 2 × SDS-PAGE loading buffer and separated by SDS-PAGE. The gels were either stained with Coomassie brilliant blue R-250 to visualize the protein bands or transferred onto a nitrocellulose (NC) membrane. After transfer, the membrane was incubated with the mouse anti-His-tag monoclonal antibody and horse radish peroxidase (HRP)-conjugated goat anti-mouse IgG antibody in turn. After incubated

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