



Roles of phagocytosis activating protein (PAP) in *Aeromonas hydrophila* infected *Cyprinus carpio*

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ABSTRACT

Cyprinus carpio (koi) is one of the most popular ornamental fish. A major problem for *C. carpio* farming is bacterial infections especially by *Aeromonas hydrophila*. Previously studies had shown that the Phagocytosis Activating Protein (PAP) gene was involved in the innate immune response of animals. Therefore, we attempted to identify a role for the PAP gene in the immunology of *C. carpio*. The expression of the PAP was found in *C. carpio* whole blood and increased when the fish were stimulated by inactivated *A. hydrophila*. In addition, PAP-phMGFP DNA was injected as an immunostimulant. The survival rate and the phagocytic index were significantly increased in the *A. hydrophila* infected fish that received the PAP-phMGFP DNA immunostimulant. A chitosan-PAP-phMGFP nanoparticle was then developed and fed into fish which infected with *A. hydrophila*. These fish had a significantly lower mortality rate than the control. Therefore, this research confirmed a key role for PAP in protection fish from bacterial infection and the chitosan-PAP-phMGFP nanoparticle could be a good prototype for fish immunostimulant in the future.

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1. Introduction

One of the most popular ornamental fish is koi carp, *Cyprinus carpio haematopterus*, because of its fancy color and ability to survive and adapt to many climates and water conditions. Koi is one of subspecies of the common carp, *C. carpio*. Unlike another subspecies, koi shows variety color. Therefore, koi carp is a popular fish for ornamental. Recently, the hobby of keeping koi eventually spread worldwide. However, koi ornamental still has some problems. One of the major problems is infection by bacterium.

Aeromonas hydrophila is one of the most bacterium infected in koi carp. *A. hydrophila* causes disease in fish known as “Motile Aeromonas Septicemia” (MAS), “Hemorrhagic Septicemia,” “Ulcer Disease,” or “Red-Sore Disease.” *A. hydrophila* has been categorized as an opportunistic pathogen. However, the term “opportunistic pathogen” conveys that *A. hydrophila* always is capable of

producing disease if given the chance (Cipriano, 2001). Fish infected with *A. hydrophila* shown many different symptoms. These range from sudden death in otherwise healthy fish to lack of appetite, swimming abnormalities, pale gills, bloated appearance, and skin ulcerations. The skin ulcers may occur at any site on the fish and often are surrounded by a bright rim of red tissue. Infection by *C. carpio* results in a distended abdomen and the scales bristle out from the skin (Jagruthi et al., 2014).

Ribosomal protein L26 (RPL26) is a ribosomal protein in the 60S subunit of the ribosome. The RPL26 gene has been named as a phagocytosis-activating protein (PAP) because there is some evidence that PAP is involved in an immune response. PAP has been found in the black tiger shrimp, *Penaeus monodon* infected with white spot syndrome virus (WSSV) (Deachamag et al., 2006). In addition, it was shown that PAP stimulated the immune response of the Pacific white shrimp, *Litopenaeus vannamei*. PAP also protected the white shrimp against several pathogens such as WSSV, Yellow head virus (YHV) and *Vibrio harveyi* (Khimmakthong et al., 2011). There is also some evidence that the entry of PAP into phagocytic cells may be facilitated by the $\alpha 2M$ protein (Chotigeat et al., 2007). Moreover, the PAP gene was activated in a mouse macrophage cell

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line when it was treated with silica, LPS and IFN γ (Segade et al., 1996). It was of interest, that in a previous study, we produced a complex of chitosan-PAP-phMGFP nanoparticles and this complex protected shrimp from pathogens (Khimmakthong et al., 2013).

Chitosan is a copolymer of N-acetyl-D-glucosamine and D-glucosamine, obtained by the alkaline deacetylation of chitin. Various studies have referred to the use of chitosan as a gene carrier because of its low toxicity, low immunogenicity, excellent biocompatibility and high positive charge density (Shu and Zhu, 2002; Lee et al., 2005). A chitosan-based formulation for delivery of DNA has been published (Mao et al., 2010). There has been some research to indicate that a chitosan-DNA nanoparticle can also help to protect fish (Asian seabass, *Lates calcarifer*) from pathogens. A gene that encoded for an outer membrane protein (OMP) of *Vibrio anguillarum* was used to construct a DNA immunostimulant using pcDNA 3.1. The constructed plasmid was encapsulated in chitosan and orally vaccinated to *L. calcarifer* with a chitosan-OMP complex. This product showed a moderate protection against an experimental infection with *V. anguillarum* (Kumar et al., 2008).

Therefore, in this study we attempted to investigate a role for the PAP gene in the immune response of *C. carpio*. Inactivated *A. hydrophila* was used to stimulate the immune response of *C. carpio* and then the PAP expression was measured. In addition we injected PAP to *C. carpio* with the PAP-phMGFP plasmid and fed the chitosan-PAP-phMGFP nanoparticles then checked the survival rate after challenging with *A. hydrophila*.

2. Materials & methods

2.1. Animals

C. carpio, average total length 13 cm and average total body mass 25 ± 3 g were purchased from a farm at Ratchaburi province. Fish were reared in a rectangular tank saturated with oxygen. Fish were fed with 3% commercial feed per body weight, twice a day.

2.2. RT-PCR

Total RNA was extracted from fish blood using Trizol reagent (Invitrogen, Carlsbad, CA, USA) following the manufacturer's protocol. Four hundred nanograms of total RNA was incubated with 200 ng of random primers at 70 °C for 5 min and cooled on ice for 5 min. This mixture was added to 1 $\times$ avian myeloblastosis virus (AMV) buffer, 1 mM deoxynucleotide triphosphate, and 10 U of AMV reverse transcriptase (Promega, Madison, WI, USA) in a 25 μ L reaction mixture and incubated at 48 °C for 2 h. The cDNA was used as a template. The primers for the PAP gene were designed from the conserved regions including the forward primer (PAP F: 5' CAATGTCCGTGCCATGC 3') and a reverse primer (PAP R: 5' CCGACCAGCAGCCTTGTT 3'). PCR was initiated with a first denaturation step of 5 min at 95 °C, followed by 40 cycles of 94 °C 2 min, annealing at 50 °C for 1 min, and extension at 72 °C for 1 min. Negative controls consisted of reactions with no template cDNA. The PCR products were detected after separation by electrophoresis on agarose gel.

2.3. PAP sequence analysis

The PAP gene was amplified from the blood of *C. carpio* which includes serum, red blood cell and white blood cell using PAP-F and PAP-R primers. The PCR product was cloned into the pGEM-T Easy (Promega, Madison, WI, USA) and sequence analysis was performed using the ABI prism 377 apparatus. The obtained nucleotide sequence was aligned with sequences in the GenBank databases using the BLASTx search program from NCBI (<http://www.ncbi.nlm.nih.gov>) to confirm the gene identity. PAP sequences from many organisms were obtained from GenBank. All sequences were aligned and analyzed using the CLUSTAL X version 2.7 to determine their similarity.

2.4. Preparation of inactivated *A. hydrophila*

A. hydrophila was grown in Tryptic-Soy-Broth (TSB) at 28 °C until an OD of 0.8–1.0 at 610 nm was reached. This culture was boiled at 100 °C for 30 min and centrifuged at 4000 rpm for 15 min. The supernatant was discarded. The pellet, that contained inactivated *A. hydrophila*, was resuspended in PBS until an OD of 0.8–1.0 was reached. The inactivation was confirmed by culturing the resuspended cells in Tryptic Soy Agar (TSA) at 28 °C for 48 h. The inactivated cells did not grow in the medium. The inactivated *A. hydrophila* was used as an immunostimulant.

2.5. Stimulation of the fish immunity by inactivated *A. hydrophila*

The 0.1 mL (1×10^9 CFU) of inactivated *A. hydrophila* was injected into the carp. After injection at 1, 2, 5 and 7 days, blood samples were collected. The total RNA was extracted and reversed to cDNA. The mRNA expression level was investigated by RT-PCR. The beta-actin gene was used as an internal control. The primers for the beta-actin gene were Beta-F: (5' CAGATCATGTTYGAGACCTTC 3') and Beta-R: (5' GATGTCCACGTCCACTTCAT 3'). The PCR products were separated by electrophoresis on a 1.5% agarose gel. The density of the DNA bands was calculated by the Scion image program. The expression levels for each group were compared statistically using a one-way ANOVA of the SPSS program at a 95% confidence level ($p < 0.05$).

2.6. PAP-phMGFP plasmid preparation

The *E. coli* Top 10 that containing phMGFP or PAP-phMGFP plasmids were received from Khimmakthong et al., 2011. The plasmids were extracted following the protocol of Green & Sambrook's (Green and Sambrook, 2012). Briefly, the bacteria were culture in Luria Bertaini (LB) contained 80 μ g/mL of ampicillin. The culture was incubated at 37 °C for 16–18 h and centrifuged at 10,000 $\times$ g for 1 min. The pellet was then resuspended with 100 μ L of solution I (50 mM glucose, 25 mM Tris-HCl pH 8.0 and 10 mM EDTA) and incubated at room temperature for 5 min. Solution II (0.2 N NaOH and 1% SDS) was added and the tube was stored on ice for 5 min. The solution III (50 M potassium acetate and glacial acetic acid) was added and then the tube was stored on ice for 30 min. The bacterial lysate was centrifuged at 12,000 $\times$ g for 15 min. The supernatant was transferred to a new tube. The plasmid was precipitated by adding one volume of ethanol at room temperature. The precipitated plasmid was harvested by centrifugation at 12,000 $\times$ g for 15 min. Then the pellet was washed with 70% ethanol, and sediment at 12,000 $\times$ g for 5 min. The pellet was dried by vacuum. Finally, the plasmid was dissolved in deionized water. The quality and quantity of the plasmid were checked by electrophoresis on an agarose gel and detected using a spectrophotometer at 260 and 280 nm.

2.7. Protection efficiency

C. carpio was divided into eight groups, and each group was consisted of 27 fish (the experiment was done in triplicate, 1N = 9). Group 1, 2 and 3 were injected intraperitoneally with 200, 400 and 600 μ g of PAP-phMGFP, respectively. Another three groups were injected intraperitoneally with 200, 400 and 600 μ g

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