Vaccine 33 (2015) 5662–5669

Contents lists available at ScienceDirect

Vaccine

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

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

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ARTICLE INFO

Article history:
Received 28 June 2015
Received in revised form 6 August 2015
Accepted 11 August 2015
Available online 22 August 2015

Keywords:
Chinese giant salamander
Iridovirus
MCP
Pichia pastoris expression
Protective immunity

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.

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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

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http://dx.doi.org/10.1016/j.vaccine.2015.08.054
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used for preventing and reducing the economic impact of diseases in aquaculture [11–13]. Inactivated vaccines are the most common 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

with 3,3-diaminobenzidine tetrahydrochloride (Sigma, USA), the membrane was analyzed for any reactions that had occurred.

2.8. Electron microscopy of the negatively stained O-MCP

The purified O-MCP was adsorbed onto carbon-coated copper grids and negatively stained with 2% ammonium phosphotungstic acid for 1 min. The grids were then examined using an H-7650 electron microscope (Hitachi, Japan) at 80 kV. Meanwhile, the purified GSIV was negatively stained and observed as a control.

2.9. Immunization and collection of blood samples

The 120 healthy Chinese giant salamanders were randomly assigned into two equal groups. The immunized group was inoculated intramuscularly with 0.1 ml of 20 μg O-MCP and the control group received the same volume of PBS (Sigma, USA). Before blood samples were collected, the experimental animals were anaesthetized for 20–30 min in MS-222 at a concentration of 1 mg/ml. Blood samples were collected from three animals of each treatment group randomly by tail venipuncture at days 1, 3, 5, 7, 14, 21, 28, and 35 post-immunization. The blood samples were allowed to clot at 4 °C for 10 h and then centrifuged at 1500 × g for 10 min. Serum was harvested from each sample and used in serum neutralization assay (SN) for the detection of specific antibody.

2.10. Blood parameters

Blood samples used for counting erythrocytes were diluted with ringer solution and the blood samples used for counting leukocytes were diluted with an acetic acid gentian violet solution. The blood cells were counted in a cell counting chamber using microscopy. For the differential leukocyte count (DLC), fresh blood smears on glass slides were stained by Wright Giemsa, and then 100 leukocytes were randomly counted and classified under oil immersion microscopy.

2.11. Serum neutralization antibody assay

The serum neutralization antibody titer was measured by using the GSIV and EPC cells according to the method of Yao et al. [26]. Briefly, the sera were heated at 56 °C for 30 min and serial twofold (1:2–1:512) dilutions were made in 0.5 ml of DMEM. An equal volume of GSIV with a titer of 100 $TCID_{50}$/ml was added to each serum dilution and the mixtures were incubated at 25 °C for 1 h. After incubation, the virus/serum mixtures were inoculated and incubated 1 h at 25 °C in wells (0.1 ml per well) of a 96-well microplate containing EPC cell monolayers. The mixtures were gently aspirated and 0.2 ml of fresh DMEM supplemented with 2% FBS were added back to each well. The 96-well microplate was incubated at 25 °C for 5–7 days. The viral cytopathic effect (CPE) was monitored and the neutralizing antibody titers of the sera were calculated by the Reed–Muench method [27].

2.12. Evaluation of the protection against GSIV challenge

400 μl of live GSIV (1.0×10^8 $TCID_{50}$/ml) was injected intraperitoneally into 30 animals each in the immunized and control groups at day 15 post-immunization. Mortality was recorded for 14 days. The relative percent survival (RPS) was calculated according to Amend [28]:

$$\text{RPS} = \{1 - (\text{mortality in vaccinated Chinese giant salamanders})/(\text{mortality in control Chinese giant salamanders})\} \times 100\%$$

2.13. Statistical analysis

The results were expressed as the mean ± standard deviation (SD) and all statistical analyses were done using SPSS 17.0 package. The least-significant-difference test was used for between-group comparisons.

3. Results

3.1. Synthesis of the codon-optimized gene

The analysis of the original O-MCP gene DNA sequence was done by using the Graphical Codon Usage Analyzer. The rare codons in the GSIV MCP gene appeared to have a lower level of expression in *P. pastoris*, which were replaced with more frequently used ones. The GC content of the GSIV MCP gene was adjusted from 59.55% to 43.03% (Fig. 1). The Codon Adaptation Index (CAI) parameter increased from 0.6 to 0.84.

3.2. Screening of multi-copy recombinant plasmids in P. pastoris

The O-MCP gene was cloned into the expression vector pPICZα B between the frames of the α-factor and the 6 × His-tag, that yielded the recombinant plasmid, pPICZα B-O-MCP. The recombinant plasmid was integrated into the chromosomal DNA of *P. pastoris* strain KM71 by electroporation. The transformants were detected by PCR using the AOX1 sequencing and the O-MCP primers (Table 1), and two bands were amplified with the sizes of approximately 2.0 and 1.4 kb, consistent with the predicted sizes of the intact AOX1 structural gene and the specific O-MCP gene, respectively (Fig. 2A). The positive clones, which were resistant to 1000 μg/ml zeocin, were subjected to further selection in order to obtain a high expression clone.

3.3. Expression and characterization of O-MCP

Expression was performed in BMGY and BMMY cultures and the expressed protein was purified by the His-bind resin procedure. The results of the Bradford protein assay indicated that the concentration of O-MCP was approximately 40 μg/ml in culture supernatants.

The SDS-PAGE analysis indicated that the O-MCP extracted from the precipitate of the culture supernatant had a molecular weight of about 66 kDa (Fig. 2B). The recombinant O-MCP became detectable 24 h after induction and the concentration of the protein increased with increased incubation (data not shown). The result of Western blotting revealed that the recombinant O-MCP combined with a His-specific MAb (Fig. 2B), but no similar sign was observed in the negative control strain Km71/pPICZα B (mock plasmid).

Electron microscopy analysis demonstrated that the purified O-MCP could self-assemble into virus-like particles (VLPs). The electron microscopy image of the negatively stained sample showed the presence of intact VLPs, that were similar to the negatively stained GSIV particles in size and morphology (Fig. 2C and D).

3.4. Blood parameters

In the immunized group, the number of erythrocytes increased beginning at day 3 and peaked at day 5 post-immunization compared to the control group. The number of erythrocytes in control group was maintained at about 6.83×10^7/ml throughout the analysis (Fig. 3). The leukocytes in the immunized salamanders increased significantly at day 3 and reached a peak at day 5 compared to the control group. A significant increase in the

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Optimized  1     ATGTCTAGTGTTACTGGTTCTGGTATTACTTCTGGTTTTATTGATTTGGCAACTTATGAC
Original   1     ATGTCTTCTGTAACCGGTTCAGGTATCACAAGTGGTTTCATCGACTTGGCCACTTATGAC
                  M  S  S  V  T  G  S  G  I  T  S  G  F  I  D  L  A  T  Y  D

Optimized  61    AACTTGGAGAGAGCTATGTATGGAGGATCCGATGCTACTACATACTTCGTCAAGGAACAT
Original   61    AATCTTGAGAGAGCAATGTACGGGGGTTCAGATGCCACCACGTACTTTGTCAAGGAGCAC
                  N  L  E  R  A  M  Y  G  G  S  D  A  T  T  Y  F  V  K  E  H

Optimized  121   TATCCAGTTGGTTGGTTTACTAAGCTTCCTTCTTTGGCTGCCAAAATGTCCGGTAACCCA
Original   121   TACCCCGTGGGGTGGTTCACCAAGCTGCCGTCTCTGGCTGCCAAGATGTCGGGTAACCCG
                  Y  P  V  G  W  F  T  K  L  P  S  L  A  A  K  M  S  G  N  P

Optimized  181   GCCTTCGGACAACAGTTTTCAGTTGGTGTCCCTAGAAGTGGAGATTACATTTTGAATGCT
Original   181   GCTTTCGGGCAGCAGTTTTCGGTCGGCGTTCCCAGGTCGGGGGATTACATCCTCAACGCC
                  A  F  G  Q  Q  F  S  V  G  V  P  R  S  G  D  Y  I  L  N  A

Optimized  241   TGGTTGGTCCTTAAGACTCCAGAAGTTAAATTGCTTGCAGCTAACCAATTGGGTGACAAT
Original   241   TGGTTGGTGCTCAAGACCCCCGAGGTCAAGCTCCTGGCCGCAAACCAGCTGGGAGACAAC
                  W  L  V  L  K  T  P  E  V  K  L  L  A  A  N  Q  L  G  D  N

Optimized  301   GGAACCATTAGATGGACTAAAAACCCTATGCACAATATCGTTGAGAACGTCAATCTTTCA
Original   301   GGCACCATCAGGTGGACAAAGAACCCCATGCACAACATTGTGGAGAACGTCAACCTCTCA
                  G  T  I  R  W  T  K  N  P  M  H  N  I  V  E  N  V  N  L  S

Optimized  361   TTCAACGATATTTCAGCTCAGAGTTTTAATACAGCTTACTTGGACGCCTGGAGTGAATAT
Original   361   TTCAACGACATCAGCGCCCAGTCCTTTAACACGGCATACCTGGACGCCTGGAGCGAGTAC
                  F  N  D  I  S  A  Q  S  F  N  T  A  Y  L  D  A  W  S  E  Y

Optimized  421   ACCATGCCAGAGGCTAAGAGAATTGGTTACTATAACATGATCGGAAATACTTCTGATTTG
Original   421   ACCATGCCAGAGGCCAAGCGCATAGGCTACTATAACATGATAGGCAACACCAGCGATCTC
                  T  M  P  E  A  K  R  I  G  Y  Y  N  M  I  G  N  T  S  D  L

Optimized  481   ATCAACCCAGCCCCTGCAACAGGTCAAAATGGAGCCAGAGTCTTGCCTGCTAAGAACTTG
Original   481   ATCAACCCCGCCCCGGCCACAGGCCAGAACGGAGCCAGGGTCCTCCCGGCCAAGAACCTG
                  I  N  P  A  P  A  T  G  Q  N  G  A  R  V  L  P  A  K  N  L

Optimized  541   GTTCTTCCATTGCCATTTTTCTTTTCCAGAGATTCCGGTCTTGCTTTGCCAGTTGTCTCT
Original   541   GTTCTTCCCCTCCCATTCTTCTTCTCCAGAGACAGCGGCCTGGCCCTGCCAGTCGTCTCC
                  V  L  P  L  P  F  F  F  S  R  D  S  G  L  A  L  P  V  V  S

Optimized  601   TTGCCTTACAACGAGATTAGAATCACTGTTAAGCTTAGAGCAATTCAAGACTTGCTTATC
Original   601   CTCCCCTACAATGAGATCAGGATAACAGTCAAGCTGAGGGCCATCCAGGACCTCCTGATC
                  L  P  Y  N  E  I  R  I  T  V  K  L  R  A  I  Q  D  L  L  I

Optimized  661   TTGCAGCATAATACCACTGGTGCTATTTCCCCAATCGTTGCCGCAGATCTTGAAGGTGGA
Original   661   CTCCAGCACAACACCACAGGGGCAATCAGCCCCATCGTGGCCGCCGACCTCGAGGGAGGT
                  L  Q  H  N  T  T  G  A  I  S  P  I  V  A  A  D  L  E  G  G

Optimized  721   TTGCCTGACACAGTTGAGGCTAATGTCTATATGACAGTTGCCTTGATTACCGGAGATGAA
Original   721   CTCCCCGACACCGTCGAGGCCAACGTCTACATGACCGTCGCCCTCATCACCGGGGACGAG
                  L  P  D  T  V  E  A  N  V  Y  M  T  V  A  L  I  T  G  D  E

Optimized  781   AGACAAGCTATGTCTTCCACTGTTAGAGACATGGTTGTCGAGCAAGTTCAGGCTGCCCCA
Original   781   AGGCAGGCCATGAGCAGCACAGTCAGGGACATGGTCGTGGAGCAGGTGCAGGCCGCCCC
                                                      A
                  R  Q  A  M  S  S  T  V  R  D  M  V  V  E  Q  V  Q  A  A  P

Optimized  841   GTTCATATGGTCAACCCTAGAAATGCAGCTACATTCCATACCGATATGAGATTTTCTCAC
Original   841   GTCCACATGGTCAACCCCAGGAACGCGGCCACCTTCCACACCGACATGCGGTTCTCACAC
                  V  H  M  V  N  P  R  N  A  A  T  F  H  T  D  M  R  F  S  H

Optimized  901   GCCGTTAAGGCATTGATGTTCATGGTTCAAAACGTCACCCACCCATCAGTCGGTAGTAAT
Original   901   GCAGTCAAGGCCCTGATGTTTATGGTGCAGAACGTCACACACCCTTCCGTCGGCTCCAAT
                  A  V  K  A  L  M  F  M  V  Q  N  V  T  H  P  S  V  G  S  N

Optimized  961   TACACTTGTGTTACACCTGTTGTCGGTGTCGGAAACACTGTTCTTGAACCAGCTTTGGCC
Original   961   TACACCTGCGTCACTCCCGTCGTGGGAGTCGGCAACACGGTCCTGGAGCCAGCCCTGGCG
                  Y  T  C  V  T  P  V  V  G  V  G  N  T  V  L  E  P  A  L  A

Optimized  1021  GTTGATCCTGTCAAATCAGCTAGTCTTGTTTATGAGAATACAACCAGATTGCCAGACATG
Original   1021  GTGGATCCCGTCAAGAGCGCCAGCCTGGTGTACGAAAACACCACAAGGCTCCCCGACATG
                  V  D  P  V  K  S  A  S  L  V  Y  E  N  T  T  R  L  P  D  M

Optimized  1081  GGTGTCGAATACTATTCATTGGTTGAGCCATGGTACTATGCCACTTCTATTCCTGTTTCC
Original   1081  GGAGTCGAGTACTACTCGCTGGTGGAGCCCTGGTACTATGCCACCTCCATCCCAGTCAGC
                  G  V  E  Y  Y  S  L  V  E  P  W  Y  Y  A  T  S  I  P  V  S

Optimized  1141  ACAGGACATCACTTGTACTCATATGCACTTAGTTTGCAAGACCCACACCCTTCTGGTTCC
Original   1141  ACCGGGCACCACCTCTACTCCTATGCCCTCAGCCTACAGGACCCCCACCCATCCGGATCC
                  T  G  H  H  L  Y  S  Y  A  L  S  L  Q  D  P  H  P  S  G  S

Optimized  1201  ACCAACTACGGAAGATTGACTAACGCTTCTCTTAATGTTACTTTGTCCGCAGAAGCTACT
Original   1201  ACCAATTACGGCAGACTGACCAACGCCAGCCTTAACGTCACCCTGTCCGCTGAGGCCACC
                  T  N  Y  G  R  L  T  N  A  S  L  N  V  T  L  S  A  E  A  T

Optimized  1261  ACAGCCGCAGCTGGTGGAGGTGGAGATGACTCTGGATACACCACTGCACAGAAATATGCT
Original   1261  ACGGCCGCCGCAGGAGGCGGAGGTGACGACTCTGGGTACACCACCGCCCAAAAGTACGC
                                                      C
                  T  A  A  A  G  G  G  G  D  D  S  G  Y  T  T  A  Q  K  Y  A

Optimized  1321  CTTATCGTTTTGGCTATCAATCACAACATTATCAGAATTATGAACGGTAGTATGGGTTTT
Original   1321  CTCATCGTTCTGGCCATCAACCACAACATTATCCGCATCATGAACGGCTCGATGGGATTC
                  L  I  V  L  A  I  N  H  N  I  I  R  I  M  N  G  S  M  G  F

Optimized  1381  CCAATCCTTTAG
Original   1381  CCAATCTTGTAA
                  P  I  L  *
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Fig. 1. Nucleotide sequences and deduced amino acid sequence of codon-optimized and wild-type GSIV MCP gene. Bases modified to optimize codons are in red and wild-type bases are in black. A total of 305 out of 464 codons (almost 66%) were substituted.

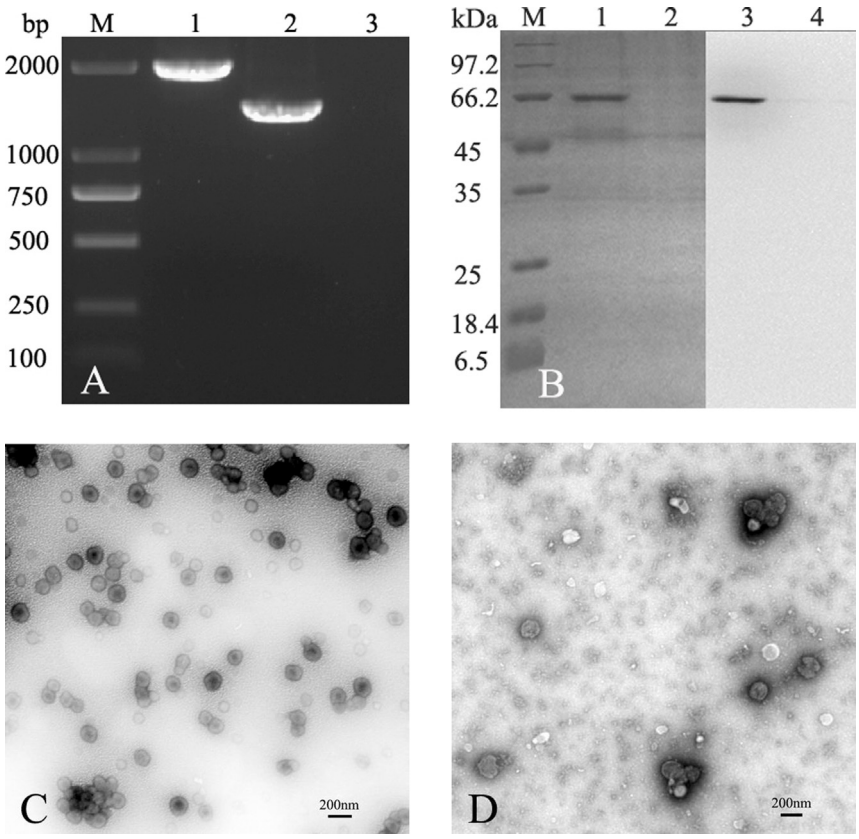

Fig. 2. Construction of the O-MCP plasmid and the expression and electron microscope observation of the O-MCP protein. (A) Verification of the transformant by PCR analysis. Lane M, DNA marker; lane 1, PCR products using 5′- and 3′-AOX1 primers; lane 2, PCR products of using O-MCPF and O-MCPR primers; lane 3, PCR products of the negative control. (B) SDS-PAGE and Western blot analysis of the O-MCP that was expressed in *Pichia pastoris*. The O-MCP was linked with the mouse anti-His-tag monoclonal antibody and horse radish peroxidase (HRP)-conjugated goat anti-mouse IgG antibody in turn. Lane M, protein marker; lane 1, the culture supernatant of Km71/pPICZα B-O-MCP; lane 2, the culture supernatant of the negative control (Km71/pPICZα B); lane 3, Western blot result of the culture supernatant of Km71/pPICZα B-O-MCP; lane 4, Western blot result of the culture supernatant of the negative control (Km71/pPICZα B). (C) Electron micrograph of negatively stained O-MCP was similar to that of the negatively stained GSIV particles (D) in size and morphology.

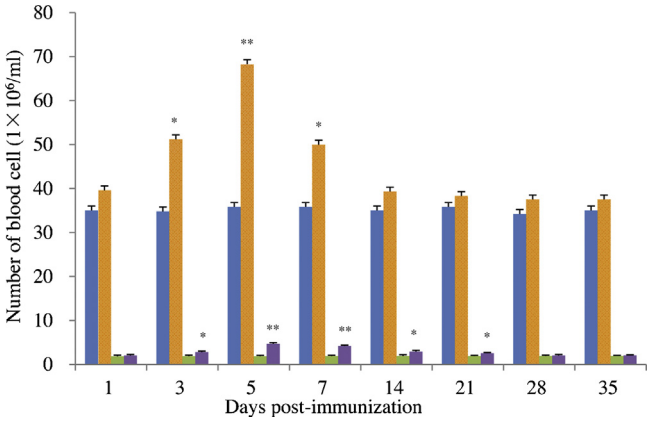

Fig. 3. The changes of the erythrocyte and leukocyte numbers in the peripheral blood of the Chinese giant salamanders immunized with the O-MCP. The results included: erythrocyte numbers of O-MCP immunized group (orange bar) and PBS group (blue bar), leukocyte numbers of O-MCP immunized group (purple bar) and PBS group (green bar). Data are presented as mean ± S.E. ($n = 3$). *$P < 0.05$; **$P < 0.01$.

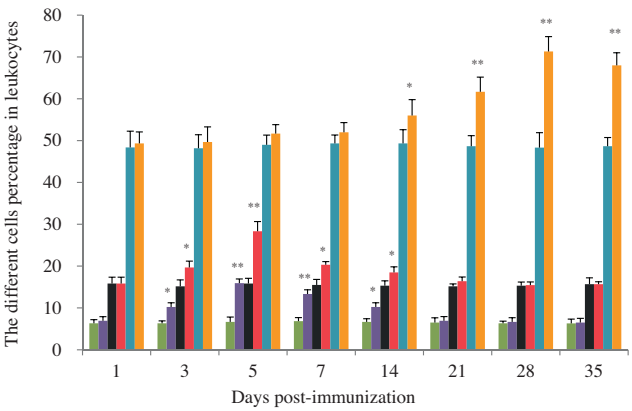

Fig. 4. The changes in the differential leukocyte counts of monocytes, neutrophils and lymphocytes in the peripheral blood of Chinese giant salamanders immunized with the O-MCP. The results included: monocyte percentages of O-MCP immunized group (purple bar) and PBS group (green bar), neutrophil percentages of O-MCP immunized group (red bar) and PBS group (black bar), lymphocyte percentages of O-MCP immunized group (orange bar) and PBS group (blue bar). Data are presented as mean ± S.E. ($n = 3$). *$P < 0.05$; **$P < 0.01$.

leukocyte numbers was also observed at day 21 post-immunization (Fig. 3).

The percentage of neutrophils began to increase on day 3 post-immunization and peaked at day 5 post-immunization (28.33 ± 2.32%). At day 7 post-immunization, the percentage declined slightly but it was significantly higher than that of the control group (Fig. 4). The percentage of monocytes increased significantly at day 4 and reached a peak of 15.92 ± 1.15% at day 7 post-immunization (Fig. 4). In the immunized group, the percentage of lymphocytes remained at basal levels throughout the

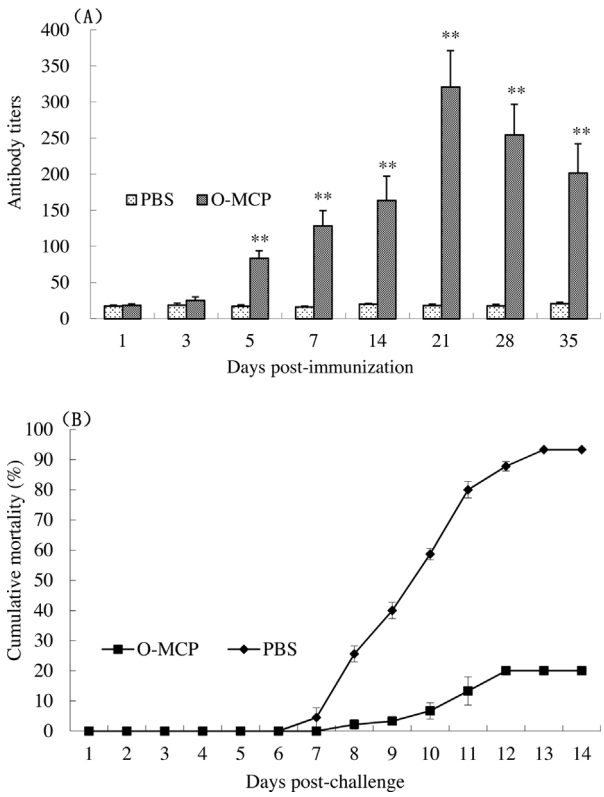

Fig. 5. Serum neutralization antibody assay and GSIV challenge test. (A) The changes of the serum antibody titers in Chinese giant salamanders immunized with O-MCP. The Chinese giant salamanders in immunized groups were vaccinated with the O-MCP at doses of 20 μg O-MCP and the mock immunized control group received the same volume of PBS. Blood samples were collected from three animals from each treatment group randomly at days 1, 3, 5, 7, 14, 21, 28, and 35 post-immunization. Data are presented as mean ± S.E. ($n = 3$). $^{**}P < 0.01$. (B) The cumulative mortality curves of vaccinated and control Chinese giant salamanders that were challenged with GSIV. Each symbol is shown as the mean of triplicate groups. The challenge test was initiated at day 15 post-immunization. The Chinese giant salamanders were inoculated intramuscularly with 0.1 ml of 20 μg O-MCP (■), while the negative control received the same volume of PBS (♦).

first 7 days and increased gradually, peaking at 71.33 ± 3.53% at day 21 post-immunization. The percentage decreased slightly but was still significant at day 35 compared to the control group (Fig. 4).

3.5. Serum neutralization assay

The results of serum neutralization assay (SN) indicated that the O-MCP vaccinated Chinese giant salamanders produced neutralizing antibodies against the GSIV. The mean of SN antibody titers of immunized group was 321.2 ± 50 at day 21 post-immunization. By day 35 post-immunization, the antibody titer was still highly significantly in the immunized group compared to the controls (Fig. 5A).

3.6. Protection of immunized Chinese giant salamanders against GSIV challenge

The Chinese giant salamanders vaccinated with O-MCP had a higher survival rate after the GSIV challenge than control group (Fig. 5B). At day 14 after the GSIV challenge, cumulative mortalities of the immunized Chinese giant salamanders injected with 20 μg of O-MCP were about 20%, while the cumulative mortality of the control group was 93%. The RPS value was 78%.

4. Discussion

The MCP is a well-known immunogenic protein of iridoviruses [29–32], and it has been used as a primary immunogen in several vaccine trials [10,24,33–35]. Recent studies indicate that the siRNA-based silencing of the GSIV MCP gene can effectively inhibit GSIV replication in FHM cells [23]. Additionally, the antiviral effect observed in a serum neutralization test confirmed the crucial role of the MCP gene in GSIV replication [23].

The native GSIV MCP gene was cloned into the expression vector pPICZα B, but it was difficult to detect the integrity of the MCP protein (data not shown). To improve expression of heterologous proteins, the codon optimization technique has been extensively used to improve the expression of heterologous proteins and typically results in a 10- to 50-fold increase in the target protein output [21]. In this study, the GC content of the O-MCP gene from the GSIV was adjusted from 59.55% to 43.03%, which was thought to be a more appropriate range for the *Pichia* system. Additionally, the Codon Adaptation Index (CAI) parameters increased from 0.6 to 0.84. A parameter of 1.0 is considered as the best for the expression organism, and a CAI of >0.8 is good in terms of the gene expression level that can be obtained.

To enable the gene cassette to carry a higher copy number of the optimized gene (O-MCP), the putative multi-copy insert transformants were selected using a high concentration of zeocin (1000 μg/ml), since the multi-copy gene generally had a positive effect on highly effective expression [36,37]. In order to simplify the purification process, the O-MCP gene was inserted the downstream of the α-factor secretion signal so that the secreted O-MCP protein should take the most of the total protein and be obtained easily from the medium [38]. The SDS-PAGE analysis of the methanol induced expression of Km71/pPICZα B-O-MCP showed that the recombinant O-MCP had a molecular mass of 66 kDa. The predicted size of O-MCP was 63 kDa, that suggested that eukaryotic post-translational modifications slightly increased the molecular weight of the protein. To detect the complete expression of O-MCP, we performed a Western blot using an anti-His antibody to identify the 6 × His-tag fused to the O-MCP protein. A band at about 66 kDa was observed and this molecular weight was identical with the protein observed by SDS-PAGE.

The virus-like particles (VLPs) resembled the complete GSIV in terms of its morphology, protein composition, and capsid structure, which could induce high-titer of antiviral neutralizing antibodies in experimental animal [38]. The VLPs have already been developed as a licensed vaccine for a human papillomavirus [39] and HBV [40]. In the results reported herein, the O-MCP produced from yeast exhibited a similar morphology to GSIV [23] and induced high concentrations of virus neutralizing antibodies. This suggested that the O-MCP could be a potential vaccine against GSIV in the Chinese giant salamander.

The health status and immunological responses of aquatic animals can be monitored by using hematology [41]. In this study, the numbers of erythrocytes and leukocytes from immunized Chinese giant salamanders increased significantly at day 3 and reached a peak at day 5 compared to the control salamanders. These results suggested that erythrocytes and leukocytes were involved in the early innate immune response of the Chinese giant salamanders after vaccination with the O-MCP. Additionally, the numbers of monocytes and neutrophils in the peripheral blood from the immunized Chinese giant salamanders also increased and reached a peak at day 5 post-immunization. However, the number of the lymphocytes did not change the first week post-immunization but increased significantly at day 14 and reached a peak at day 21 post-immunization. At the same time, the numbers of monocytes and neutrophils were decreasing. These results suggest that the monocytes and neutrophils were involved in the early innate immune

response of the Chinese giant salamanders vaccinated with the O-MCP, while the lymphocytes increased during the adaptive stage of the immune response. The hematological changes observed were similar to Chinese giant salamanders that were immunized with an inactivated iridovirus vaccine [3].

Neutralizing antibody plays a crucial role in providing protection against some viral diseases [42]. The SN antibody titers reached a peak at day 21 post-immunization both in this study and in a previous study that tested an inactivated GSIV vaccine. However, in this study the SN titers detected were slightly lower than those observed for the inactivated GSIV vaccine (341 ± 9.52) [3]. The Chinese giant salamanders vaccinated with the O-MCP exhibited significant protection compared to the control group (RPS, 78%). The RPS of the vaccinated group was higher in this study than that reported for the study that used the inactivated vaccine (RPS, 72%) [3]. The *in vivo* experiment did indicate that the O-MCP was very immunogenic and was able to induce a strong protective immune response against GSIV. Currently, all the epidemic strains of the GSIV appear to be the same genotype in China [43]. The low-cost of production of the recombinant O-MCP reported herein and the strong immune dose that was elicited suggest that there may be an application for this vaccine. Additional studies are required to further define the effective dose and time before protective immunity is elicited in different life stages of the Chinese giant salamander.

5. Conclusion

In summary, the MCP of the GSIV was successfully expressed in *P. pastoris* through optimization of the MCP codons and the recombinant protein exhibited a similar morphology to GSIV. Additionally, the O-MCP stimulated a protective immune response in the Chinese giant salamander suggesting that the O-MCP is a potential candidate for the development of a vaccine for the control of disease caused by the Chinese giant salamander iridovirus.

Acknowledgement

This work was supported by the Special Fund for Agro-scientific Research in the Public Interest (201203086).

Conflict of interest statement

All authors declare no conflict of interest.

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