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Comparative proteomic analysis of *Litopenaeus vannamei* gills after vaccination with two WSSV structural proteins

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ABSTRACT

White spot syndrome virus (WSSV) is one of the most devastating viral pathogens of cultured shrimp worldwide. Recently published papers show the ability of WSSV structural protein VP28 to vaccinate shrimp and raise protection against the virus. This study attempted to identify the joining proteins of the aforementioned shrimp quasi-immune response by proteomic analysis. The other envelope protein, VP36B, was used as the non-protective subunit vaccine control. Shrimp were intramuscularly injected with rVPs or PBS on day 1 and day 4 and then on day 7 their gill tissues were sampled. The two-dimensional electrophoresis (2-DE) patterns of gill proteins between vaccinated and PBS groups were compared and 20 differentially expressed proteins identified by mass spectrometry, some of which were validated in gill and hemocyte tissues using real-time quantitative RT-PCR. Many of identified proteins and their expression levels also linked with the shrimp response during WSSV infection. The list of up-regulated protein spots found exclusively in rVP28-vaccinated shrimp include calreticulin and heat shock protein 70 with chaperone properties, ubiquitin, and others. The two serine proteases, chymotrypsin and trypsin, were significantly increased in shrimp of both vaccinated groups compared to PBS controls. The information presented here should be useful for gaining insight into invertebrate immunity.

1. Introduction

The Pacific white shrimp, *Litopenaeus vannamei*, is one of the most important penaeid shrimp species cultivated worldwide. As the aquaculture industry has developed over time, infectious diseases of shrimp have also been revealed, some of which cause economic losses [1,2]. Among these, white spot syndrome virus (WSSV; Nimaviridae: *Whispovirus*), one of the most devastating viral pathogens, is a large, enveloped, ellipsoid, double-stranded DNA virus that also attacks crabs, crayfishes, and many other crustaceans [3–5]. In cultured penaeid shrimp, WSSV can cause a cumulative mortality of up to 100% within 3–7 days of infection. Molecular diagnosis techniques have been utilized to ensure good biosecurity practices to avoid WSSV outbreaks [6,7]. Invertebrates are believed to possess only an innate immune system that lacks true adaptive immune responses. However, a quasi-immune response has been demonstrated in kuruma shrimp (*Marsupenaeus japonicus*), in that previous exposure to WSSV protects shrimp from future challenges with the virus [8]. Much has been published within the last decade about the practical issues of vaccination, indicating that shrimp and other crayfish vaccinated with either inactivated virus or the WSSV envelope protein VP28, as subunit vaccine revealed higher survival rate for WSSV infection, rather than targeting elucidation of the mechanisms involved in this immune response [9–12].

One clue about the nature of the quasi-immune response comes from the onset and duration of conferred protection. Studies to date show that enhanced survival in virus-challenged shrimp appears as early as one day after vaccination and persists for a limited period, but not for the organism's entire lifespan [13]. The protection

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conferred by recombinant protein vaccines was found to be relatively short-lived, and thus need to be extended by a booster [14]. In contrast, DNA vaccines offer long-lasting antigen expression eliciting longevity in its protective efficacy [15,16]. We suggested in a previous report [17] that a structure- and dose-dependent protective response incorporated with the elevated innate immunity in shrimp following a protein-based vaccination might confer resistance to WSSV. There have been no related experiments to examine specifically whether the protective activity triggered by rVP28 also works with other pathogenic challenges. To summarize these observations, there should be essential differences in the mechanisms between a quasi-immune response and the vertebrate adaptive immune system [18].

The field of proteomics encompasses several principal applications including protein-expression profiling, protein-network mapping, and post-translational modification. Comparative proteomic analysis coupled with two-dimensional gel electrophoresis and mass spectrometry (2-DE/MS) are widely used to identify protein variations in specific tissues under given conditions. Comparing protein expression profiles facilitates the discovery of complicated changes in the proteome induced by all kinds of biological events. Detailed genome sequencing and annotation of most species of crustaceans are not yet available, notably excepting a water flea (*Daphnia pulex*) and the salmon louse (*Lepeophtheirus salmonis*). Based on the published sequence database and expressed sequence tag (EST), this proteomic strategy has been used in many studies on the immune responses of shrimp against infections from viruses and bacteria such as WSSV [19,20], Taura syndrome virus [21], Yellow head virus [22], and *Vibrio harvei* and *Vibrio anguillarum* [23–25] in order to identify the responsive proteins.

Our previous report found that the effective dosage and scheme of administering the WSSV structural protein rVP28 but not rVP36B triggered resistance against WSSV [17]. In the current study, we report for the first time a proteomic analysis of *L. vannamei* gill tissues to elucidate crustacean quasi-immune responses at the protein level after vaccination. The shrimp gill is the main site where WSSV infections and abundant hemocytes are found. Proteins whose expressions changed after vaccination were identified in order to obtain an overview of the effects of recombinant protein vaccines. In addition, the transcript levels of some identified proteins were further assessed by real-time quantitative PCR after vaccination, also in comparison with WSSV infected shrimp.

2. Materials and methods

2.1. Shrimp culture

Shrimp (*L. vannamei*) were obtained from the Tungkang Biotechnology Research Center, Fisheries Research Institute (Pingtung, Taiwan) and maintained in 100 L tanks with aerated filtered seawater (26–30 °C). Shrimp were kept in these tanks for acclimation and quarantine prior to experiments. During acclimation and experiments, shrimp were fed once daily with commercial shrimp feed (at 5% of the shrimp's body weight). Then each group of shrimp was confirmed to be WSSV-free by a nested polymerase chain reaction (PCR) using primers designed by Lo et al. [6]. Only animals proven to be healthy were used for experiments.

2.2. Preparation and administration of recombinant VP28 and VP36B

PCR fragments representing the full length of the coding regions of WSSV structural proteins VP28 and VP36B were amplified using primer sets and cloned into the pET-28b vector. The resulting pET constructs were transformed into BL21 (DE3) *Escherichia coli* cells after sequencing. The details of protein expression and purification were described previously by Yang et al. [17]. The buffer of eluted fractions was changed to PBS with a PD10 desalting column (GE Healthcare, USA). Protein concentrations were determined using a Quant-iT™ protein assay kit (Invitrogen, USA). Healthy shrimp (body weight 15–20 g) were randomly divided into three groups for exposure experiments. Two groups were injected with 100 μl rVP28 or rVP36B (5 μg/g shrimp) and the control group received an equivalent volume of PBS. Three days after initial administration, shrimp were boosted by injection again with the same amount of protein.

2.3. Protein sample preparation

Three days after the second injection with rVPs or PBS, gills from three individuals were combined as a sample for each 2-DE, with 5 replicates being sampled from each group. Each gill sample was homogenized thoroughly in 40 mM Tris buffer with 1 mM PMSF at pH 6.8 and an equivalent volume of TCA/acetone mixture (final concentration: 10% TCA, 0.035% DTT, 1 mM PMSF, and 50% acetone). After refrigeration at −20 °C for 45 min and then centrifuging (2000 × *g*, 15 min, 4 °C), the supernatant was discarded and the pellet resuspended in acetone containing 0.07% DTT. The sample was refrigerated at −20 °C for 30 min and spun again (2000 × *g*, 15 min, 4 °C). The acetone resuspension procedure was repeated three times and the pellet dried under vacuum. The protein precipitate was then dissolved in rehydration buffer (9.8 M Urea, 2% CHAPS), and after a final centrifugation (14000 × *g*, 45 min, 4 °C), the supernatant was used as a 2-DE sample. Protein concentrations of 2-DE samples were determined by a 2D-Quant Kit (GE Healthcare, USA).

2.4. Two-dimensional electrophoresis (2-DE)

A 750 μg solution of total protein in 250 μl rehydration buffer (containing 9.8 M urea, 2% CHAPS, 0.5% IPG buffer, 1% DeStreak Reagent and 0.004% bromophenol blue) was used to rehydrate the linear immobilized pH gradient (IPG) strip (Immobiline™ DryStrip, pH 4–7, 13 cm, GE Healthcare, USA) using the Ettan IPGphor II system (GE Healthcare) overnight (14 h at 30 V). Isoelectric focusing (IEF) was subsequently performed as follows: 1 h at 500 V, 1 h at 1500 V, 3500 V for 3500 Vh, 2 h at 3500 V, 8000 V for 16000 Vh, and 1 h at 8000 V. All the above procedures were carried out at 20 °C. Focused strips were then reduced with 1% DTT and alkylated with 2.5% iodoacetamide in equilibration buffer (50 mM Tris–HCl pH 8.8, 6 M urea, 30% glycerol, 2% SDS). For the second dimensional separation, paired rVP and PBS equilibrated IPG strips were separately sealed on top of 12% Tris-glycine SDS polyacrylamide gel with a 0.5% agarose stack containing trace bromophenol blue and simultaneously run at 150 V and 16 °C for 6 h using a SE600 Ruby electrophoresis set (GE Healthcare, USA).

2.5. Gel analysis

Following 2-DE, protein gels were stained with sypro ruby (Invitrogen, USA) and protein images of gels were captured using Gel Doc™ XR (Bio-Rad, USA). Melanie 7.0 (GeneBio, Switzerland) was used to perform image analysis, including matching spots, quantifying spots, and identifying differences in spot intensity. The percentage intensity (% vol) of each matched spot was used for comparisons between groups.