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メタデータ	言語: eng 出版者: 公開日: 2020-03-30 キーワード (Ja): キーワード (En): 作成者: Boonyakida, Jirayu, Xu, Jian, Satoh, Jun, Nakanishi, Takafumi, Mekata, Toru, Kato, Tatsuya, Park, Enoch Y. メールアドレス: 所属:
URL	http://hdl.handle.net/10297/00027213

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].

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

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

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

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

103

104 ## 2. Materials and Methods

105 *2.1. Cloning of WSSV-VP Encoding Genes for Expression in E. coli*

106 Based on the genome sequence of WSSV (WSSV-CN01, NCBI, accession number:
107 NC_003225), five ORFs (codon-optimized for insect expression system) encoding VP15
108 (*wsv214*), VP19 (*wsv414*), VP24 (*wsv002*), VP26 (*wsv311*) and VP28 (*wsv421*) were
109 synthesized with a C-terminal FLAG®-tag (DYKDDDDK) fused of each VP gene, respectively
110 (Genewiz, Suzhou, China). The genes were amplified by M13F (-20) and M13R with the
111 following amplification protocol consisted of pre-denaturation at 94°C for 2 min followed by
112 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
113 min by KOD-PLUS-NEO (Toyobo, Japan). The corrected products were then purified and
114 treated with *Bam*HI before ligating with pGEX-6P-1 (GE Healthcare, USA). The vector was
115 prepared by treating with *Xho*I, T4 DNA polymerase for blunting, and finally treated with
116 *BamH*I. The primers used were listed in **Table 1**. The plasmids were extracted from positive
117 colonies and verified by Sanger DNA sequencing.

118

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

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

148

149 *2.3. Generation of Recombinant Bacmid Encoding VP15 and VP19 for Protein Expression in*
150 *Silkworm Larvae (Bombyx mori)*

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

162

163 *2.4. Expression and Purification of VP15 and VP19 from Silkworm Fat Body*

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

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

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

177

178 *2.4. Quantitative real-time PCR of WSSV copy number*

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

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

192

193 *2.5. Kuruma Shrimp, WSSV Inoculum Preparation, Vaccination and Intramuscular (IM)*
194 *Challenging Experiment*

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

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

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

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

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

240

241 *3.2. Evaluation of the Purified WSSV-VPs as a Protective Agent against WSSV*

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

253

254 *3.3. Expression and Purification of VP15 and VP19 from Silkworm Fat Body*

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

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

263

264 *3.4. Evaluation of Protective Effect of the Silkworm-derived VP15 and VP19*

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

277

278 # 4. Discussion

279 In our study, we surveyed five major viral proteins of WSSV in activating the immunogenic
280 response in Kuruma shrimp. Injection of VP19 failed to protect shrimp against WSSV in this
281 study. A different result was observed in experiments by Witteveldt *et al.* (2004) in which a
282 recombinant VP19 was used for coating fed food pellets and fed to *P. monodon* shrimp. After
283 14 dpi, the group of shrimps fed with VP19-coated feed showed cumulative mortality of 83%

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

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

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

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

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

329 In this study we showed firstly that VP15-vaccination could protect shrimp against WSSV
330 infection, and the effect was retained for at least 20 days. However, there is a noticeable
331 difference of RPS resulted from *E. coli*-derived VP15 and silkworm-derived VP15. We
332 hypothesized that this difference was due the stability and the solubility of GST-fused VP15
333 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].

Author Contributions Conceptualization, T.K. and E.Y.P.; Formal Analysis, J.X. and J.B.; Funding Acquisition, E.Y.P.; Investigation, J.B., J.X.; Methodology, J.B., J.X. and J.S.; Writing – Original Draft Preparation, J.B., J.X.; Writing – Review & Editing, J.B., J.X., T.K. and E.Y.P.

Funding Information This work was supported by Marine Industry Cluster Council of Shizuoka City.

Compliance with Ethical Standards

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

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510

511 # Figure legend

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

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

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

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

Fig. 1

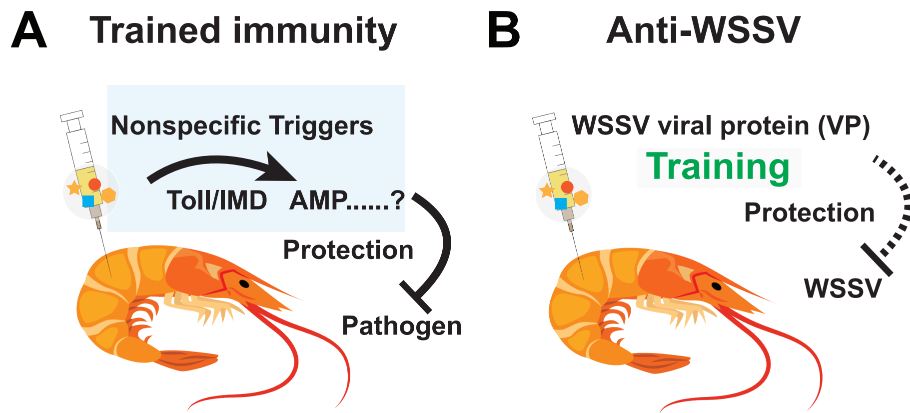

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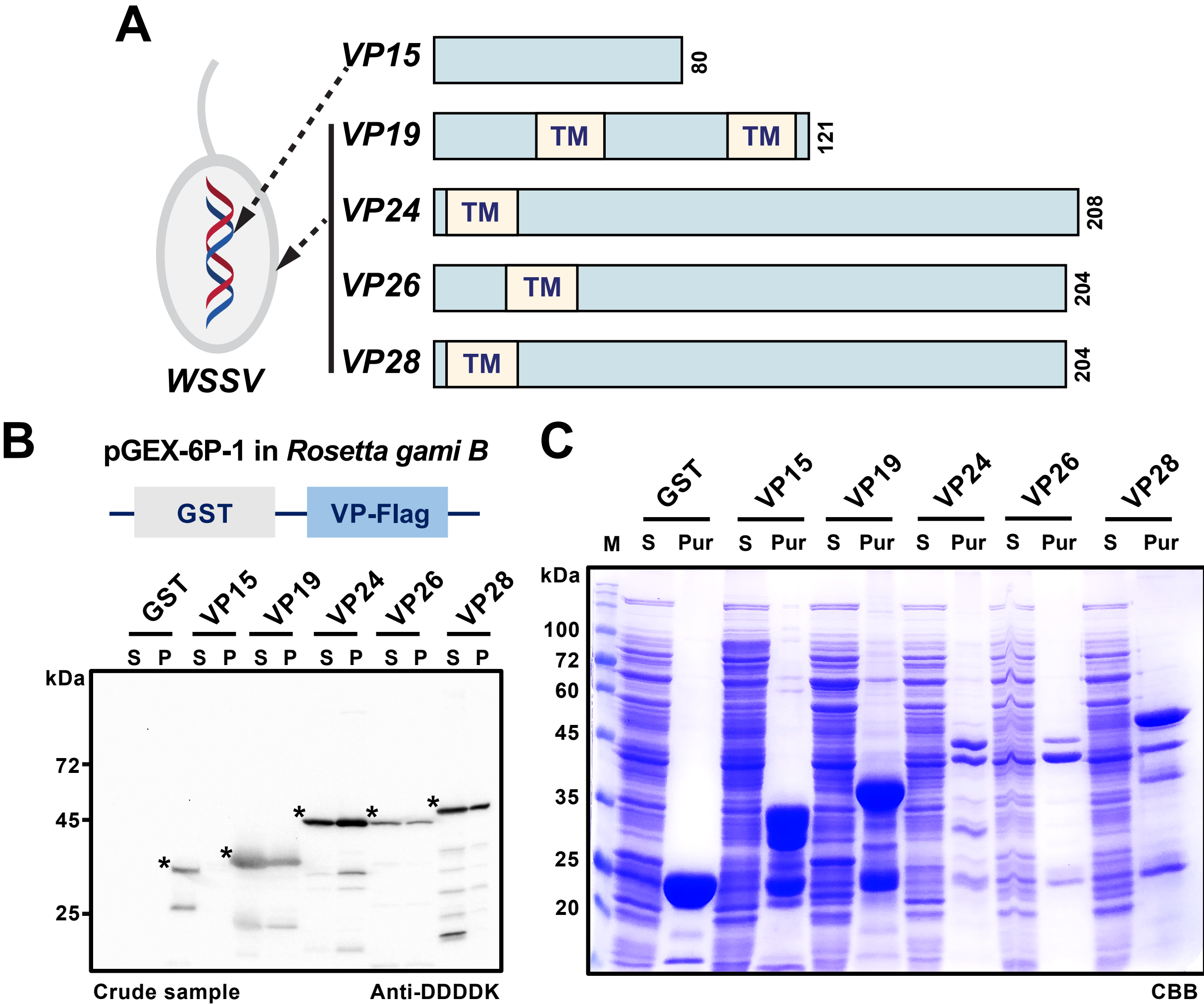
Fig. 2



Boonyakida et al., 2019

Fig. 3

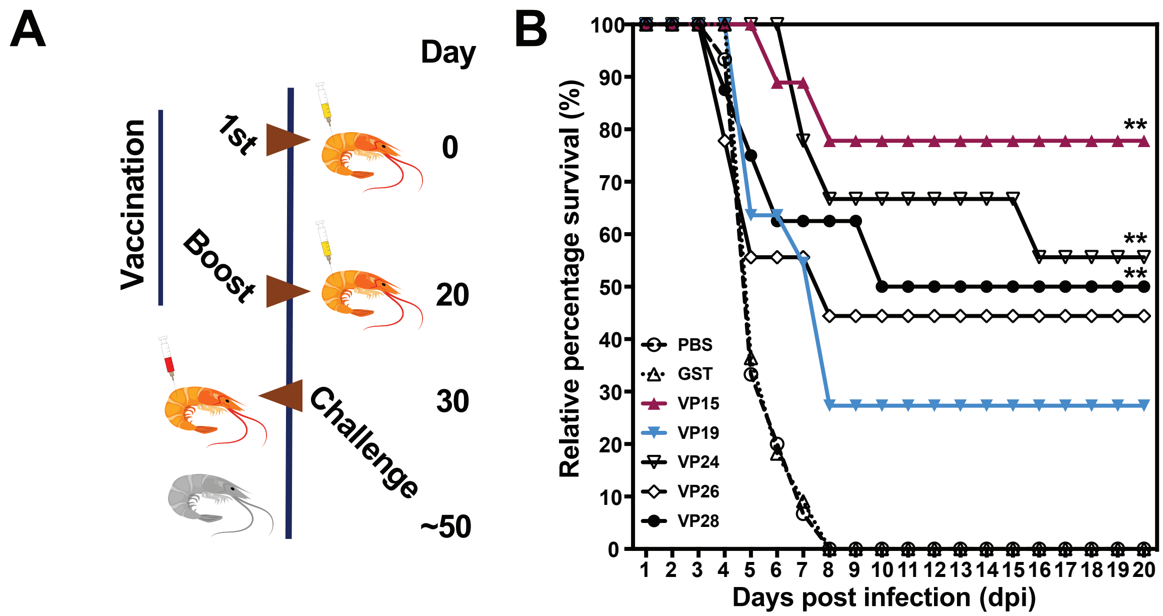


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Fig. 4

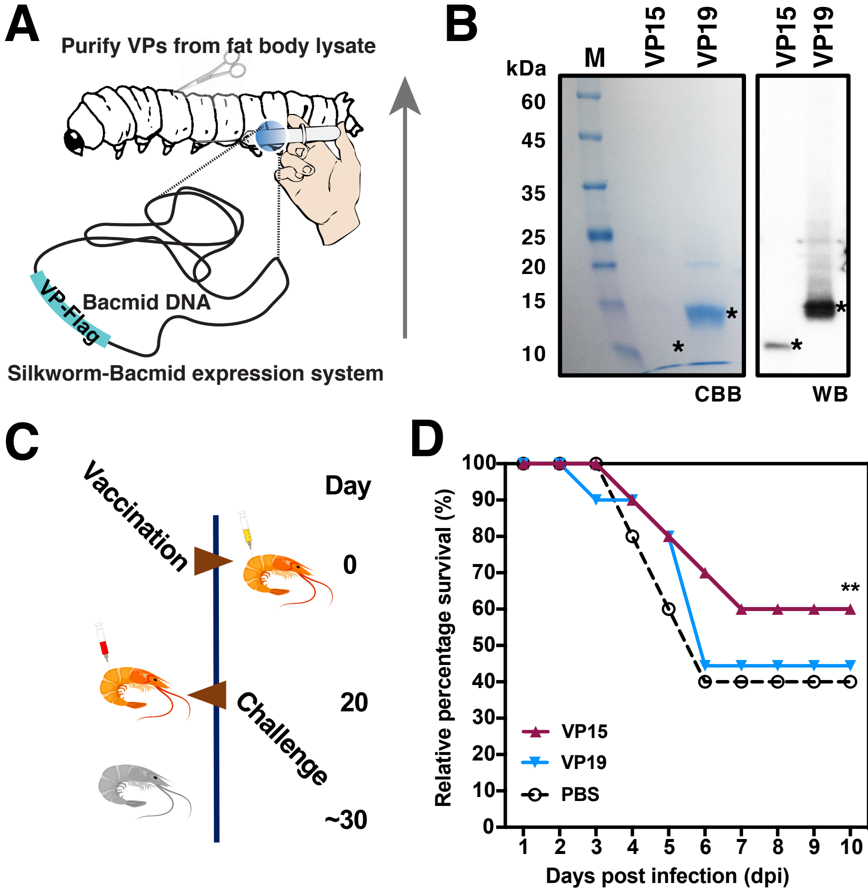

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Table 1. Primers used in this study

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