Prokaryotic Expression of Largemouth Bass Virus MCP and Preparation and Characterization of Its Polyclonal Antibody

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Abstract

In order to further study the main epitope and antigenic properties of the major capsid protein (MCP) of largemouth bass virus (LMBV), this experiment based on the LMBV-MCP gene sequence number in GenBank (entry number: FR682503. 1) performed PCR amplification by designed region-specific primers. The target fragment was inserted into the prokaryotic expression vector pET22b to construct the prokaryotic expression plasmid of pET22b-MCP. The plasmid was transformed into E. coli (DE3) competent cells and induced by IPTG. The expression of the fusion protein was identified by SDS-PAGE. The target protein was purified by Ni-NTA column and identified. The purified protein was mixed with the adjuvant and emulsified as an immunogen. The polyclonal antibody of MCP protein was prepared by immunizing New Zealand white rabbit and detected its titer by ELISA. The results showed that after induction and expression of the recombinant plasmid, 4.5 mg of recombinant MCP protein with a purity of >85% was obtained by SDS-PAGE and WB verification. The ELISA titer test results showed that the titer of polyclonal antibody of MCP was ≥512000. This indicates that the high purity LMBV-MCP protein was successfully obtained in this study, and the prepared MCP polyclonal antibody has high reactivity and specificity.

Keywords

Largemouth bass virus (LMBV); MCP protein; prokaryotic expression; polyclonal antibody.

1. Introduction

The largemouth bass virus is also known as the California prion, the largemouth bass frog virus. In the years after 1995, the Santee-Cooper Reservoir of South Carolina and other states, USA, had serious fish disasters. They were identified as largemouth bass viruses by experts and classified as iridoviridae [1,2]. In 1999, Mao et al analyzed the viral protein, Restriction Fragment Length Polymorphisms (RFLP), DNA Methyltransferase (DMet) and Major Capsid Protein (MCP). The largemouth bass virus was classified into the species of the genus Ranavirus in the family iridoviridae [3]. Each member of the iridoviridae virus family has roughly the same structure, which is composed of an outer capsule, a liposome double-layer capsid, and DNA core. The liposome double capsid, also known as the Major Capsid Protein (MCP), is the main component of the icosahedral configuration of the iridovirus. Its molecular weight is about 48-55 kDa, accounting for about 40% of the total viral protein [4,5]. As the outer shell of the virus, MCP not only protects the viral nucleic acid, but also has its own virus-specific surface antigen, which can not only mediate the binding between the virus and the host but also cause a specific immune response [6]. The MCP gene of iridovirus is highly conserved, and the phylogenetic relationship between different iridovirus strains can be analyzed by MCP gene sequence or its encoded amino acid sequence alignment. In this study, we constructed a prokaryotic expression plasmid expressing the LMBV-MCP protein, and immunized the purified target protein as an antigen to immunize the rabbit, and finally obtained a polyclonal antibody against MCP. It can also lay the foundation on further study of the MCP in the process of innate immunity.Organization of the Text

2. Materials and methods

2.1 Materials

Rosstta (DE3) Competent Cells, IPTG, Pre-stained Protein Marker, Protein Concentration Quantitation Kit, TMB Colorimetric Kit, Horseradish-Labeled Goat Anti-Rabbit IgG, Incomplete Reagent, Complete Reagent, etc.

2.2 Construction of recombinant prokaryotic expression vector

According to the LMBV-MCP gene sequence on NCBI, Primer Premier 5.0 was used to design specific primer pairs, and the restriction sites BamHI and NdeI were inserted at the 5' end of the upstream and downstream, respectively. The expected length of the amplified fragment was 1392 bp. The primer sequence was: upstream primer LMBV-MCP-F: 5'-GGAATTCC ATATGATGT CTTCT GTTACGGGTTCTGG-3'; downstream primer LMBV-MCP-R: 5'-CGGGAT CCAG GATG GG GA AACCCATG-3'. Using the laboratory-preserved original strain LMBV as a template, PCR amplification was carried out by using the above-mentioned specific primers The PCR reaction system was 50 μL: 2 μL for each of the upstream and downstream primers (10 μmol/L), 25 μL for 2× PCR Mix, 1.5 μL for the template, and 19.5 μL for ddH2O. PCR reaction conditions: pre-denaturation at 94 ° C for 5 min; denaturation at 94 ° C for 30 s, annealing at 58 ° C for 30 s, extension at 72 ° C for 1 min 30 s, total of 35 cycles; 72 ° C extension for 10 min. The MCP amplification product was recovered by gelatinization, and the recovered fragment was digested by BamHI and NdeI. Then, MCP was ligated with the same double-digested and purified pET-22b vector at 16 °C overnight, and the ligated product was transformed into E. coli DH5α competent cells. For the cells, monocolones were picked and cultured in LB medium, and cultured overnight at 37 ° C with shaking. The bacterial liquid containing the target band was identified by PCR and sent to the biotechnology company for sequencing. The correct bacterial extract plasmid was the positive recombinant expression plasmid pET22b-MCP, and the recombinant plasmid map is shown in Fig. 1.

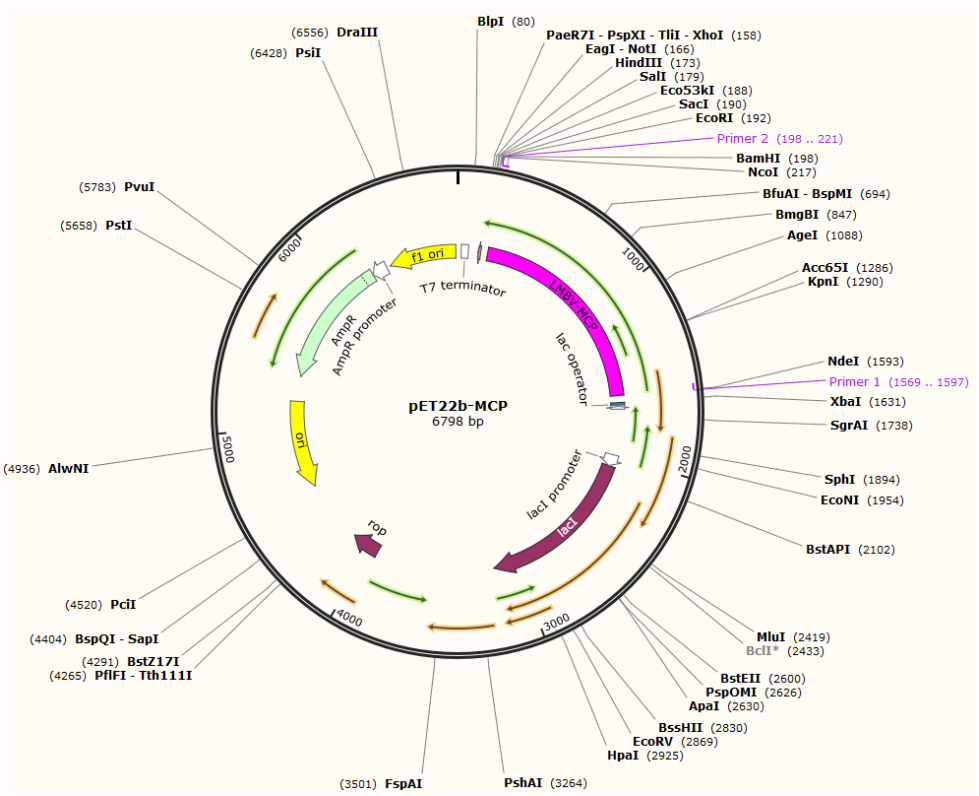

Fig.1 The map of the recombinant plasmid pET22b-MCP.

2.3 Expression of the target protein

The correct recombinant plasmid was re-transformed into E. coli DE3 competent cells. Monoclones were picked and cultured in liquid medium containing antibiotics. When the OD value of liquid reached at 0.6, IPTG was added and cultured at 20 ° C for 16 h and 37 ° C for 4 h, respectively. The liquid without IPTG added was acted as a negative control (NC). The cells were collected and then ultrasonic crushed. The supernatant and the precipitate were separately taken for SDS-PAGE gel analysis.

2.4 Purification and analysis of the target protein

A large amount of induced protein was induced according to the above-mentioned induction conditions. The thallus was subjected to ultrasonicate and the supernatant crude protein was subjected to affinity chromatography with a Ni-NTA column. The crude protein, effluent sample and elution sample were separately processed and sampled. The degree of purification was determined by SDS-PAGE. Six groups of better purity were dialyzed into 1 x PBS, 0.1% SKL, 2 mM DTT, pH 7.4 buffer. After the end of dialysis, the protein was concentrated with PEG20000 and filtered through a 0.22 μm filter, and then dispensed into 1 mL/tube stored at -80 °C. Finally, the molecular weight of the protein was determined by SDS-PAGE and the protein was verified by WB.

2.5 Preparation of polyclonal antibodies

The purified MCP protein was used as an antigen to routinely immunize New Zealand white rabbits. The primary immunizing antigen is a protein antigen mixed with an equal volume of Freund's complete adjuvant. After 3 weeks, the same immunization dose was emulsified in the same volume with the incomplete adjuvant, and then subjected to secondary immunization. After 5 weeks, the third enhancement is performed. Thus, 1 mL of ear vein blood collection were performed one week after booster immunization, and the antiserum titer was detected by ELISA. After 7 weeks, the 4th immunization was performed and the blood was taken through vena 1 week after the immunization. The antiserum titer was detected by ELISA. The whole body blood was collected by the carotid artery after the titer reached the requirement.

2.6 Determination of antibody titer

Antibody titer determination was performed by indirect enzyme-linked immunosorbent assay (ELISA). The obtained MCP polyclonal antibody was diluted 1:1000, and then diluted by the ratio. The enzyme plate was coated with 2 μg/mL of the purified MCP protein. The rabbit serum before the MCP protein immunized was acted as a negative control. The diluted MCP polyclonal antibody was used as the primary antibody, and the horseradish peroxidase-labeled goat anti-rabbit IgG was diluted 1:8000 as the secondary antibody. After tetramethylbenzidine (TMB) was developed, the absorbance at a wavelength of 450 nm was measured to determine the antibody titer.

3. Results

3.1 Construction and identification of recombinant plasmid

The recombinant plasmid pET22b-MCP was identified by PCR and found to be about 1400 bp in length (Fig. 2), which was consistent with the expected fragment size. The sequence obtained by sequencing was subjected to BLAST analysis. The results showed that the MCP gene was highly homologous to the sequence in GenBank, which matched 99%. The reading framework was also correct.

3.2 Expression of fusion protein

The recombinant prokaryotic expression plasmid pET22b-MCP was transformed into E. coli DH5α competent cells and induced by IPTG. The cells were cultured at 20 °C for 16 h and cultured at 37 °C for 4 h, respectively. The supernatant and precipitate were taken for SDS-PAGE electrophoresis. The bacteria without IPTG was acted as control. The results are shown in Figure 3. It can be seen from Fig. 3 that the fusion protein MCP can be efficiently expressed in the host bacteria in a soluble form after induction at 20 ° C for 16 h and at 37 ° C for 4 h. The expression amount is mainly concentrated

in the precipitate. Partial expression was also observed in the supernatant at 20 °C. The molecular weight of the fusion protein was approximately 52.6 kDa and the results were as expected.

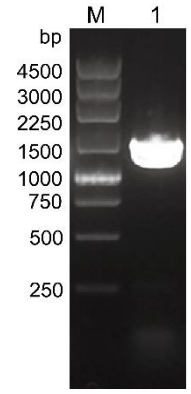

Fig.2 The PCR of MCP.
M: DNA Marker, 1: The specific amplification of MCP of recombinant plasmid pET22b-MCP.

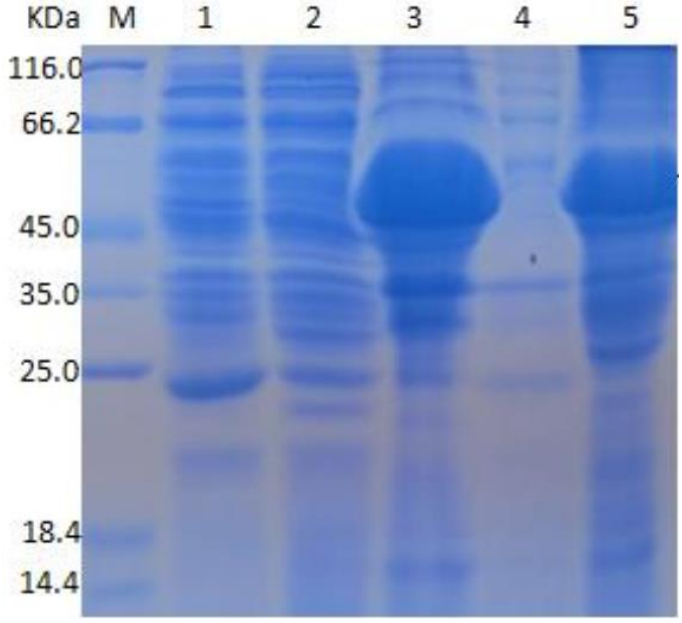

Fig.3 SDS-PAGE analysis result of pET22b-MCP recombinant plasmid fusion protein. M: protein marker; 1: protein before induction; 2: the supernatant of 20 ° C; 3: the precipitation of 20 ° C; 4: the supernatant of 37 ° C; 5: the precipitation of 37 ° C.

3.3 Purification and analysis of prokaryotic expression proteins of MCP

The affinity chromatography was carried out by Ni-NTA and the separated samples were processed. The purification effect was verified by SDS-PAGE. The results are shown in Fig. 4. After the crude protein being eluted by imidazole, the target protein band can be clearly detected. The 20 mM imidazole concentration was effectively to remove the heteroprotein. As the concentration of imidazole increases, the purity of the protein of interest gradually increases. A single target band can be obtained at an imidazole concentration of 500 mM. The purified target protein was subjected to SDS-PAGE. As shown in Fig. 5, the MCP protein produced a distinct target band around 53 kDa, and the size was consistent with the expected result. The WB was used to further confirm the purified target protein, and the results showed that a distinct band appeared at the corresponding position, indicating that the protein was the target protein (Fig. 6). After purification, the MCP protein was concentrated to 1 mL/tube, and the protein concentration was determined by a non-interfering protein quantification kit. From the measurement results, the purified protein concentration of the protein was 0.75 mg/mL, and the total amount was about 6 mL, for a total of 4.5 mg. (Detailed results were not shown).

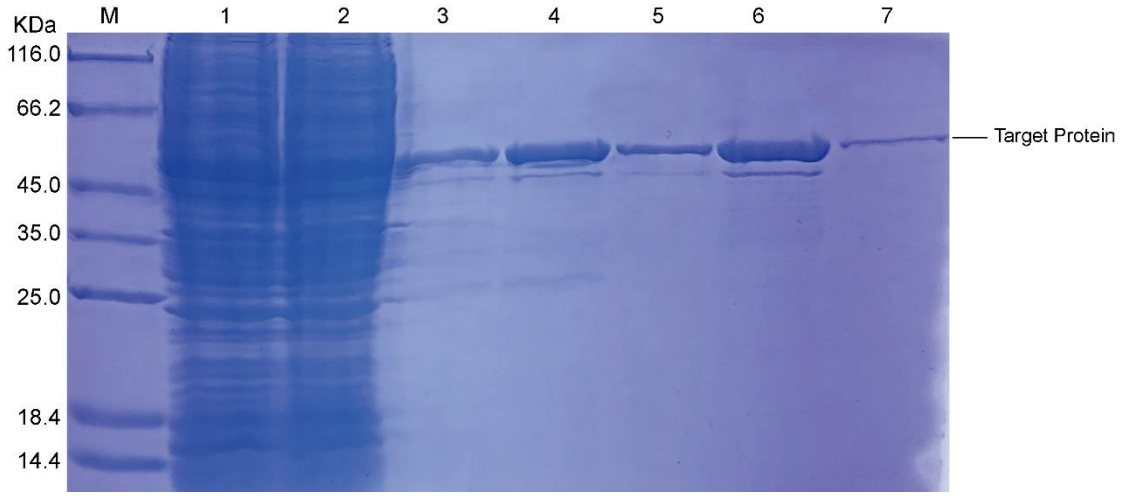

Fig.4 The SDS-PAGE analysis map of fusion protein affinity chromatography.
M: Protein marker; 1:crude protein; 2: the sample of impure efflux; 3: 20 mM imidazole eluting fraction; 4-5: 50 mM imidazole eluted fraction; 6-7: 500 mM imidazole eluted fraction.

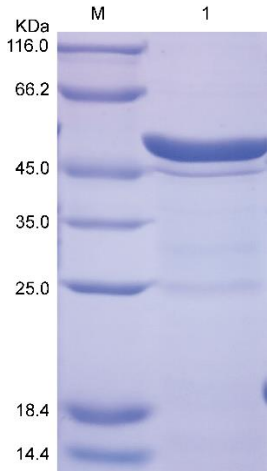

Fig.5 SDS-PAGE analysis result of target fusion protein.
M: protein marker; 1: MCP protein with His flag

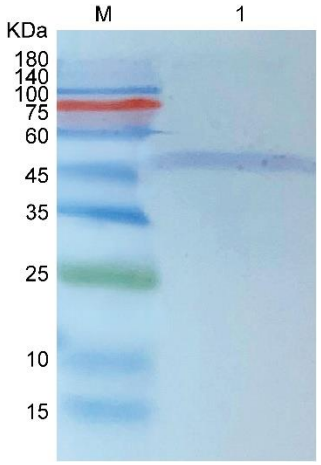

Fig.6 Western blot analysis of fusion protein.
M: protein marker; 1: MCP protein with His flag

3.4 Identification of polyclonal antibody titers

Serum titers and polyclonal antibody titers were determined by ELISA using the serum of rabbits after booster immunization. The white rabbit serum before immunization of the MCP protein was used as a negative control (NC), and no serum treatment was used as a blank control. The results were shown at Tab.1. Antibody efficacy value ≥ 2.5 × negative value, antibody titer ≥ 512000.

Tab.1 Titer determination result of MCP polyclonal antibody.

No.	NC	Blank	1K	2K	4K	8K	16K	32K	64K	128K	256K	512K
A	0.063	0.051	2.249	2.324	2.272	2.237	2.237	2.175	2.057	1.995	1.176	1.082
B	0.056	0.046	2.141	2.176	2.044	2.195	2.176	2.05	2.15	1.993	1.427	1.53

4. Discussion

Largemouth bass virus (LMBV) is a member of the the species of the genus Ranavirus in the family iridoviridae, which is extremely lethal to largemouth bass. As an important structural protein of LMBV, MCP protein is the main binding site of serum antibodies and has good immunogenicity [6]. The LMBV virus in this study was derived from a largemouth bass infected with LMBV in Foshan City, Guangdong Province, which has high homology with LMBV from the German strain on NCBI. By constructing the recombinant prokaryotic expression plasmid pET22b-MCP for expression of the target protein, it was found that a single target band appeared in the target region after purification, and the band size was consistent with the expected result. The WB results further confirmed that the collected and purified protein was the target protein MCP. The purified target protein was used as an antigen to immunize New Zealand white rabbits. After a round of booster immunization, the serum antibody titer of the rabbits was tested and the total antibody was extracted after the titer was determined to reach the required value. From the results, the titer of the final antibody was as high as 1:512,000. Thus, the highly expressed LMBV-MCP protein can be obtained by using the pET22b expression system, and the polyclonal antibody developed by the protein can be used for establishing the LMBV detection method, especially for the double-anti-sandwich ELISA detection method.

Acknowledgement

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