Asia-Pacific Journal of Science and Technology: Volume: 27. Issue: 02. Article ID.: APST-27-02-08. Research Article

APST

Asia-Pacific Journal of Science and Technology

https://www.tci-thaijo.org/index.php/APST/index

Published by the Research and Graduate Studies,
Khon Kaen University, Thailand

White spot syndrome virus (WSSV) recombinant envelope protein VP28 activates shrimp phagocytic activity

Naing W. Htut[1,2,3], Suparat Taengchaiyaphum[2], Kallaya Sritunyalucksana[2], Siripong Thitamadee[1,3,*]

[1]Department of Biotechnology, Faculty of Science, Mahidol University, Bangkok, Thailand
[2]Aquatic Animal Health Research Team, Integrative Aquaculture Biotechnology Research Group, National Center for Genetic Engineering and Biotechnology (BIOTEC), National Science and Technology Development Agency (NSTDA), Bangkok, Thailand
[3]Center of Excellence for Shrimp Molecular Biology and Biotechnology (Centex Shrimp), Faculty of Science, Mahidol University, Bangkok, Thailand
[*]Corresponding author: siripong.thi@mahidol.ac.th

Received 23 June 2021
Accepted 13 September 2021
Revised 21 September 2021

Abstract

Diseases constitute a fundamental limiting factor for stable and reliable production of cultured shrimp. Successful vaccination against white spot syndrome virus in shrimp by using the viral recombinant envelope protein VP28 has been previously reported. We hypothesized that the mechanism of the subunit vaccine protection might be via an increase of phagocytic activity in vaccinated shrimp. To test this hypothesis, monomeric recombinant VP28 protein (rVP28) was prepared and injected into the shrimp at the concentration of 25 µg/shrimp. Then, hemolymph was collected at different as 15, 30, and 60 min post injection (p.i.) to determine the total hemocyte count (THC) and phagocytic activity. Results revealed that THC and phagocytic activity increased significantly at 30 min post injection, but that THC subsequently declined by 60 min post injection. The findings indicated that rVP28 could induce an increase in circulating hemocyte numbers and activate phagocytic activity. The data obtained provides a baseline for further work on improving vaccination efficacy for protection against white spot syndrome virus (WSSV).

Keywords: *Penaeus vannamei*, Cellular immune response, Hemocytes, Phagocytosis, Monomeric recombinant VP28 protein (rVP28)

1. Introduction

Viral disease outbreaks constitute a major constraint for sustainable aquaculture. There is currently no effective treatment for viral infections in shrimp. Like other crustaceans, shrimps use innate immunity to fight against pathogenic organisms [1]. Shrimp hemocytes play an essential role in both cellular and humoral, innate immune responses. These responses include production of prophenoloxidase (proPO), phagocytosis, encapsulation, nodule formation and synthesis of antimicrobial peptides and lysozymes [2-4]. Hemocyte phagocytosis involves ingestion and digestion of invading pathogens as well as clearance of dead cells [3,5]. Study of shrimp white spot syndrome virus (WSSV) revealed that the viral envelope protein VP28 is involved in viral entry into shrimp cell targets. Thus, many studies have focused on production of VP28-based vaccines to protect shrimp against WSSV or, at least, to improve their survival rate upon infection by either intramuscular injection or oral administration [6-8]. We hypothesized that VP28 protection against WSSV might involve the mechanism of VP28-induced phagocytosis. The experiments were divided into two parts. The first part involved development of a cellular immune assay to determine phagocytic capacity in shrimp. The second part involved injection of shrimp groups with recombinant VP28 protein (rVP28) in buffer or with buffer alone followed by total hemocyte counts and analysis of phagocytosis between the two groups. The results gave a better understanding of the effect of VP28

on shrimp cellular response and also provided a baseline for further work on improving vaccination efficacy for protection against WSSV.

2. Materials and methods

2.1 Experimental shrimp

A batch of juvenile white leg shrimp (*Penaeus vannamei*) of 3 g fresh body weight were acclimatized in artificial seawater at a salinity of 10 g/kg for two days under laboratory conditions at 28℃ with continuous aeration before any experiments were done. They were fed with commercial feed daily prior to being used in experiments.

2.2 Viral subunit protein preparation and experimental injection

rVP28 was synthesized using the *Escherichia coli* system harboring pET17b + VP28 recombinant plasmid as described by previous reports [9,10]. Briefly, the transformed bacterial were cultured in Luria broth (LB) medium at 37℃, 250 rpm agitation and 1 mM isopropyl β-D-1-thiogalactopryranoside (IPTG) was subsequently added to induce expression of the recombinant rVP28. The IPTG induced bacterial culture was harvested, and then rVP28 was extracted and purified by using nickel nitrilotriacetate (Ni-NTA) affinity chromatography. The purified rVP28 was examined by 12.5% sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and visualized by Coomassie blue G-250. Total protein concentration was determined by Bradford protein assay. The purified rVP28 protein was resuspended in 1 mL 1X phosphate-buffered saline (PBS) pH 7.4 and then 100 µL was intramuscularly injected into 24 individual shrimp for immune stimulation. For the control shrimp (n = 24), 100 µL 1X PBS buffer only was injected. At 15, 30, and 60 min post injection, 200 µL of the hemolymph from 8 individual shrimps was withdrawn at each time interval from the ventral sinus using a 1 mL syringe equipped with a 27G needle for sub-dividing to do total hemocyte counts and phagocytosis assays.

2.3 Total hemocyte count (THC)

The withdrawn hemolymph (50 µL) was immediately mixed with an equal volume of 10% formalin in modified Alsever's solution (19.3 mM sodium citrate, 239.8 mM NaCl, 182.5 mM glucose, 6.2 mM ethylenediaminetetraacetic (EDTA), pH 7.2) and incubated for 15 min at 25℃. Then, 10 µL was aliquoted and diluted in the same buffer at the ratio 1:9 in the new tube. The diluted hemolymph was loaded into a hemocytometer and the total hemocyte count was determined under the light microscope [10]. The number of hemocytes (cell/mL of original hemolymph) was calculated.

2.4 Hemocyte phagocytic activity assay

Hemolymph (50 µL) was mixed with 100 µL modified Alsever's solution prior to dividing 50 µL aliquot of hemolymph (~10^5 cells) into a 24-well plate containing 0.5 mL 2X L-15 medium (Gibco). Before the cell seeding step, sterilized-round coverslip was placed at the bottom of the plastic well to allow hemocyte attachment. The phagocytosis experiment was performed by adding 3 µm diameter latex beads at 10^6 (Sigma, USA) to each well and incubating them at room temperature for 15 min. After that, hemocytes were fixed by 10% formalin for 15 min followed by sequential staining with 1.2% Rose Bengal in 50% ethanol for 20 min and finally hematoxylin staining for 3 min. Hemocytes were washed with tap water and absolute ethanol. For slide mounting, the coverslip was collected, air-dried, and dipped into xylene solution for 5 seconds. The coverslip was placed up-side down onto a glass slide containing a drop of permount solution. The slide was air-dried prior to observing the phagocytic activity under the light microscope. The phagocytic activity was evaluated by counting the number of cells that ingested latex beads (i.e., phagocytic cell) in a total of 100-200 cells [11]. The phagocytic activity (%) was calculated: (number of bead-ingesting cells/100-200 cells of hemocytes) ×100. The schematic representation of *in vitro* phagocytosis assay is shown in Figure 1.

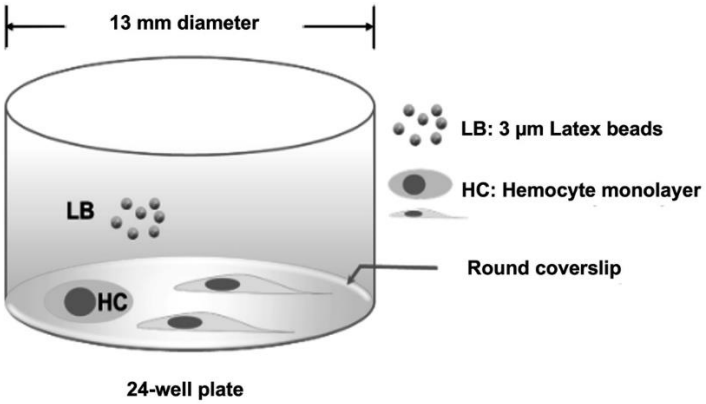

Figure 1 An outline of *in vitro* phagocytosis assay of shrimp hemocytes. Hemocytes (~10^5) were allowed to form a monolayer on the coverslip in the well of the 24-microwell plate before incubation with 10^6 latex beads. After incubation at room temperature, the excess beads were washed away and the hemocytes were fixed and stained before evaluation of the phagocytic activity under a microscope.

3. Results

3.1 Establishment of phagocytosis assay

In shrimp, three types of hemocytes have been reported including the hyaline cells (HC), the semi-granular cells (SGC), and the granular cells (GC). To develop the phagocytic assay in this study, latex beads were used since they were easy to observe microscopically in shrimps injected with and without rVP28 protein. Although all hemocytes are capable of performing this function, granule-containing hemocytes (i.e. SGC and GC) appear to have a higher phagocytic activity ratio than HC [12,13]. Based on the described technique (Figure 1), clear evidence of ingested or engulfed latex beads by hemocytes could be easily recognized, indicating that this hemocyte phagocytic activity assay might be used to assess cellular immune response in shrimp (Figure 2).

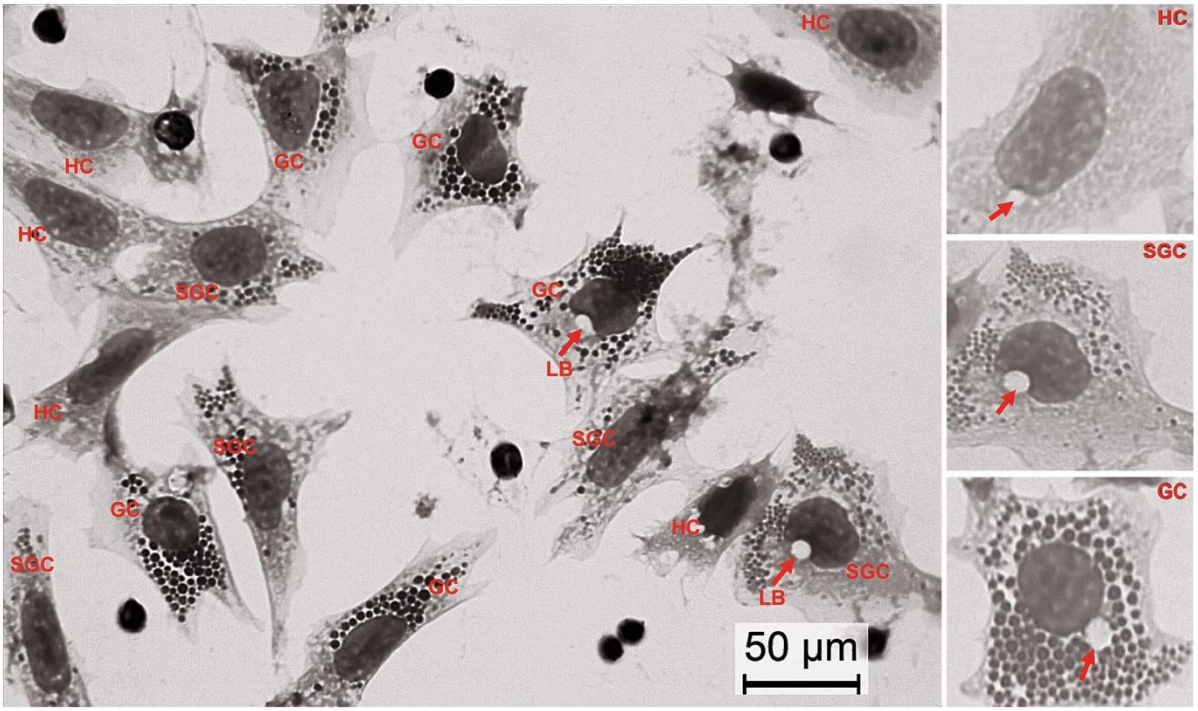

Figure 2 Microscopic examination of shrimp phagocytosed latex beads. Three types of hemocytes including - hyaline cells (HC), semi-granular cells (SGC), and granular cells (GC) are shown. The phagocytic activity was observed in all three types represented by ingested latex beads (LB, arrows).

3.2 Preparation of purified recombinant VP28

As mentioned previously, injection of subunit protein prepared from the envelope protein VP28 of WSSV could enhance shrimp tolerance to cognate virus infection [9]. We successfully purified rVP28 by Ni-NTA affinity chromatography and confirmed it production by SDS-PAGE analysis by obtaining a single protein band of 28 kDa-rVP28 protein in the eluted fraction (E1) from the lysate of bacterial cells containing the VP28-inserted plasmid (Figure 3).

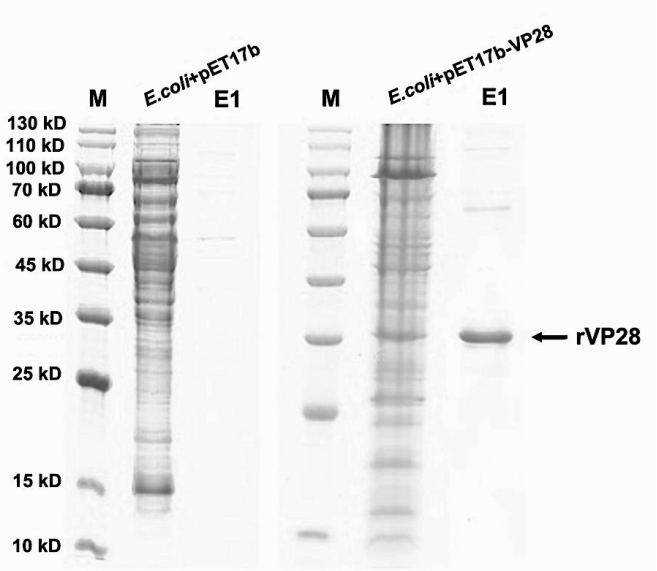

Figure 3 SDS-PAGE analysis reveals a 28-kDa protein of monomeric recombinant VP28 (rVP28). The protein profiles in the bacterial lysates of the bacterial cells harboring VP28-inserted plasmid (*E. coli* + pET17B-VP28) and those with no VP28-inserted plasmid (*E.* coli + pET17b). E1 indicates protein eluted by Ni-NTA chromatography column. M indicates protein marker.

3.3 Determination of phagocytosis activity in shrimp injected with recombinant VP28 (rVP28)

In response to shrimp injection with our monomeric rVP28 at of 25 μg/shrimp, samples were taken after injection at 15, 30, and 60 min showed a maximum increase in THC at 30 min that declined by 60 min to the same level as in the buffer injected group. However, phagocytic activity for the rVP28 group showed a significant increase in phagocytic activity by 30 min and an even higher increase by 60 min. In contrast, the shrimp group injected with PBS showed no significant differences in THC or phagocytic activity at the same time intervals and no difference to the counts obtained from shrimp prior to injection (0 min), as shown in Figure 4. These findings revealed that rVP28 injection can activate phagocytic activity of shrimp hemocytes. Further study is needed to determine whether induction of phagocytic activity by VP28 is associated with higher shrimp survival upon challenge with WSSV.

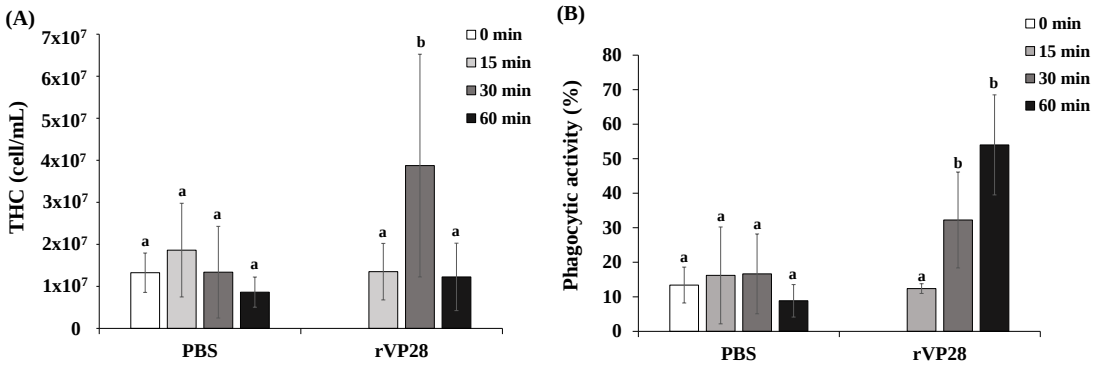

Figure 4 Bar graphs represent the total hemocytes count (A) and phagocytic activity (B) comparing between control (PBS injection) and recombinant VP28 protein (rVP28) injection in shrimp. Different letters (a and b) denote statistically significant differences of phagocyte activity comparing among time-point ($p<0.05$) by One-Way ANOVA test and bars represent mean values with SD.

4. Discussion

The innate immune response in invertebrates including shrimp is a generic, non-specific, fast, and efficient defence mechanism that can defend against invading pathogens at the site of infection [14,15]. It is comprised of cellular and humoral immune reactions**.** The cellular immune responses are phagocytosis, nodule formation, encapsulation, and apoptosis, whereas the humoral immune responses include the prophenoloxidase cascade, the clotting cascade, antimicrobial peptides (AMPs), lectins, the Dscam (Down syndrome cell adhesion molecule) pathway, the Toll pathway and the immune deficiency (IMD) pathway [3,16]. Phagocytosis is a crucial component of innate immunity in multicellular species. It is one of the endocytic mechanisms by which cells engulf or ingest exogenous materials such as pathogens and cell debris via the development of endocytic vesicles [5,17]**.** In brief, the phagocytosis process begins with binding of endogenous foreign particles or pathogens by phagocyte surface receptors. The bound particles are then uptaken and enveloped by the plasma-membrane after induction of a signaling cascade to produce a phagolysosome by fusion of the phagosome with lysosomes. Subsequently, hydrolytic enzymes break down and remove the particles or pathogens within the phagolysosome [18,19]. Hemocytes are the cells that circulate in the hemolymph and interact with foreign substances found in the open circulatory system of crustaceans [20]. Hemocytes, which play an essential role in the immune responses, are the primary source for the production of immune defence mechanisms and are responsible for the elimination of pathogens and other foreign particles [3,21-24]. Nevertheless, the elucidation of the phagocytic activity of each type of hemocyte (GC, SGC, HC) in crustaceans remains unclear since numerous studies of the closely related species have produced different results [25-27].

Previous research has clearly demonstrated that administration of recombinant viral proteins of WSSV can boost shrimp immunity against WSSV infection [6-9,28], For example, shrimp vaccinated with rVP292 protein resulted in a 52% survival rate from WSSV challenge compared to zero survival without [29]. Also, shrimp injected with the recombinant VP28 showed relative percent survival (RPS) of 67% compared to the untreated shrimp [9]. Our study aimed to better understand the mechanism involved with protection efficiency of recombinant WSSV protein administration against WSSV infection. We hypothesized that the cellular response of phagocytosis might play a major role for viral clearance. We have developed the protocol to evaluate the hemocytes phagocytosis in shrimp injected with rVP28 protein. After injection of rVP28, the number of THC dramatically increased at 30 min post injection and it corresponded to an increase in phagocytic activity. This is possible due to recognition of VP28 as non-self of phagocytosis. At 60 min post rVP28 injection, while the THC level decreased to the baseline level, the phagocytic activity was significantly maintained at a high level. It is speculated that the phagocytes become a major proportion of the total hemocyte population that is produced by 60 min post rVP28 injection. It might also be possible that the cells, not phagocytes, might accumulate at the site of injection or degranulate to release active immune molecules to degrade foreign particles, which in this case, latex beads and consequently, the non-phagocyte population is decreased. Shrimp SGC and GCs are induced to degranulate by foreign substances such as β-glucan and thus release several proteins including proPO, prophenoloxidase activating enzyme (ppA), peroxinectin (PX), proteinase inhibitors and lysozyme that can destroy invading pathogens and stimulate phagocytosis [30]. It remains to be determined whether there was a selective change in the hemocyte subpopulations that would be associated with a high-level phagocytic activity. Hence, the results from our study suggest the effect of vaccination to improve the number of total hemocytes and phagocytic activity in shrimp. The information will guide us to optimize the appropriate amount and frequency for rVP28 use in increasing shrimp resistance to WSSV.

5. Conclusions

Injection of rVP28 protein of WSSV results in activation of shrimp phagocytosis. The effect is rapid since both induction of THC and phagocytic activity were revealed as early as 30 min post injection of rVP28. The ongoing direction of this study is to optimize rVP28 therapy to maximize phagocytic activity in shrimp in their fight against the viral infection.

6. Acknowledgements

We would like to thank the Thai Agricultural Research and Development Agency (ARDA) for supporting this work via Grant no. 18570 to Dr. Suparat Taengchaiyaphum and “Mahidol University under the New Discovery and Frontier Research Grant 2022”. We would also like to thank Prof. T.W. Flegel for assistance in reviewing and editing the draft manuscript.

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