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Evaluation of Recombinant Viral Inhibitor Protein for Whiteleg Shrimp Resistance Against White Spot Syndrome Virus (WSSV)

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ABSTRACT Viral inhibitor protein (VIP) contributes to shrimp resistance against virus infection. VIP can be used as a new strategy to control white spot syndrome virus (WSSV) infection in shrimp culture by inhibiting its action. This study aimed to evaluate the application of VIP on the whiteleg shrimp (*Litopenaeus vannamei*) to inhibit the WSSV infection. Shrimp at an average body weight of 6.66±0.08 g was injected by recombinant VIP with two different concentrations (1 µg/g and 10 µg/g) and challenged with WSSV. As a positive control was the shrimp not injected with recombinant VIP and challenged with WSSV, and as a negative control was the shrimp not injected with recombinant VIP and not challenged with WSSV. The results showed that the survival rate of shrimp that were injected with recombinant VIP was significantly increased after being challenged with WSSV ($P<0.05$). The survival and immune response of shrimp that were injected with recombinant VIP were higher than the positive controls ($P<0.05$). In this study, the application of recombinant VIP can significantly increase the shrimp resistance against WSSV up to 95.65% higher than the positive control.

Keywords: *Litopenaeus vannamei*; recombinant; viral inhibitor protein; white spot syndrome virus (WSSV)

INTRODUCTION

White spot syndrome virus (WSSV) is a disease agent that can cause white spot disease and its virulence causes outbreak to happen very fast (Li *et al*., 2013). If not prevented and treated, WSSV infection can cause mass mortality to shrimp up to 100% mortality after 3 to 10 days of infection (Leobert *et al*., 2017; Taengchaiyaphum *et al*., 2017). One of the attempts that is often done is to increase the shrimp immune response. The method that is commonly used to increase shrimp immune response is the application of immunostimulants. However, the application of immunostimulants can't directly inhibit WSSV activity and depends a lot on the increase in the shrimp immune response due to the administration of immunostimulants. Shrimp responses to immunostimulants are varies greatly depend on dose, active substance and performance or genetic quality of shrimp (Ding *et al*., 2020). Therefore, an alternative method must be developed to directly inhibit WSSV infection in shrimp.

Viral inhibitor protein (VIP) is known to play a role in shrimp resistance against viral infection (Luo *et al*., 2003). Based on the in-silico study, it is assumed that there is an interaction between the VIP and RING finger domain on WSSV that forms a complex binding which causes direct inhibition of WSSV activity (Gajula *et al*., 2013). Based on these facts, VIP can be used as a new strategy to control WSSV infection in shrimp culture. One of the technologies that can be used to utilize VIP potential is a recombinant protein. VIP protein molecule can be produced by inserting expression vector of VIP-encoding gene into bacteria so that VIP can be mass-produced rapidly, taking advantage of the high replication rate of bacteria (Holifah, 2015). Therefore, this study aimed to evaluate the production of recombinant VIP and its application on the whiteleg shrimp (Litopenaeus vannamei) to inhibit the WSSV infection.

MATERIALS AND METHODS

Test subject

Subject used for this experiment are whiteleg shrimp from PT. Suri Tani Pemuka with average body weight of 6.66± 0.08 g. Shrimp was reared in aquarium with water volume of 30 l, temperature of 27–28 °C, salinity of 30 ppt for one week and fed with commercial feed. Shrimp was tested for WSSV infection status with nested PCR to ensure that the shrimp was free from WSSV infection (Sinthujaroen *et al*., 2015).

Experimental design

Recombinant VIP was delivered to shrimp through intramuscular injection with different dose for each treatment (Table 1). Experimental design used in this research consist of four treatments with six replicates. Three replicates used for survival rate observation, and the other replicates used for immune response observation.

Table 1. Experimental design.

Treatment Code	Treatment
K	Negative control; Shrimp was injected with PBS.
W	Positive control; Shrimp was injected with WSSV.
AV1	Shrimp was injected with recombinant VIP 1 µg/g and 24 hour later injected with WSSV.
AV10	Shrimp was injected with recombinant VIP 10 µg/g and 24 hour later injected with WSSV.

WSSV isolate preparation and lethal dose 50 tests

WSSV filtrate that used in this experiment was isolated

from WSSV positive whiteleg shrimp (Xie *et al*., 2005). *Lethal dose* 50 (LD_{50}) tests were done by injecting 100 µl of WSSV filtrate to shrimp. Doses that used for injection was based on serial dilution (10^{-2}, 10^{-3}, 10^{-4}) from WSSV filtrate stock. As negative control, shrimp was injected with *phosphate buffer saline* (PBS). *Lethal dose* 50 (LD_{50}) test was done with two replicates and 15 shrimp was used for each replicate. Mortality was observed after 5 days post infection.

In vivo test of recombinant VIP

Shrimp was reared at aquarium tank with 30 l sea water and divide for two parts; (1) survival test and (2) immunity response after WSSV infection. 15 shrimp was used for each replicate and observed until day 5 post infection. Shrimp was tested for WSSV infection status with nested PCR after day 5 post infection (Sinthujaroen *et al*., 2015). Primer sequences and annealing temperature (Ta) (Nunan & Lightner, 2011) that used for nested PCR are listed in Table 2.

Cumulative survival rate observation

Cumulative survival rate was observed every day after WSSV infection. Survival rate was evaluated at day 5 post infection. Survival rate was counted with this formula:

$$SR(\%) = \left(\frac{N_t}{N_o}\right) x100$$

SR : Survival rate
Nt : Total shrimp count before treatment (individual)
No : Total shrimp count after treatment (individual)

Immune response observation

Immune response was observed from total hemocyte count (THC) and prophenoloxydase (proPO) activity. Sample was collected at 6, 12, 24, 72 and 120 hour post infection. Sample for total hemocyte count was collected from shrimp hemolymph and diluted with anticoagulant (30 mM trisodium citrate, 0.34 M sodium chloride, 10 mM EDTA, 0.12 M glucose, pH 7.55) by 1:1 ratio. Hemocyte was count with hemacytometer and observed under the microscope with 400x magnification. Hemocyte was counted using this formula:

$$THC\left(^{cell}/_{ml}\right) = \frac{\Sigma \text{observed haemocyte cell}}{\Sigma \text{observed box}} \times 25 \times \frac{1}{v} \times f$$

THC : Total hemocyte count
V : Hemacytometer volume
f : Dilute factor

Sample for prophenoloxydase activity was collected from shrimp hemolymph and diluted with anticoagulant by 1:4 ratio. Prophenoloxydase activity was done with spectrophotometry according to Huang *et al*. 2010 by measure the optical density at 492 nm after the formation of dopachrome from l-3,4-dihydroxyphenylalanine (L-DOPA).

Statistical analysis

Analysis of variance (ANOVA) at a 5% significance level was used to test the data obtained for survival rate and immune response of whiteleg shrimp. Post hoc test was done by using least significant different (LSD) test.

RESULTS AND DISCUSSION

Survival rate

Whiteleg shrimp survival rate after WSSV infection shown that at the AV1 (100%) and AV10 (100%) treatment have a significantly higher rate than the positive control treatment (51.11±3.85%) (Figure 1). This result, indicated that there is an increase at shrimp resistance against WSSV after the administration of recombinant VIP. The role of VIP gene in shrimp resistance against WSSV infection has been observed before by Parenregi *et al*. (2021) at black tiger shrimp *Penaeus monodon*. VIP gene in black tiger shrimp was enhanced by overexpression and shown that after WSSV infection, shrimp with overexpressed VIP gene have a 24.5% higher survival rate than the normal shrimp. In this experiment, survival rate of whiteleg shrimp after the administration of recombinant VIP shown 95.65% higher than the positive control treatment.

Table 2. Primer sequences and PCR program for WSSV infection status confirmation in whiteleg shrimp.

Primer	Sequences (5'-3')	T_a	Product length
146F1	CTACTAACTTCAGCCTATCTAG	62 °C	941 bp
146R1	TAATGCGGGTGTAATGTTCTTACGA		
146F2	GTAACTGCCCCTTCCATCTCCA	62 °C	
146R2	TACGGCAGCTGCTGCACCTTGT		

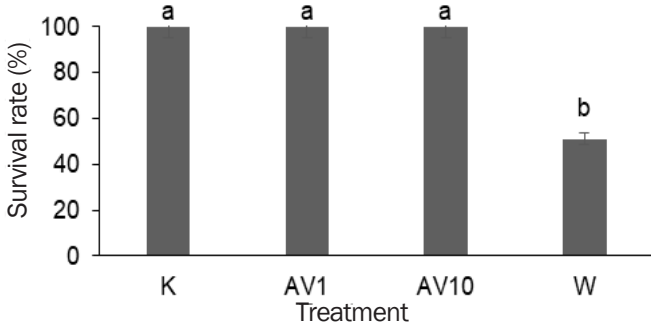

Figure 1. Whiteleg shrimp survival rate at day 5 after WSSV infection. (W) positive control, (AV1) recombinant VIP 1 µg/g, (AV10) recombinant VIP 10 µg/g, (K) negative control. Different superscripted letter shown the significantly difference treatment ($p<0.05$).

Immune response

Shrimp immune response after pathogen infection was highly depend on nonspecific immune system. Shrimp immune response such as cellular and humoral response are abundant in shrimp hemolymph (Xu *et al*., 2014). There are hemocyte cells in shrimp hemolymph that play a role in shrimp immunity response. Hemocyte cells play a role in pathogen recognition process, phagocytosis, cytotoxicity, and release of proPO system (Xian *et al*., 2016). In this experiment, the role of hemocyte cells can be observed

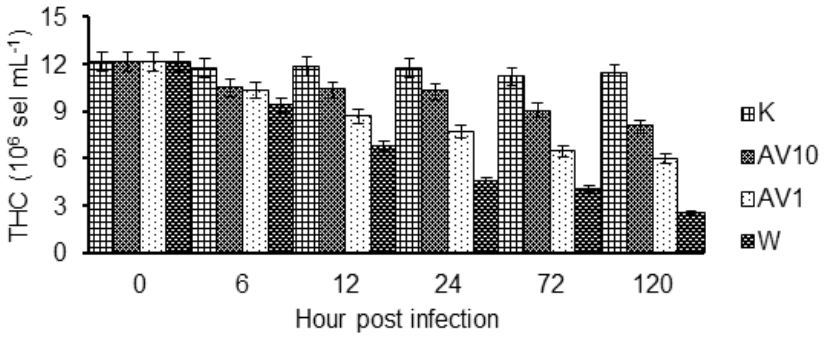

Figure 2. Whiteleg shrimp total hemocyte count at day 5 after WSSV infection. (W) positive control, (AV1) recombinant VIP 1 µg/g, (AV10) recombinant VIP 10 µg/g, (K) negative control. Different superscripted letter shown the significantly difference treatment ($p<0.05$).

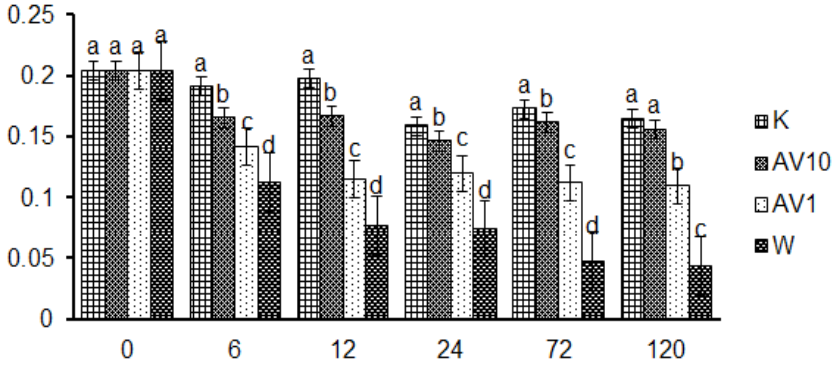

Figure 3. Whiteleg shrimp prophenoloxydase activity at day 5 after WSSV infection. (W) positive control, (AV1) recombinant VIP 1 µg/g, (AV10) recombinant VIP 10 µg/g, (K) negative control. Different superscripted letter shown the significantly difference treatment ($p<0.05$).

from total hemocyte count (Figure 2). After WSSV infection, total hemocyte count in infected shrimp are significantly lower than the healthy shrimp. The reduction of total hemocyte count due to migration process of hemocyte cells to the WSSV infected cells (Van De Braak *et al*., 2002). Total hemocyte count in recombinant VIP treatment (VIP1 and VIP10) are significantly higher ($p<0.05$) than the positive control treatment after WSSV infection. This result indicates that there is a decrease in the number of infected cells after the application of recombinant VIP. It is thought that the decrease in the number of infected cells due to a mechanism between VIP protein and protein in WSSV RING finger domain which causes inhibition of WSSV activity (Gajula *et al*., 2013).

In this experiment, humoral immunity response was observed from proPO activity in whiteleg shrimp hemolymph. ProPO activity begins when the pattern recognition protein (PRP) is activated due to WSSV infection which will lead to the interaction of PRP molecules with protein molecules in WSSV. PRP activation will initiate ProPO activity which will turn proPO into PO molecules (Jeswin *et al*., 2013). This activity will cause a catalysis in hydroxylation process of mono-phenol to o-diphenol and oxidation of o-diphenol to o-quinones which then leads to the formation of melanin (Huang *et al*., 2018). Melanin produced from PO activation has a role as an anti-pathogen that will coat pathogens and cells that have been damaged by infection and form a protective barrier for healthy cells (Cerenius & Söderhäll, 2021). In this experiment, proPO activity from the WSSV infected treatment are significantly lower ($p<0.05$) than the negative control treatment (Figure 3). ProPO activity in positive control are significantly lower ($p<0.05$) than the recombinant VIP treatment (VIP1 and VIP10) after WSSV infection. The lower proPO activity in the positive control are caused by the decrease in the total hemocyte count in hemolymph. In penaeid shrimp, proPO activity located in hemocyte cells and the mRNA expression of proPO is reported only occurs in hemocyte cells (Amparyup *et al*., 2013). The synthesis of enzymes involved in the proPO system occurs in granular and semi-granular cells of hemocyte. These enzymes will later be released into the hemolymph when infection from pathogens occurs (Cerenius *et al*., 2008).

Confirmation of WSSV infection status

WSSV infection status confirmation was shown at Figure 4. Before treatment (0), shrimp was confirmed free of WSSV infection. After WSSV infection, positive control, AV1 and AV10 treatment was confirmed positive of WSSV infection

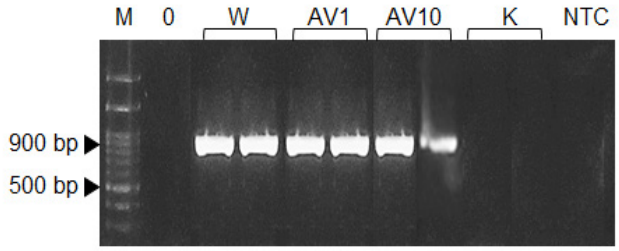

Figure 4. WSSV infection status confirmation in whiteleg shrimp after WSSV infection. (M) DNA marker, (0) before treatment, (W) positive control, (AV1) recombinant VIP 1 µg/g, (AV10) recombinant VIP 10 µg/g, (K) negative control, (NTC) no template control.

while negative control treatment was confirmed free of WSSV infection. This infection status confirmation indicates that the cause of shrimp mortality was indeed from WSSV infection.

CONCLUSION

The application of recombinant VIP in whiteleg shrimp with dose of 10 µg/g can significantly increase the shrimp resistance against WSSV up to 95.65% higher than the positive control.

AUTHOR'S CONTRIBUTIONS

AAR doing the research, data collecting and analysis, and written manuscript. AMD contributing in idea development, data evaluation, manuscript evaluation and funding. AOM contributing in idea development, data evaluation and manuscript evaluation. SNR contributing in idea development, data evaluation and manuscript evaluation.

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