Vibrio anguillarum組換えヘモリジンのヒラメにおける免疫効果

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Immunoprotection of Japanese Flounder Paralichthys olivaceus by Recombinant Vibrio anguillarum Hemolysin

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ABSTRACT—The gene vah4 encoding Vibrio anguillarum hemolysin was cloned and heterologously expressed. The purified recombinant VAH4 protein was then injected into Japanese flounder Paralichthys olivaceus as an immunogen. Activities of superoxide dismutase, catalase, alkaline phosphatase and acid phosphatase in liver became higher in the immunized group. However, these improvements vanished in 72 h after the injection. VAH4-specific antibodies were detected at a titer of 1×10²⁴ by ELISA on the 10th day after the injection. In a separate experiment, fish were immunized with VAH4 twice and challenged with V. anguillarum. The immunization with the VAH4 protected fish from V. anguillarum infection.

Key words: Vibrio anguillarum, vah4 gene, hemolysin, Paralichthys olivaceus

Vibrio anguillarum, usually attached to suspended substance in sea water, is considered the major infectious agent in fishes and brings enormous loss to aquaculture every year¹⁻⁶. The bacterium includes 16 serotypes according to Grisez’s classification based on the different lipopolysaccharides⁷. Its pathogenic mechanisms are complicated, including endotoxin, exotoxin, iron uptake system mediated by plasmid and the resistance to serum anti-bacterium activities⁸⁻¹⁰. The hemolysin is considered to be one of the major pathogenic factors among them by contributing to hemorrhagic septicemia and diarrhea. To date, five hemolysin genes (vah1–5) of V. anguillarum have been identified¹¹.

Previously, some studies indicated that the V. anguillarum whole-cell vaccines were competent to protect marine fishes from V. anguillarum infection¹²⁻¹³. The objective of this study is to clone the vah4 gene of V. anguillarum, then express and purify the VAH4 protein, and finally to determine the immunoprotective effect of VAH4 protein on Japanese flounder Paralichthys olivaceus.

Respiratory burst of aerobic organisms caused by engulfment results in oxygen free radicals and other reactive oxygen species which lead to a bactericidal effect, including superoxide anion, hydroxyl radicals, singlet oxygen and hydrogen peroxide. All aerobic organisms contain the antioxidant defense system to prevent the endogenous metabolism of reactive oxygen free radicals. The key components of this system include superoxide dismutase and catalase¹⁴. Activities of these two enzymes may reflect whether a toxin can activate immune system of the Japanese flounder¹⁵. A variety of hydrolytic enzymes also play an equally important role in the process of engulfement, such as acid phosphatase and alkaline phosphatase. In this study, these enzyme activities in Japanese flounder were monitored after the VAH4 hemolysin injection.

Materials and Methods

Fish

Japanese flounder were bought from Lianyungang Eastern Aquaculture Company, and reared in sea water at 14⁻¹⁶°C for 320 days. The fish were fed once a day with commercial pellet feed (Meiyan Fish Feed Factory). The average length and weight of the fish were 29 cm and 280 g, respectively.

Bacterial strains and culture condition

V. anguillarum strain CAVA23 and V. parahaemolyticus stain CAVP6 were provided by Dr. Huang (Chinese Academy of Fishery Sciences), and statically cultured in LB medium at 28°C for 24 h. Escherichia coli strain BL21 (DE3; Novagen) were cultured in LB medium at 37°C for propagation and 28°C for induced expression of recombinant protein.

Gene cloning and plasmid construction

Gene vah4 was amplified by PCR using primers GCGAATTCATGAAAACCATACGCTCAGCAT and CGAAGCTTTCACGCTTGTTTTTGGTTTAAA (the enzyme recognition sites for EcoR I and Hind III were shown in bold). Genomic DNA of V. anguillarum was used as the template. The PCR parameters were as follows: one cycle of 94°C for 2 min, then 35 cycles of 94°C for 20 s, 55°C for 40 s, 72°C for 40 s, followed by an additional extension at 72°C for 7 min. All PCR regents used were bought from Takara Bio Inc. The PCR product was purified using Gel DNA purification Kit
Expression and purification of VAH4 hemolysin

The host strain BL21 (DE3) was transformed with the verified pET-vah4 plasmid and incubated in LB medium at 37°C until OD$_{600}$ of the suspension reached 0.6. The culture was then added with IPTG to a final concentration of 1 mM and incubated at 20°C for another 10 h. Then the cells were collected by centrifugation at 5,000 rpm for 5 min at room temperature. The collected cells were rinsed two times with PBS (pH 7.4) and then resuspended in 5 mL column buffer (50 mM Tris-HCl pH 8.0, 0.5 mM NaCl, 20 mM imidazole) per 0.1 g of cells in wet weight, followed by sonication in short pulses of 15 s in ice-water bath. The lysate was centrifuged at 9,000 x g at 4°C for 10 min. The supernatant was loaded to His-Tag Affinity Resins column (Novagen) at a flow rate of 1 mL/min. The fusion protein VAH4 was eluted with elution buffer (50 mM Tris-HCl pH 6.0, 0.3 mM NaCl, 250 mM imidazole) and then stored at -80°C. The protein concentrations were determined in triplicate with the Bradford assay (Bio-Rad) using bovine albumin as a standard.

Analysis of protein expression and purification

The IPTG-induced E. coli cells were resuspended in distilled water, and then added with equal volume of 2 x loading buffer for SDS-PAGE. The mixtures were boiled for 10 min, and then centrifuged at 12,000 rpm for 10 min. Ten microliter of the supernatant was withdrawn and run on SDS-PAGE (10%) gel. Homogeneity of the purified VAH4 protein was verified by running 1 μL of it on SDS-PAGE. Uninduced E. coli cells harboring pET-vah4 plasmid were used as parallel controls.

Immunization of Japanese flounder with the recombinant VAH4 protein

Purified VAH4 recombinant protein solution was mixed with the same volume of incomplete adjuvant (lanolin 10 g and paraffin 30 mL) and then injected intraperitoneally into 70 Japanese flounder (8 μg protein/g of fish). VAH4 protein was replaced by equal amount of sterile saline in the control group (n = 70). Fish were reared at 14–16°C with commercial pellet feed (Meiyan Fish Feed Factory) for 6 weeks.

Analysis of enzyme activities in liver of the immune flounder

Liver samples (n = 3) of fish from each immunized and control group were collected at 5, 12, 24, 48 and 72 h after injection and homogenized in 0.85% saline. After centrifuged at 12,000 rpm at 4°C for 20 min, the supernatant was stored at -20°C until used. Activities of alkaline phosphatase (AKP), acid phosphatase (ACP), superoxide dismutase (SOD) and catalase (CAT) in the supernatant were measured using respective detection kits (Nanjing Jiancheng Bioengineering Institute) following the manufacturer's instructions.

Detection of antibody specific to VAH4

Rabbit antiserum against serum of Japanese flounder was raised by two injections of mixture of the inactivated (55°C, 30 min) serum and Freund's complete adjuvant into New Zealand white rabbits. VAH4 protein solution was dialyzed against 10 mM PBS (pH 7.2) with a dialysis tubing (20 kD MWCO; Spectrum Laboratories). One hundred microliter of the dialysate was added into wells of microtiter plate and then incubated at 4°C overnight. After washing the plate three times with PBS, the wells were blocked with 200 μL blocking buffer (1% non fat dry milk in PBS) per well. The plate was incubated at 4°C overnight and then washed twice with PBS. Serial two-fold diluted fish antiserum (n = 5) at 10 days post-immunization, rabbit anti-fish serum, and HRP-labeled Goat Anti-Rabbit IgG (H + L, Kirkegaard & Perry Laboratories Inc) were sequentially added to the wells and incubated at room temperature for 2 h, each followed by washings with PBS. Finally 100 μL of tetramethylbenzidine in PBS was added into the wells. The plate was incubated at room temperature for 30 min, and subjected to spectrophotometric analysis at a wavelength of 655 nm. Serum samples (n = 5) from non-immune flounder were used as negative control.

Challenge test by V. anguillarum

At 3 weeks after the first injection with the incomplete adjuvant-VAH4 recombinant protein mixture as described above, Japanese flounder were immunized again in the same manner. Control fish received PBS in the same manner. At 6 weeks after the first immunization, fish (each n = 40) was challenged with 1 mL of live V. anguillarum stain CAVA23 cells suspended in 0.5% saline (7 x 10$^6$ CFU/mL) by intraperitoneal injection. After bacterial challenge, fish were observed at 14–16°C for 20 days.

Results

Heterologous expression and purification of VAH4

SDS-PAGE analysis of the VAH4 expression and purification was shown in Fig. 1. An extra band could be observed in IPTG-induced E. coli BL21 (DE3) cells harboring the plasmid pET-vah4 when compared with the uninduced cells. The apparent molecular weight of
the extra band was about 45 kDa, which was consistent with the calculated molecular weight of VAH4 protein (lane 1). No band of the same molecular weight was observed in the control group (lane 2). The purified protein was also in good agreement with the deduced molecular weight (lane 3). The concentration of the purified VAH4 protein was 1.4 mg/mL.

Activation of the immune related enzymes and protection of fish by immunization with VAH4

To investigate the immune activation of the fish after the VAH4 injection, SOD, CAT, AKP and ACP activities were determined. Compared with the non-injected fish, higher SOD activity was detected in the VAH4 group at 24 and 48 h after the injection (Fig. 2A). In the VAH4 group, CAT activity was about 4-fold of that of the control group at 12 h after the injection (Fig. 2B). Higher AKP activity was observed in the VAH4 group at 5, 12 and 24 h after injection (Fig. 2C) and higher ACP activity was detected at 12, 24 and 48 h after the injection (Fig. 2D).

VAH4-specific antibody titer of the immune fish serum, which was collected at 10 days post-injection, was determined as 1:10,240 (average, n = 5). In challenge experiment, 40 individuals of Japanese flounder were challenged by virulent V. anguillarum strain CAVA23. As shown in Fig. 3, survival rates of fish immunized with the recombinant VAH4 protein and the control group previously received PBS were 80% and 0%, respectively. Cause of mortality in the untreated control group (survival rate: 85%) remains unsolved.
Fig. 3. Survival rates of Japanese flounder in challenge test by virulent *Vibrio anguillarum*. *V. anguillarum* strain CAVA23 cell suspension was injected to fish (n = 40) previously immunized with VAH4 protein (VAH4+Vibrio group —) and fish (n = 40) previously injected with PBS (PBS+Vibrio group —). The untreated fish group (n = 40) was served as a control.

**Discussion**

Immune system is extremely complex, as the mechanisms of both the host defense to foreign invaders and the tissue damage repairing exist in primary and advanced biological organisms. The defense mechanisms in fishes include innate and adaptive immunity caused by a variety of external substances. The type of most of the non-specific immune response is similar to the phagocytosis of invertebrates, such as the molluscs and arthropods blood cells responsible for the phagocytosis of foreign antigens.

In the present study, we showed the activation of the immune related enzymes in serum of Japanese flounder after injection of the recombinant VAH4. AKP activity in Japanese flounder increased 5 h after injection of the recombinant *V. anguillarum* VAH4 and reached maximum at 24 h and then decreased gradually. ACP activities increased in 12 h and also reached the maximum at 24 h and then decreased gradually. These enzyme activities of the experimental group were higher than those of the control group. CAT removes hydrogen peroxide, thereby protecting the animals from its harmful effects and maintaining normal physiological activity. CAT activity was also enhanced in this experiment, in particular at 12 h when the activity was nearly 4 times higher than that of the control. The results indicated that VAH4 can stimulate the immune system associated enzymes in Japanese flounder. This could thus provide a new strategy for protecting the mariculture fish from *V. anguillarum* infection.

In consistent with the results in analysis of the enzyme activities, injection of the VAH4 recombinant protein induced antibody production at a high level, the antibody titer being 1:10,240. The results indicated that injection of the VAH4 protein significantly stimulated the immune response of Japanese flounder. Furthermore, the VAH4 as an immunoogen protected Japanese flounder from the challenge of live *V. anguillarum*. The results indicated that the recombinant VAH4 protein possesses immunoprotective effect on the flounder against *V. anguillarum*. The VAH4 recombinant protein as an immunogen is useful in preventing *V. anguillarum* infections in aquatic animals, and particularly valuable in developing effective vaccines against vibriosis for commercial use.

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