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Antigenic Properties of VP15 from White Spot Syndrome Virus in Kuruma Shrimp *Marsupenaeus japonicus*

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Abstract

White spot syndrome virus (WSSV) is known as one of the most lethal pathogenic viruses in shrimp causing massive damage to shrimp aquaculture industries. To date, no effective treatment or prevention has been found. In this study, five recombinant viral proteins VP15, VP19, VP24, VP26, and VP28 were expressed and purified in *E. coli*, which were employed as candidates against WSSV in Kuruma shrimp *Marsupenaeus japonicus*. *In vivo* antiviral assay in this study newly revealed that VP15 of major nucleocapsid protein, being known as a DNA-binding protein provided the substantial protection against the viral infection when pre-injected into shrimps. Furthermore, we also verified the immunogenic effects of purified VP15 and VP19 proteins produced in a silkworm-bacmid expression system. Taken together, our study identified VP15 as an effective candidate against WSSV infection in the Kuruma shrimp. It is interesting to uncover why and how VP15 is involved in the immune memory in shrimp in the future study.

Keywords VP15 · White Spot Syndrome Virus · Trained immunity · Shrimp · Silkworm

1. Introduction

White spot syndrome virus (WSSV) is a circular double-stranded DNA (dsDNA) virus which is classified in a group I of Baltimore classification system and is the only member of the genus *Whispovirus*, which is also an only genus in family *Nimaviridae* [1-3]. The virus has an ellipsoid to bacilliform morphology with a tail-like appendage at one end in which a function is unknown [4, 5]. Each virion consists of nucleocapsid covered by a trilaminar envelope. The nucleocapsid has a size of about 60–70 nm in diameter and 300–350 nm in length [6]. Xie, Xu and Yang [7] reported that WSSV contains at least 5 major virion proteins, such as VP15, VP19, VP24, VP26, and VP28, as analysis by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and N-terminal sequencing. Also, VP19, VP24, VP26, and VP28 were located at envelope fraction, while VP15 was located at nucleocapsid fraction [7]. These virion proteins are suggested as the key elements for infections of WSSV in shrimp and other aquatic crustaceans [3, 8-10].

WSSV causes white spot disease (WSD), is considered as one of the most lethal viral pathogens in cultured shrimp especially in penaeid shrimp [11]. Crustaceans suffering from WSD show a clinical sign such as lethargy, reduced food consumption, reddish discoloration, reduced preening activity and appearance of white calcified spots on its exoskeleton [2, 12]. The first report of this virus was in the 1990s from Taiwan, Republic of China [13]. Since then, the WSSV has caused a huge economic loss in shrimp aquaculture industries each year worldwide. Besides, the cumulative mortality in farmed shrimp is rapid and could be increased up to 90 to 100 percent within 1 week after the first infection [3, 11]. For immunizing in shrimp against the WSSV, viral subunit vaccines are appealing to many scientists. The viral subunit is a part of pathogen and can be produced by heterologous expression systems. For example, viral proteins such as VP19, VP24, VP26, VP28, VP292, and VP466 may enhance shrimp survival when applied as a monovalent vaccine [14-18] or as a polyvalent vaccine [19-22].

The fact that multicellular organisms have developed a system throughout the evolution to recognize pathogens such as bacteria and viruses, known as the "immune system". Unlike vertebrates, invertebrates such as insects and crustaceans are known to lack an adaptive immune system. Therefore, they solely rely on their innate immune system [11, 23, 24]. Generally, the innate immune response from invertebrates is initiated by activation of pattern recognition receptors including Toll and immune deficiency (IMD) pathways of nuclear factor-κB (NF-κB)-mediated signaling pathways which leads to an activation of transcription factors such as *Dorsal* and *Relish* leading to transcription and translation of antimicrobial peptides (AMPs) [24-26] (**Fig. 1A**). Recently, emerging evidence indicates that invertebrates like shrimp have "trained immunity" within the innate immune system [27, 28]. Several studies reported that pre-challenged with inactivated pathogenic bacteria such as *Vibrio harveyi*, *V. alginolyticus,* and *V. anguillarum* could enhance shrimps' protection against Vibriosis by enhancing phagocytic activity and the protections were effective for 3 weeks after immune priming [29, 30]. Also, it has demonstrated that when pre-vaccinated with attenuated/killed WSSV [27, 31] or certain WSSV structure subunits, later WSSV-challenged shrimps showed higher survival rates (**Fig. 1B**).

It is known that the *Escherichia coli* expression system is the most common tool in many laboratories as gene manipulation can be easily done and the protein expression process is simple. Despite that, disadvantages of the system are such as lack of post-translational processes, degradation of expressed protein inside the cells, accumulation of inclusion bodies resulted in a difficulty in purification step and even contamination of endotoxin [32, 33]. However, for expression of more complex proteins such as proteins requiring post-translational modification, eukaryote cell expression system such as insect cell or silkworm larvae is required. The silkworm expression system is a low-cost and easy-to-handle system, which

provides a large amount and stable expression of protein-of-interest as well as that this system also enables complex post-translational modifications similar to mammalian cells [34, 35].

In this study, employing the *E. coli* expression system, we intend to express and purify five major WSSV structure proteins, VP15, VP19, VP24, VP26, and VP28 to be used for vaccination in shrimps against WSSV infections. Here, we demonstrated that the WSSV major nucleocapsid protein, VP15, could provide a promising protective effect in Kuruma shrimp (*Marsupenaeus japonicus*) against the WSSV. Moreover, we also employed silkworm larvae as a protein production platform for VP15 and to validate a protective effect against the infection of the WSSV.

2. Materials and Methods

2.1. Cloning of WSSV-VP Encoding Genes for Expression in E. coli

Based on the genome sequence of WSSV (WSSV-CN01, NCBI, accession number: NC_003225), five ORFs (codon-optimized for insect expression system) encoding VP15 (*wsv214*), VP19 (*wsv414*), VP24 (*wsv002*), VP26 (*wsv311*) and VP28 (*wsv421*) were synthesized with a C-terminal FLAG®-tag (DYKDDDDK) fused of each VP gene, respectively (Genewiz, Suzhou, China). The genes were amplified by M13F (-20) and M13R with the following amplification protocol consisted of pre-denaturation at 94°C for 2 min followed by 32 cycles of 98°C for 10 s, 57°C for 30 s, and 68°C for 20 s and final-extension at 68°C for 7 min by KOD-PLUS-NEO (Toyobo, Japan). The corrected products were then purified and treated with *Bam*HI before ligating with pGEX-6P-1 (GE Healthcare, USA). The vector was prepared by treating with *Xho*I, T4 DNA polymerase for blunting, and finally treated with *BamH*I. The primers used were listed in **Table 1**. The plasmids were extracted from positive colonies and verified by Sanger DNA sequencing.

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119 *2.2. Expression and Purification of Recombinant Proteins from E. coli*

120 The correct plasmids were transformed into *E. coli* Rosetta gami-B (Novagen, Inc., USA) by
121 electroporation transformation for expression of recombinant proteins. Transformed *E. coli*
122 Rosetta gami-B cells were inoculated in LB broth containing 50 μg/ml ampicillin and incubated
123 at 37°C overnight followed by further inoculation in baffled flasks containing 250 ml of the
124 same medium at 37°C and vigorous shaking at 150 rpm until the absorbance reaches 0.6 at 600
125 nm (OD_{600}). Protein expression was induced by adding isopropyl β-D-1-thiogalactopyranoside
126 (IPTG) at the final concentration of 0.5 mM to the cultured medium and continued incubation
127 at 16°C for 16 h. After its induction, the cells were collected by centrifugation (6,000 × g, 4°C,
128 15 min). The pellets were washed with phosphate-buffered saline (PBS, pH 7.3) and were re-
129 suspended in PBS containing 1 × proteinase inhibitor (cOmplete™, Mini, EDTA-free Protease
130 Inhibitor Cocktail, Sigma-Aldrich, USA) and 10 μg/ml lysozyme. The cells were disrupted on
131 ice by sonication (Vibra-Cell™ Ultrasonic Liquid Processors, Sonics & Materials Inc, USA).
132 The whole-cell lysates were centrifuged (10,000 × g, 4°C, 10 min) and the supernatants were
133 collected. Supernatants and precipitates were used for analyzing expressed recombinant
134 proteins by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) followed
135 by Coomassie brilliant blue (CBB) staining. Western blotting using anti-FLAG (DYKDDDK)
136 antibody (1:5000, MBL, Tokyo, Japan) was carried out to detect the expressed proteins before
137 subjected for purification.

138 For purification, the clear supernatant containing GST-fused proteins was loaded onto the
139 GST affinity chromatographic column (Glutathione Sepharose 4 Fast Flow, GE Healthcare,
140 USA) according to the supplier's protocol. In brief, the glutathione resin was pre-equilibrated
141 with 10 × bed volumes of PBS (pH 7.3) before applying a cell lysate containing GST-fused

recombinant protein. Then, wash the resin with 10 × bed volumes of PBS and elute the protein with elution buffer containing 10 mM glutathione in 50 mM Tris-HCl (pH 8.0). After a purification process, purified proteins were concentrated and dialyzed against PBS using Amicon Ultra-15 30K Centrifugal Filter Unit (Merck Japan, Tokyo, Japan). The protein concentration was measured by bicinchoninic acid (BCA) assay (Pierce BCA Protein Assay Kit, Thermo Fisher Scientific, USA) after purification.

2.3. Generation of Recombinant Bacmid Encoding VP15 and VP19 for Protein Expression in Silkworm Larvae (Bombyx mori)

The codon-optimized DNA sequences of WSSV-VP15 and VP19 for insects with C-terminal DYKDDDDK-tag were synthesized by Genewiz (Suzhou, China). The DNAs were amplified using KOD-PLUS-NEO (Toyobo, Japan) with the primers listed in **Table 1**. The amplicons were cloned into pFastBac1 vector (Thermo Fisher Scientific, Japan) and confirmed for the insert DNAs via Sanger DNA sequencing. The correct recombinant plasmids were designated as pFastBac-VP15 and pFastBac-VP19. Each plasmid was transformed into *E. coli* BmDH10Bac to generate the recombinant *Bombyx mori* Nucleopolyhedrovirus (BmNPV) bacmid, then the bacmid was used for transfecting into silkworm larvae. The silkworms used in this study were purchased from Ehime Sansyu (Ehime, Japan) and were reared with Silkmate S2, an artificial diet (Nosan, Yokohama, Japan) under a controlled environment (25°C, 65±5% relative humidity).

2.4. Expression and Purification of VP15 and VP19 from Silkworm Fat Body

At 4 days after transfection, the fat body from silkworm larvae was collected and resuspended in a lysis buffer (100 mM Tris–HCl pH 8.4, 0.15 M NaCl, 1 mM EDTA 0.1% NP-40) with an

addition of the EDTA-free protease inhibitor. The suspensions were sonicated, centrifuged, and the clear supernatants were obtained for analysis by SDS-PAGE followed by CBB staining and by western blotting with the anti-DYKDDDDK antibody.

For purification of the DYKDDDDK-tagged protein, the recombinant proteins were subjected to DDDDK-tagged protein purification gel (MBL, Nagoya, Japan); an affinity chromatography. In brief, the column was pre-equilibrated with 10 × bed volumes of washing buffer containing 300 mM NaCl in 50 mM Tris-HCl (pH 7.5) before applying the fat body lysates, then eluted the proteins by an elution buffer comprised of 0.17 M glycine-HCl buffer (pH 2.3). The eluate was immediately neutralized with PBS (pH 7.3) and concentrated using Amicon Ultra-15 3K Centrifugal Filter Unit (Merck Japan, Tokyo, Japan). The protein concentration was measured by bicinchoninic acid (BCA) assay.

2.4. Quantitative real-time PCR of WSSV copy number

Real-time PCR was performed using a total volume of 20 μL (Adjust to 20 μL with ddH_2O), containing 70 ng template DNA and 2× Thunderbird Probe qPCR Mix 10 μL (TOYOBO, Japan). For WSSV quantification, 0.25 μM TaqMan probe [WSV079 Pr (5'-FAM-TGGGTGATTACGATTCG -BHQ-3')] and 0.3 μM of each WSSV specific primer [WSV079 Fw (5'-GAAACCGTCGCCGTTGAT-3') and WSV079 Rv (5'-AACACGGCATCCCTCCATT -3')] were used in the total volume. Thermal cycling consisted of an initial denaturation step at 95°C for 60 s, followed by 45 cycles of 95°C for 15 s and 60°C for 45 s annealing and extension steps on the CFX Connect (Bio-Rad., USA). The quantity of each sample was determined using CFX Operating Soft-ware version 4.0. The copy number of the target amplicon in the plasmid was estimated and 10-fold serial dilutions were made for use as absolute standards for quantification. The viral copy number was normalized on a

nanogram (ng) genomic DNA basis, or on an mL basis for water. For each new run, at least 2 NTCs (non-template control) were performed as negative control.

2.5. Kuruma Shrimp, WSSV Inoculum Preparation, Vaccination and Intramuscular (IM) Challenging Experiment

Kuruma shrimp (*Marsupenaeus japonicus*) of mean body weight (MWB) of 3.1 to 6.8 g were obtained from the National Research Institute of Aquaculture and were maintained in flow-through dechlorinated-electrolyzed seawater (33.05 ± 0.13 parts-per-trillion) at 24 ± 1.8°C using double-bottomed tanks with sand beds. Shrimp were fed with a commercial diet (Shrimp feed, Juveniles P-2, Maruha, Tokyo, Japan) at 3% of body weight per day. Adult *M. japonicus* (MBW 78 g), were intramuscular (IM)-inoculated with 50 μL of a 10^{-3} dilution of a virus source (prepared from naturally WSSV-infected juveniles of *M. japonicus*). The hemolymph was withdrawn with a four-fold volume of sterile PBS from moribund prawns 3 days after inoculation, divided into 0.3 ml aliquots, and stored at –80℃. Prior to each experiment, an aliquot of stored virus was thawed and centrifuged at 1500 × g at 4℃ for 10 min. The resultant supernatant was diluted with PBS to $10^{-4.8}$ for challenge tests [5].

The first experiment was done with the use of recombinant proteins purified from *E. coli* cell lysates. Kuruma shrimp (MBW 3.16 g, n = 12) were divided into seven groups and each shrimp of these groups was IM-vaccinated with GST, VP15, VP24, VP19, VP26, or VP28, respectively. The dose of recombinant proteins was 0.05 mg/g shrimp. The shrimp was immunized again at 20 days post-vaccination (dpv), then IM-challenged with WSSV at a dose of 2.69×10^{3} DNA copies/shrimp at 30 dpv. Shrimp mortality was observed for 20 d at 24 h interval and relative percent survival (%, RPS) was calculated with the formula proposed by Amend [36] as follow,

214 $$RPS = \left\{1 - \left(\frac{\%\ \text{mortality in immunized group}}{\%\ \text{mortality in PBS}-\text{injected group}}\right)\right\} \times 100.$$

215

216 To validate an anti-viral effect of VP15 expressed in silkworm, the purified VP15 was
217 used for the assay. In brief, Kuruma shrimp (MBW 3.16 g, n = 12) were divided into three
218 groups and IM-injected with PBS, VP15 or VP19 without boosting. Vaccinated shrimp were
219 challenged with WSSV at the dose of 2.69×10^3 DNA copies/shrimp at 20 dpv. The dose of
220 recombinant proteins was determined as 0.05 mg/g shrimp. Shrimp mortality was observed for
221 10 d at 24 h interval and an RPS was calculated by the same formula.

222

223 ## 3. Results

224 *3.1. Expression and Purification of WSSV-VPs from E. coli Lysate*

225 In this study, pGEX-6P-1, a glutathione S-transferase (GST, molecular weight; 26 kDa) gene
226 fused system was employed to express the GST-fused recombinant VPs (**Fig. 2A**). The
227 recombinant proteins were expressed in *E. coli* by IPTG induction. As the addition of the GST
228 tag would increase the sizes of recombinant proteins to approximately 35, 39, and 48 kDa for
229 VP15, VP19, and VP28, respectively, while VP24 and VP26 would show a similar MW of
230 about 49 kDa. The presence of recombinant proteins expressed in *E. coli* cells was confirmed
231 by western blot analysis with anti-DYKDDDDK antibody (**Fig. 2B**), and the proteins were
232 further purified using GST affinity chromatography and analyzed by SDS-PAGE followed by
233 CBB staining (**Fig. 2C**). As illustrated, the purified GST protein was loaded as a control and
234 other GST-VPs were successfully purified. The amounts of purified proteins were 2.7, 4.0, 2.5,
235 2.7, and 3.3 mg for VP15, VP19, VP24, VP26, and VP28, respectively. There are reports that
236 some VPs are difficult to dissolve with detergents [7] or recombinantly expressed as an

inclusion bodies when fusion tag is presence [15], therefore, it is necessary to use GST as a protein fusion partner while expressing a protein in *E. coli*, since the tag can improve protein solubility and facilitate protein folding thus reduce a chance of inclusion bodies forming.

3.2. Evaluation of the Purified WSSV-VPs as a Protective Agent against WSSV

To evaluate an anti-viral effect of the partially purified recombinant proteins in our study, the proteins were administered to shrimp via IM-injection and the experiment was done accordingly to the illustration in **Fig. 3A**. PBS and GST served as a negative control. As expected, a group of shrimps injected with only PBS or GST showed a dramatically decreasing survival rate at 5 days post-infection (dpi) with WSSV and showed 100% of cumulative mortality at 8 dpi. Throughout the experiment period of WSSV challenging assay using GST-fused VPs (GST-VPs) revealed that the RPS in groups of shrimps vaccinated with GST-fused VP15, VP19, VP24, VP26, and VP28 were 78%, 27%, 56%, 44%, and 50% at 20 dpi, respectively (**Fig. 3B**). The shrimps immunized with VP15, VP24, and VP28 showed a significantly higher survival rate than the groups of VP19 and VP26 ($p < 0.05$). The VP15 showed the highest RPS in this study.

3.3. Expression and Purification of VP15 and VP19 from Silkworm Fat Body

Since WSSV infects invertebrates such as shrimp, we consider it might be better to process VP15 in a eukaryotic protein expression system, *e.g.* silkworm expression system. Given the high productivity of silkworm larva-based expression system, we then generated VP15 and VP19-containing recombinant bacmids, as illustrated in **Fig. 4A**. To further test VP15 for immunization in shrimp against WSSV, the silkworm expression system was employed in this study. Purified proteins using DYKDDDDK affinity chromatography were verified by SDS-

PAGE and western blotting. As demonstrated in **Fig. 4B**, expressed and purified VP15 and VP19 (9 and 13 kDa) proteins showed corrected molecular weight as expected, respectively.

3.4. Evaluation of Protective Effect of the Silkworm-derived VP15 and VP19

Subsequently, to evaluate the silkworm-derived VP15 and VP19 in shrimp against the WSSV, the experiment was designed as illustrated in **Fig. 4C**. In the current study, PBS-injected shrimp showed 40% of RPS, while the VP19-injected group showed about 45% of RPS similar to the RPS from PBS control group at 10 dpi, indicating that silkworm-derived VP19 also cannot protect effect against the WSSV. Consistently, silkworm-derived VP15 did provide significant protection ($p < 0.05$) in Kuruma shrimp against the WSSV with 60% of the RPS (**Fig. 4D**), indicating VP15 is a true candidate employed by shrimp against WSSV infection. Since *E. coli*-derived VP19 couldn't provide any substantial protection against WSSV (**Figs. 2 and 3**), silkworm-derived VP19 was employed as a negative control to demonstrate a consistency in protective effect against WSSV as well as to support our result from *E. coli*-derived VP19. As speculated, our results indicated that VP19 from both systems could not provide a significant effect against WSSV in tested shrimp.

4. Discussion

In our study, we surveyed five major viral proteins of WSSV in activating the immunogenic response in Kuruma shrimp. Injection of VP19 failed to protect shrimp against WSSV in this study. A different result was observed in experiments by Witteveldt *et al.* (2004) in which a recombinant VP19 was used for coating fed food pellets and fed to *P. monodon* shrimp. After 14 dpi, the group of shrimps fed with VP19-coated feed showed cumulative mortality of 83%

(RPS of 17%) [37]. However, vaccination of maltose-binding protein (MBP)-fused VP19 via IM-injection provided a protection against WSSV with an RPS of about 33% and 57% after challenging at 2 and 25 dpv [14]. Our result of vaccination in shrimp using VP19, which gave RPS of 27%, was similar to the latter experiment that fusion-VP19 gave an RPS of 33% at 2 dpv. According to their experiment, maltose-binding protein was able to induce a minimum immune response, unlike GST that could not elicit any immune response. Moreover, Witteveldt, Vlak and van Hulten [14] suggested that an immune response of VP19 was specific since vaccination of shrimp with MBP-VP19 could yield protection against WSSV even after challenging at 25 dpv. The function of VP19 remains unclear but was thought to be the first contact to host cells along with VP28 [10] and was found to be essential for a complete virion assembly [38]. These results suggested that the protective effect from VP19 could be achieved only by injection into shrimp.

Even though VP24 is one of the major WSSV envelope proteins and the role of VP24 is still inadequate, but was recently identified as a chitin-binding protein [39]. Only a few attempts using recombinant VP24 (rVP24) as a subunit vaccine has been demonstrated for instance oral administration of rVP24-coated feed-in *Penaeus monodon* [15]. Our result also demonstrated a protective effect provided by GST-fused VP24 showing an RPS of about 67% at 15 dpi, however, RPS was decreased to 56% at 16 dpi.

VP26 is acting as a tegument protein associated with both envelopes by anchoring its N-terminal at the membrane and nucleocapsid by interacting with VP51 suggesting an important role in the envelopment of WSSV virion [40]. However, the study about vaccination using VP26 as a protective agent is still limited. Up to date, only a few studies demonstrated that VP26 could protect shrimps from WSSV infections such as intramuscularly injection of recombinant VP26 (rVP26) once or twice [31]. An oval vaccination using rVP26 expressed from *E coli* soaked with a commercial feed and shrimp were challenged with WSSV [16].

Another interesting research about vaccination using VP26 has been demonstrated by the use of spores from gram-positive *Bacillus subtilis* displaying CotC::VP26 and the spores were coated with food pellets before fed to *Litopenaeus vannamei* at larvae state [22]. Our result of VP26 injection for shrimp immunization against WSSV gave RPS value of 44% in *M. japonicus* in which the degree of immune response was lower than the results done by Namikoshi, Wu, Yamashita, Nishizawa, Nishioka, Arimoto and Muroga [31] and Satoh, Nishizawa and Yoshimizu [16]. This might be due to i) the dose of WSSV for challenging and/or ii) toxicity of the proteins.

Many studies have been focusing on VP28 since the VP28 was found to be involved in the initial step of the infection in shrimp by acting as an attachment protein and facilitating virus entry [10, 41]. Various studies demonstrated a protective effect of recombinant VP28 either oral vaccination or intramuscularly vaccination against WSSV and even using DNA vaccine encoding VP28 [16, 19, 31, 42]. Displaying technology of VP28 such as using the baculovirus expression system and *B. subtilis* spore-displaying was employed for shrimp and crayfish vaccination but could provide a significant protection against WSSV [21, 22, 43]. Our result of using VP28 as a protective agent and IM-injected into Kuruma shrimp showed RPS of 50% supported the above experiments that VP28 could induce shrimp immunity against the WSSV. As VP28 plays a vital role in the initial step of WSSV infection in shrimp by attaching the virus to host cells, thus using rVP28 as a vaccine could protect shrimp from the WSSV infection by binding competitively to VP28 receptors.

In this study we showed firstly that VP15-vaccination could protect shrimp against WSSV infection, and the effect was retained for at least 20 days. However, there is a noticeable difference of RPS resulted from *E. coli*-derived VP15 and silkworm-derived VP15. We hypothesized that this difference was due the stability and the solubility of GST-fused VP15 are better than solo VP15 without GST fusion partner. From previous results, shrimp

vaccination using VP15 has been barely conducted simply because VP15 is located inside of virion but not displayed on the surface. The VP15 is an 80 amino acid histone-like basic DNA-binding protein showing some homology to putative baculovirus DNA binding proteins whose functions mainly remain unclear, however, the protein is found to be a major nucleocapsid of the virus and involves virus genome packaging into the capsid [44-46]. To our knowledge, only one experiment using VP15 has been attempted as a DNA vaccine of VP15 but failed to provide a protective effect in shrimp [42]. The discrepancy could be explained by the expression level and the efficacy of the DNA vaccine in the previous study. DNA vaccine encoding VP15 might not be effective due to the intrinsic properties of VP15. VP15 is a nucleoprotein with a low solubility when overexpressed in eukaryotic cell such as cultured silkworm cells. In our study, VP15 was produced in silkworm larvae and mainly detected in precipitations, which is consistence to the fact that the major structural protein VP15 was found in the pellet fractions of WSSV virions [46]. We also tried to conduct an additional study to improve a protein solubility using various detergents such as Triton X-114 and NP-40 but the results were not significant (data not show). Another possibility is that when used as immunostimulants, the VP15 will be targeted directly by immune cells, such as hemocytes of shrimp hemolymph and be recognized as a nonself compartment, triggering a humoral or cellular immune response. However, the intracellular expression of VP15 might be too low and thus not such sufficient to induce the receptor-based immune responses. The detailed mechanism of how shrimps could confer protection against WSSV after VP15-vaccination is yet to be cleared. However, we are shortly suggesting that the VP15 could interact with a highly acidic host receptor, gC1qR, via charge-charge interaction of proteins and trigger the early defense responses against viral infections [47, 48].

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Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflict of interest.

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Figure legend

Fig. 1. Schematic diagram of shrimp immunity and major structural proteins of WSSV. (A) Shrimp's trained immunity within the innate immunity which is triggered after exposing to nonspecific triggers such as non-self-proteins resulting in activations of Toll and/or IMD signaling pathways leading to a production of AMPs for combating pathogens. (B) By injecting WSSV-VP into shrimp, shrimp could develop a protective mechanism against a specific pathogen such as WSSV.

Fig. 2. Expression and purification of five recombinant WSSV-VPs from *E. coli* expression system. (A) The five major WSSV structural proteins used in this study. VP15 is identified as a DNA-binding protein and major nucleocapsid protein. VP19, VP24, and VP28 are known as membrane proteins while VP26 is found to be a tegument protein. TM: a transmembrane region. (B) Construction of plasmids used for recombinant protein expression and expression verification of the GST-fused recombinant WSSV-VPs in *E. coli*. GST: glutathione-S-transferase as a fusion tag and for affinity purification; Flag: DYKDDDDK epitope tag for western blot analysis; M: marker; S: supernatant; P: precipitate or cell pellet. (C) The SDS-PAGE analysis of the partially purified proteins from *E. coli* cell lysates. M: marker; S: supernatant; Pur: purified protein.

Fig. 3. Evaluation of five recombinant WSSV-VPs expressed in *E. coli* for a protective effect against WSSV. (A) Time-schedule of shrimp vaccination, WSSV challenge, and observation. The prime and boost immunization strategies for seven different groups (PBS, GST, VP15, VP19, VP24, VP26, and VP28) were as shown. Briefly, shrimps were boosted at 20 dpv and challenged with WSSV via intramuscular injection at a dose of 2.69×10^3 DNA copies/shrimp at 10 days afterward. (B) Time-and-RPS relationship of WSSV-VP-vaccinated Kuruma shrimp. The mortality of shrimp was observed at 24 h interval for 20 dpi.

Fig. 4. Expression and purification of VP15 and VP19 from silkworm expression system, and evaluation for a protective effect against WSSV. (A) Schematic diagram of the silkworm expression system. In brief, silkworm was injected with a recombinant bacmid carrying VP-DKYDDDDK encoding gene, reared, then collected a fat body for purification of VPs. (B) SDS-PAGE and western blot analysis of purified VP15 and VP19 from silkworm fat body lysate. (C) Time-schedule of shrimp vaccination, WSSV challenge, and observation. The immunization approaches for three different groups (PBS, VP15, and VP19) were as illustrated. Briefly, shrimps were single vaccinated and challenged with WSSV at 10 dpv via intramuscular injection at a dose of 2.69×10^3 DNA copies/shrimp. (D) The time-and-RPS relationship of VP15, VP19, or PBS-vaccinated Kuruma shrimp in vaccination assay. The mortality of shrimp was observed at 24 h interval for 10 dpi.

Fig. 1

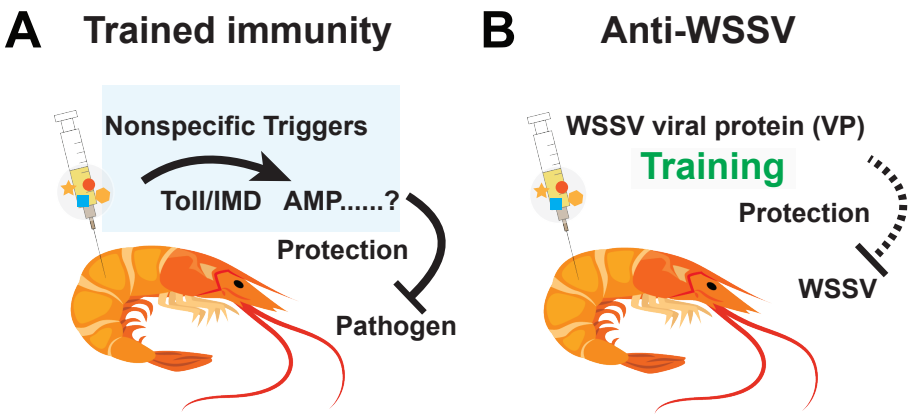

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Fig. 2

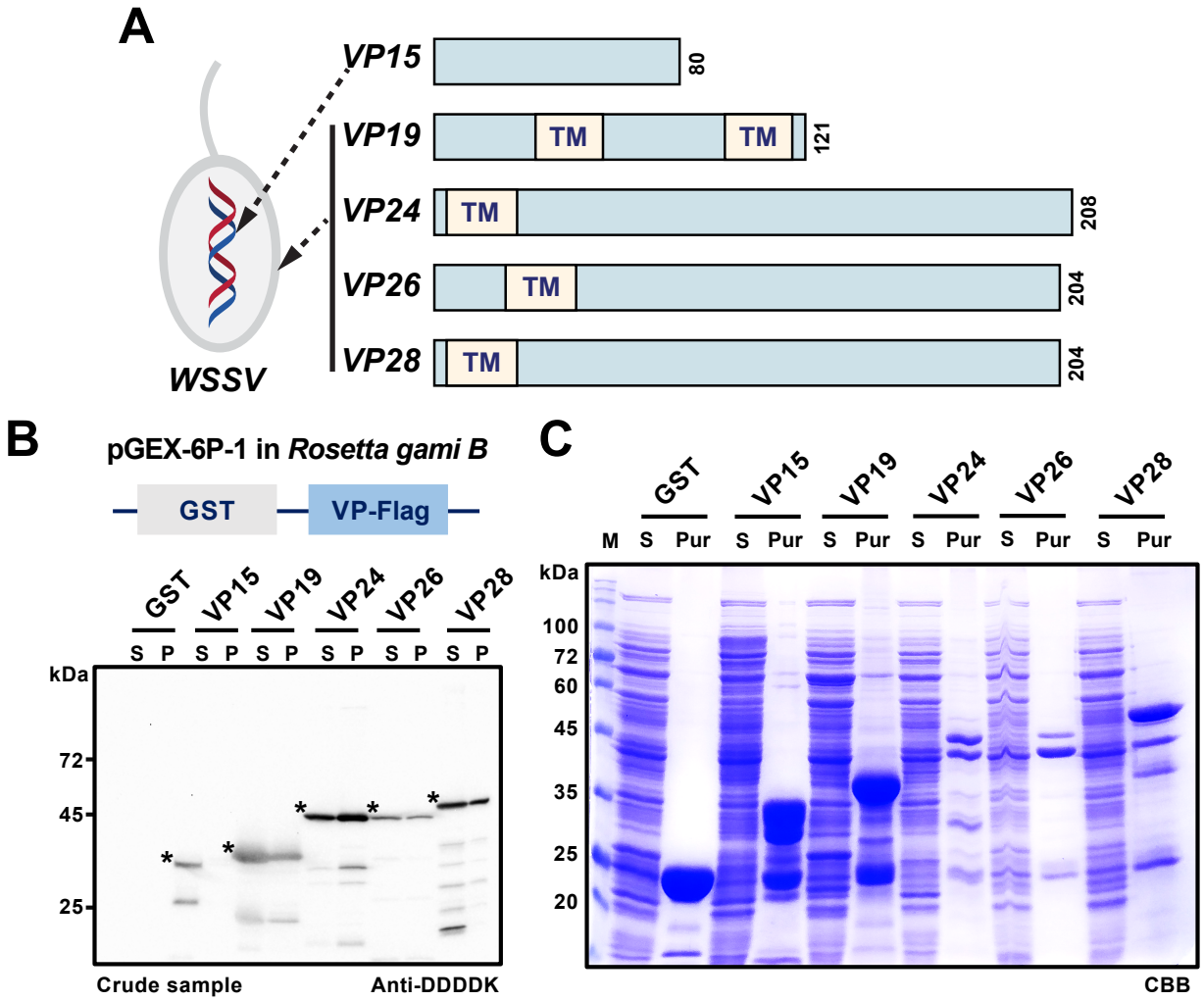

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Fig. 3

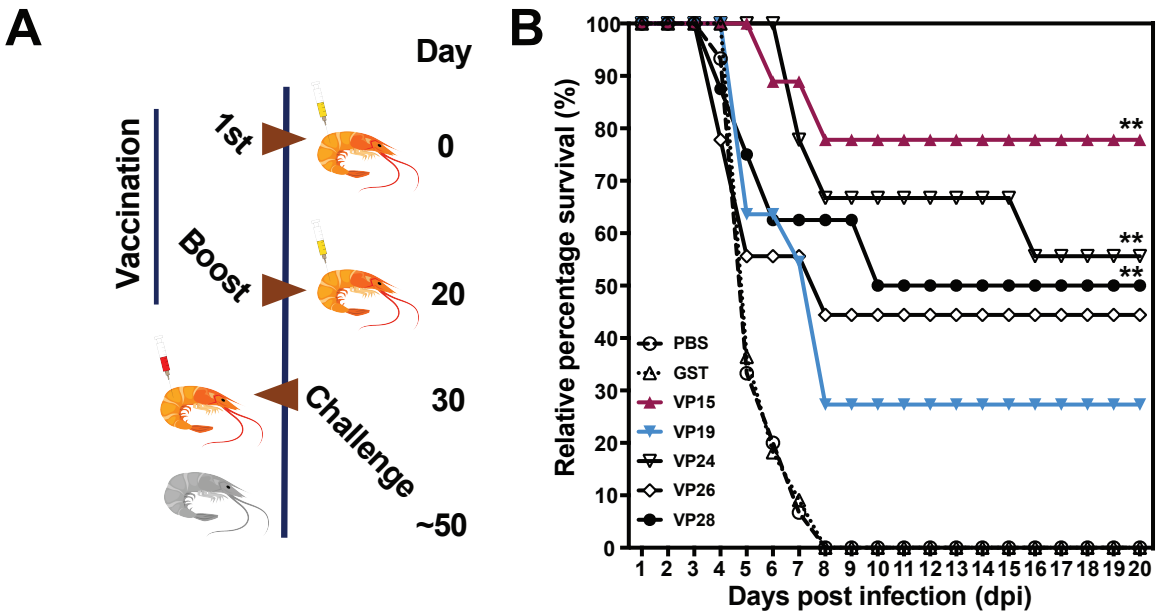

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Fig. 4

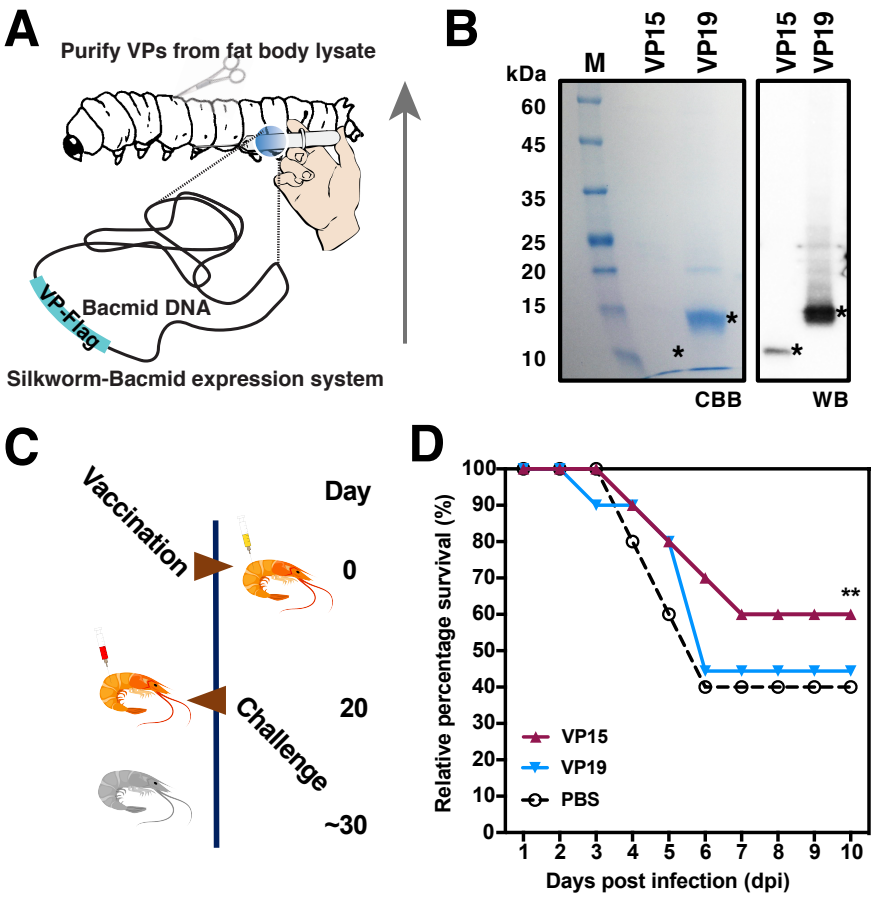

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Table 1. Primers used in this study

Primers	Primer sequences (from 5' to 3')
M13F (-20)	GTAAAACGACGGCCAG
M13R	CAGGAAACAGCTATGAC
pGEX-FW	GAAGTTCTGTTCCAGGGGCCC
pGEX-RV	AGGCAGATCGTCAGTCAGTCA
pFastBac1-FW	TATTCCGGATTATTCATACC
pFastBac1-RV	ACAAATGTGGTATGGCTGATT
VP15-FW	ATGGTTGCCCGAAGCTCCAAG
VP15-RV	TTAACGCCTTGACTTGCGGGC
VP19-FW	ATGGCCACCACGACTAACACT
VP19-RV	TTACTGCCTCCTCTTGGGGTA
VP24-FW	ATGCACATGTGGGGGGTTTAC
VP24-RV	TTATTTTTCCCCAACCTTAAACAGA
VP26-FW	ATGGAATTTGGCAACCTAACA
VP26-RV	TTACTTCTTCTTGATTTCGTCC
VP28-FW	ATGGATCTTTCTTTCACTCTTTC
VP28-RV	TTACTCGGTCTCAGTGCCAGA