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Identification of Omp38 by immunoproteomic analysis and evaluation as a potential vaccine antigen against *Aeromonas hydrophila* in Chinese breams

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ABSTRACT

Aeromonas hydrophila is a fish pathogen causing systemic infections in aquatic environments, and determining its antigenic proteins is important for vaccine development to reduce economic losses in aquaculture worldwide. Here, an immunoproteomic approach was used to identify immunogenic outer membrane proteins (OMPs) of the Chinese vaccine strain J-1 using convalescent sera from Chinese breams. Seven unique immunogenic proteins were identified by two-dimensional (2-D) electrophoresis and matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF-TOF-MS). One protein of interest, Omp38, was expressed, and its immunogenicity and protective efficacy were evaluated in Chinese breams. The two groups of fish immunized with the inactivated vaccine and recombinant Omp38 protein showed significant serum IgM antibody levels after vaccination, compared with the fish injected with PBS buffer. In addition, the superoxide dismutase (SOD) activity, lysozyme (LSZ) activity and phagocytosis activity of head kidney lymphocytes of immunized groups were significantly higher than those of the control. The fish receiving inactivated vaccine and recombinant Omp38 protein developed a protective response to a live *A. hydrophila* challenge 45 days post-immunization, as demonstrated by increased survival of vaccinated fish over the control and by decreased histological alterations in vaccinated fish. Furthermore, protective effect was better in Omp38 group than in the inactivated vaccine group. These results suggest that the recombinant Omp38 protein could effectively stimulate both specific and non-specific immune responses and protect against *A. hydrophila* infection. Therefore, Omp38 may be developed as a potential vaccine candidate against *A. hydrophila* infection.

1. Introduction

Aeromonas hydrophila is an important bacterial pathogen with a broad geographical distribution in the aquatic environment, which can infect terrestrial animals and a variety of fish species including common carp, cat fish, tilapia, eel and goldfish [1,2]. Hence, the development of a vaccine is essential both in reducing the economic losses which occur as a result of the diseases caused by this bacterium and for decreasing the use of antibiotics in the aquaculture industry. Vaccination strategies employed against *A. hydrophila* include heat-killed cells, heat or formalin inactivated bacterial extracts, live cells and biofilm [3–8]. A major problem for the development of vaccines against this pathogen is the antigenic diversity of *A. hydrophila* strains. Formalin-killed *A. hydrophila* vaccines have shown significant protection against the homologous strain but not the heterologous strain of *A. hydrophila*.

For the reasons mentioned above, there is growing interest in developing a new generation of *A. hydrophila* vaccines. The research is oriented toward identification of antigens that can induce protective immune responses and development of a recombinant subunit vaccine. Recently, the subunit vaccines for this bacterium have been mainly focused on outer membrane proteins (OMPs). The outer membrane of Gram-negative bacteria enables the bacteria to adhere to host tissues and take up nutrients from the hosts [9]. Bacterial OMPs are highly immunogenic and would be good candidates for vaccine development due to their exposed epitopes on the cell surface, which allows them to easily interact with the host immune system [10]. Because of their location at the host-bacterial interface, the OMPs of bacterial pathogens have been of particular interest with respect to host immune responses and as targets for drug therapy. They may stimulate the host to produce strong neutralization responses to microorganisms. It has been shown that some OMPs of bacteria can confer protective immunity. Rahman and Kawai [5] demonstrated that OMPs of *A. hydrophila* are immunogenic and may be useful as vaccine antigens in fish. Khushiramani

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et al. [11] suggested that recombinant OmpTS of *A. hydrophila* is highly immunogenic in Indian major carp and can protect fish from infection. In another study by Khushiramani et al. [12], the protective immune responses of Omp48 against two important warm water fish pathogens, *A. hydrophila* and *Edwardsiella tarda*, were reported. Fang et al. [8] suggested that recombinant adhesin AHA1 has the potential to be an effective vaccine in fish targeting multiple microorganisms, such as *A. hydrophila* and a freshwater *Vibrio anguillarum* strain. Guan et al. [13] showed that OMP can increase the protective immunity in European eel (*Anguilla anguilla*) against *A. hydrophila* as well as *Aeromonas sobria*. Merino et al. [14] demonstrated *A. hydrophila* AH-3 porin II as an important immunoprotective molecule for fish against either *Aeromonas salmonicida* or *A. hydrophila* strains.

Immunoproteomics, a technique involving two-dimensional (2-D) electrophoresis followed by Western blotting, has been successfully used to identify pathogen antigens for the development of new vaccines [15,16]. In this study, we applied an immunoproteomic approach to identify candidate antigens in OMPs of the Chinese *A. hydrophila* strain J-1 with convalescent sera from Chinese breams (*Megalobrama amblycephala*). An important *A. hydrophila* OMP, Omp38, was identified and evaluated for immunogenic potential and protective efficacy in Chinese breams. Antibody production and non-specific immune response parameters, such as superoxide dismutase (SOD) and lysozyme (LSZ) activities in sera and phagocytosis capacity in head kidney leucocytes, were observed at different times after immunization. This study demonstrates the feasibility of using immunoproteomics for antigen discovery and provides a new candidate for vaccine development against *A. hydrophila*.

2. Materials and methods

2.1. Bacterial strains and culture conditions

The *A. hydrophila* strain J-1, used as a vaccine strain in China, was first isolated from dead cultured cyprinoid fish in eastern China in 1989. The green fluorescent protein (GFP) labeled *A. hydrophila* J-1 strain (J-1^{GFP}) was constructed in the previous study [17]. All plasmids and their host *Escherichia coli* in the study were obtained from TaKaRa (China). *A. hydrophila* and *E. coli* were routinely cultured in Luria broth (LB) or on Luria agar (LA) plates at 28 °C and 37 °C, respectively. For all strains used in this study, kanamycin (50 μg/ml) was added as required.

2.2. Animals

This study was conducted in accordance with the guidelines established by Nanjing Agricultural University and the Guide for the Care and Use of Laboratory Animals. BALB/c mice weighing 18–22 g were supplied by the Comparative Medicine Center of Yangzhou University, China. The Chinese breams were supplied by the Freshwater Fisheries Research Center, Chinese Academy of Fishery Sciences in China.

2.3. Antibody production

2.3.1. Preparation of Chinese bream sera against A. hydrophila J-1

Chinese breams ($n = 50$) were allocated to five concrete tanks ($800 \times 200 \times 100$ cm) and acclimated for 15 days at 25 °C. They were anesthetized with tricaine methanesulfonate (MS-222) (Hangzhou Animal Medicine Factory, China) at a concentration of 95 μg/ml, and infected with 0.1 ml (1×10^8 CFU/ml) of *A. hydrophila* J-1 by dorsal intramuscular inoculation. Sera from breams were collected on days 30 and 40 after infection for immunological assays.

2.3.2. Preparation of IgM monoclonal antibodies (mAbs) from Chinese breams

Sera IgM from Chinese breams were purified by ammonium sulfate precipitation, followed by Macro-prep high Q Cartridge and Sephacryl S-300 column chromatography. The hybridomas secreting mAbs against Chinese breams serum IgM were established by fusing SP2/0 with spleen cells from BALB/c mice immunized with the purified IgM. Immunization, fusion and cloning were performed according to a previously described method [18,19]. Mouse-anti-bream IgM monoclonal antibody (M-bream-IgM mAb) was obtained as the secondary antibody for evaluating the specific IgM titer of breams.

2.4. Precipitation of OMPs

OMPs from whole cells of *A. hydrophila* J-1 were prepared with Sarkosyl as described previously [20]. OMPs were resuspended in ddH_2O and frozen at −20 °C. Protein samples were treated with the 2-D Clean-up Kit (GE Healthcare, USA) to remove impurities, and their concentrations were determined by using the Ettan™ Sample Preparation Kits and Reagents 2-D Quant Kit (GE Healthcare, USA).

2.5. 2-D electrophoresis and Western blot analysis

Precipitated OMPs were dissolved in rehydration buffer (8 M urea, 2% CHAPS, 50 mM DTT, 2% IPG buffer solution, 0.002% bromophenol blue) containing 100 μg of the protein sample in a total volume of 125 μL and centrifuged at 10,000 × g for 20 min at room temperature to remove the insoluble materials. The proteins were then absorbed onto a 13 cm immobilized pH gradient strips and isoelectric focusing (IEF) was performed using an Ettan IPGphor III and by stepwise increase of the voltage as follows: S1 30 V, 12 h; S2 500 V, 4 h; S3 1000 V, 1 h; S4 2000 V, 1 h; S5 5000 V, 1 h; S6 8000 V, 2 h 30 min; S7 8000 V, 2 h and S8 500 V, 10 h.

Prior to SDS-PAGE, each IPG strip was washed in equilibration buffer 1 (75 mM Tris–HCl at pH 8.8, 6 M urea, 2% SDS, 2% DTT, 0.002% bromophenol blue) and equilibration buffer 2 (75 mM Tris–HCl at pH 8.8, 6 M urea, 2.5% iodoacetamide, 2% SDS, 0.002% bromophenol blue) each for 15 min. SDS-PAGE and immunoblotting procedures were performed as described in a previous study [20]. Sera from Chinese breams infected with *A. hydrophila* J-1 were used as the primary antibody at a 1:200 dilution. Following incubation with M-bream-IgM mAb (1:1000 dilution) and washing, the secondary antibody peroxidase-conjugated goat-anti-mouse IgG (Boster, China) was used at a 1:2000 dilution. The antibody-bound proteins were then detected using the enhanced chemiluminescence (ECL kit) (Pierce, USA) and visualized by LAS-4000 chemiluminescence imaging analysis system. Three replicates were performed for each sample.

2.6. Protein identification and database searches

Protein spots of interest were excised from the 2-D gels and sent to Shanghai GeneCore BioTechnologies for tryptic in-gel digestion and matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF-MS) or MALDI-TOF-TOF-MS. The peak list for each protein spot was analyzed with the aid of "PMF" and "MS/MS Ion Search" engines of MASCOT software (http://www.matrixscience.com/) and/or a local MASCOT server (http://www.proteomics.cn/mascot) for sequence matches. Proteins that were in similar locations on gels and on PVDF membranes were coded with the same number. The probability score for the match, molecular weight (MW), isoelectric point (pI) and number of peptide matches were analyzed for confident spot identification. Sequences of identified proteins were submitted to a BLAST server (http://www.ncbi.nlm.nih.gov/