

# Effects of ribosomal protein S3a (RPS3a) on the survival of WSSV-infected pacific white shrimp (*Litopenaeus vannamei*)

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**ABSTRACT:** White spot syndrome virus (WSSV) causes substantial economic losses in shrimp farming. It has been reported that shrimp infected with this virus exhibits an overexpression of ribosomal protein S3a (RPS3a). Therefore, a recombinant protein, His-RPS3a, from shrimp was produced and its potential to protect shrimp from WSSV was evaluated. The purified His-RPS3a was injected at concentrations of 1–20  $\mu\text{g}/\text{shrimp}$  to determine the expression of the genes prophenoloxidase (*proPO*) and phagocytosis-activating protein (*PAP*) by real-time PCR. *PAP* expression increased 4.1-fold when His-RPS3a was injected at 1  $\mu\text{g}/\text{shrimp}$ , and *proPO* gene expression increased 5.3-fold when His-RPS3a was injected at 20  $\mu\text{g}/\text{shrimp}$ . Therefore, shrimp were injected with His-RPS3a at concentrations of 10, 20, or 40  $\mu\text{g}/\text{shrimp}$  for 3 days and then challenged with WSSV. The relative percentages of survival (RPS) of the WSSV-challenged shrimp after injection with the His-RPS3a were 23%, 27%, and 35%, respectively. Besides, RPS3a-phMGFP was prepared in the form of chitosan-DNA nanoparticles and fed at 75  $\mu\text{g}/\text{shrimp}/\text{day}$  for 7 days. *PAP* expression was found to be higher than that in the control group. Furthermore, shrimp fed with RPS3a-phMGFP DNA at 75  $\mu\text{g}/\text{shrimp}/\text{day}$  were challenged with WSSV and further cultured for 15 days. The RPS of the WSSV-challenged shrimp after feeding with RPS3a-phMGFP was 35%, whereas the control shrimp exhibited 100% death. These results demonstrated that RPS3a acts as an antigen and could delay the death of WSSV-infected shrimp by activating the general immune system; furthermore, RPS3a may be activated after WSSV infection to induce the production of proinflammatory cytokines.

**KEYWORDS:** immune-related genes, shrimp, ribosomal protein S3a, white spot syndrome virus

## INTRODUCTION

Penaeid shrimp cultivation is an economically important aspect in the food industry [1]. Several Asian countries had started cultivating the Pacific white shrimp (*Litopenaeus vannamei*) in 2002, which has emerged as a dominant cultivated species in the world. Thailand has been the leader in the production and export of shrimp, with the production being valued at more than one billion US dollars per year [2]. However, shrimp are prone to infections by microbes such as viruses, among which white spot syndrome virus (WSSV) is known to cause high mortality and potential losses of 100% within 3–10 days after infection. Epidemics of this virus have been reported to cause considerable economic losses to the shrimp industry [3, 4]. WSSV is one of the most common and destructive diseases of shrimp [5, 6]. Molecular biology has been applied in the investigation of shrimp diseases; for example,

microarray techniques have been used to analyze gene expression in the hemocytes of *Penaeus monodon* after challenge with WSSV. Upregulated gene expressions, including the 60S ribosomal proteins L34 (RPL34), L30 (RPL30), and L14 (RPL14), a 14-3-3-like protein, and the 40S ribosomal protein S3a (RPS3a), have been reported. It was assumed that these genes play an essential role in the immune system of shrimp [7].

Ribosomal protein S3a (RPS3a, S3a, or Fte-1) is a component of the 40S subunit of the ribosome [8]. A study on a gene library from Namalwa Burkitt lymphoma cells isolated from the thymus of mice reported that RPS3a was overexpressed in cancer cells and that the inhibition of the RPS3a gene induced cell death and apoptosis [9]. In addition, yeast two-hybrid screening that was performed to identify the interaction between RPS3a and the transcription factor CHOP (GADD153), an apoptosis inducer,

showed that this interaction inhibited CHOP activity and induced erythroid differentiation [10, 11]. Similarly, another research demonstrated that RPS3a interacts with poly (ADP-ribose) polymerase (PARP) and Bcl-2, which acts as an apoptosis inducer and suppressor, respectively. It was reported that Bcl-2 could inhibit PARP activity by working together with RPS3a [12]. In another study, the expression of *Bombyx mori* S3a (BmS3a) helped in slowing down the death rate of silkworms infected with *B. mori* nuclear polyhedrosis virus (BmNPV). It was observed that BmS3a inhibited the proliferation of BmNPV, and high expression of BmS3a could be involved in the regulation of cell death and inhibition of the translation of BmNPV, thus causing an anti-BmNPV infection effect in silkworms [13]. As silkworms belong to the phylum Arthropoda, to which shrimp also belong [14], the RPS3a protein of shrimp could function similarly to BmS3a and may play a role in protecting shrimp from diseases caused by viruses.

Therefore, in the present study, we produced and purified the recombinant protein His-RPS3a for injection into white shrimp to examine immune-related genes, including phagocytosis-activating protein (PAP) and phenoloxidase (PO). In addition, we investigated the effect of RPS3a on the protection of *L. vannamei* against WSSV.

## MATERIALS AND METHODS

### Production and purification of His-RPS3a protein

The recombinant plasmid pET28-RPS3a was obtained from our previous study [15] and was introduced into *E. coli* strain BL21(DE3). The bacteria were grown overnight for 16–18 h in 30 ml LB broth (containing 30 µg/ml kanamycin) at 37°C under shaking at 180 rpm. This culture was then used to inoculate 300 ml of fresh LB medium, and cells were grown at 28°C under shaking at 180 rpm. When the culture reached an OD<sub>600</sub> of 0.5–0.6, protein expression was induced by adding isopropyl β-D-1-thiogalactopyranoside (IPTG) to a final concentration of 1 mM. The cultured cells were grown at 24°C under shaking at 80 rpm for 5 h, after which the temperature was reduced to 18°C for 13 h. Next, the cells were harvested by centrifugation at 4000g for 20 min, and the bacterial pellet was suspended in 30 ml of binding buffer (50 mM Tris-HCl, 300 mM NaCl, and 5 mM imidazole, pH 12), followed by cell lysis via sonication. The resulting lysate was centrifuged at 10 000g for 20 min, and the supernatant was further purified. The His-RPS3a fusion protein

was purified in a Ni<sup>2+</sup> nitrilotriacetic acid column (Ni-NTA column) with AKTAp<sub>prime</sub> plus (GE Healthcare Bio-Sciences AB, Sweden). The column was equilibrated with 120 ml of binding buffer (50 mM Tris-HCl, 0.3 M NaCl, and 5 mM imidazole, pH 12), and then a 2 ml sample was applied. Protein was eluted with elution buffer (50 mM Tris-HCl, 0.3 M NaCl, and 500 mM imidazole, pH 8). The purified protein was dialyzed against TBS buffer (50 mM Tris-HCl and 0.15 M NaCl, pH 8) to remove imidazole. Furthermore, the purity of the protein fractions was assessed by 12% SDS-PAGE analysis, and the His-RPS3a protein was confirmed by western blotting. Briefly, the recombinant His-RPS3a protein was separated by 12% SDS-PAGE and transferred onto a nitrocellulose membrane, which was then incubated for 1 h with an anti-His antibody conjugated with alkaline phosphatase (AP) (Invitrogen, USA, diluted 1:1000). Alkaline phosphatase activity on the membrane was detected using 0.23 mM bromochloroindolyl phosphate (BCIP) and 0.37 mM nitroblue tetrazolium (NBT) in the detection buffer (0.1 M Tris-HCl, 0.1 M NaCl, and 0.05 M MgCl<sub>2</sub>, pH 7.5), in which the positive band developed a blue color. When the color was sufficiently intense, the reaction was stopped by dipping the membrane in distilled water.

### Experimental animals

Healthy *L. vannamei* white shrimp were obtained from a commercial farm in Songkhla province, Thailand. Shrimp with an average body weight of 10 g were used for the experiment. They were cultured in fiberglass tanks containing aerated, diluted natural seawater with a salinity between 10 and 15 ppt. The shrimp were fed twice-daily with a formulated shrimp diet for 1 week before the experiment, during which we randomly checked the shrimp for WSSV infection by PCR.

### Expression of RPS3a in WSSV-infected shrimp

Two healthy *L. vannamei* were challenged by an intramuscular injection of 100 µl of a 1 × 10<sup>-6</sup> dilution of WSSV in phosphate-buffered saline (PBS). Two shrimp in the control group were injected with 100 µl of PBS. After 3 days, the hemolymph and heart of both normal and infected shrimp were collected for RNA extraction using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). Total RNA (4 µg) was reverse-transcribed into cDNA at 48°C for 2 h using 2 µl of random primers (100 ng/µl) and 1 µl of avian myeloblastosis virus (AMV) reverse transcriptase (5 U/µl) (Promega, Madison, WI, USA).

PCR was performed in a final volume of 25  $\mu$ l containing 160 ng cDNA template, each of primer at 0.4  $\mu$ M, each dNTP at 0.2 mM, 1.5 mM  $MgCl_2$ , 10 mM Tris-HCl (pH 9), 50 mM KCl, 0.1% TritonX-100, and 2.5 U *Taq* DNA polymerase. A total of 30 cycles of PCR were performed with denaturation at 94 °C, annealing at 60 °C and extension at 72 °C, each step at 30 s. Cycling was initiated with 2 min of denaturation at 94 °C and terminated with 10 min of incubation at 72 °C. Table S1 shows the primers used to amplify the RPS3a and  $\beta$ -actin genes. The PCR products of the  $\beta$ -actin and RPS3a genes were used as templates of the positive control, and the expression of the  $\beta$ -actin gene was used as an internal standard. The RT-PCR products of each sample were analyzed on 1.5% agarose gel and visualized by ethidium bromide staining under ultraviolet light.

#### Expression of *PAP* and *proPO* in RPS3a-immunized shrimp

The His-RS3a fusion protein was purified with Ni-NTA resin (GE Healthcare Bio-Sciences AB, Sweden). Five shrimp weighing 10 g from each group were injected intramuscularly with 0.1, 1, and 2  $\mu$ g/g of body weight of His-RPS3a. The negative control group was injected with TBS buffer, and the entire group was not vaccinated. After 48 h, the hemolymph was withdrawn for analyzing the expression of the *proPO* and *PAP* genes. Total RNA was extracted from the hemolymph using TRIzol reagent (Invitrogen, Carlsbad, USA), and 4  $\mu$ g of RNA was reverse-transcribed into cDNA using random primers and AMV reverse transcriptase (Promega, USA). Real-time PCR was performed in a final reaction volume of 25  $\mu$ l using FastStart Universal SYBR Master Mix (Roch, Germany). The primers used to amplify the *PAP*, *proPO*, and  $\beta$ -actin genes are shown in Table S1. The expression of the  $\beta$ -actin gene was used as an internal standard. Thermal cycling and fluorescence detection were conducted using MX3000P™ (Stratagene, La Jolla, CA). Standard curves for the quantification of the *proPO*, *PAP*, and  $\beta$ -actin genes were plotted using serial dilutions of the linearized purified PCR products of their respective genes. The copy number of each amplified product was calculated according to its molecular weight and then converted into the copy number based on Avogadro's number.

#### Effect of His-RPS3a on the protection of *L. vannamei* against WSSV

White shrimp were divided into five groups of 10 shrimp. Two groups serving as controls were

injected intramuscularly with TBS buffer. The other groups were injected intramuscularly every day for 3 days with His-RPS3a at 10, 20, and 40  $\mu$ g/g of shrimp bodyweight. After the third vaccination, the shrimp were challenged with WSSV. Shrimp that did not receive His-RPS3a and were challenged with WSSV were used as the positive control, and shrimp that were injected only with PBS served as the negative control. After injection with WSSV, mortality was recorded for an additional 15-day period. The relative percentage of survival (RPS) was calculated using the following formula [16]

$$RPS = 1 - \frac{\% \text{Mortality in the test group}}{\% \text{Mortality in the positive group}} \times 100$$

#### Preparation of RPS3a DNA nanoparticles for feeding

The DNA used for nanoparticle feeding was prepared as follows. RPS3a-phMGFP was constructed from the PCR product of the RPS3a gene. The PCR product was cloned into the phMGFP vector (Promega, USA), and the recombinant plasmid was transformed into *E. coli* (Top10). *E. coli* containing the RPS3a-phMGFP or phMGFP plasmid (control) were grown overnight for 16–18 h in 5 ml LB broth (containing 80  $\mu$ g/ml ampicillin) at 37 °C. The cells were harvested by centrifugation at 10 000g for 5 min, after which the supernatant was discarded, and the bacterial pellet was extracted by alkaline lysis [17]. The plasmid DNA was suspended in a sterile tube and stored at 20 °C until use.

Low-molecular-weight chitosan was prepared according to a previously described method [18]. Chitosan was dissolved at 1% (w/v) in 1% acetic acid under magnetic stirring. Then, 0.1 M  $NaNO_2$  was dropped into the chitosan solution until the chitosan/ $NaNO_2$  molar ratio was 0.01, followed by stirring for 3 h at room temperature. The pH of the chitosan solution was adjusted to 8.0 using 1 N NaOH to precipitate chitosan. Then, the solution was centrifuged at 7500g for 2 min, after which the sample was washed 10 times with deionized water. The precipitated chitosan was dried by lyophilization.

Dried chitosan was dissolved at 1 mg/ml in 1% acetic acid at 37 °C and concentrated NaOH was added to adjust the pH of the solution to 5.6–6.96. This stock solution was filtered through a 0.2  $\mu$ m filter and diluted to 0.4 mg/ml as a working solution for the preparation of chitosan-S3a-phMGFP nanoparticles [19, 20]. RPS3a-phMGFP at 607  $\mu$ g/ml in PBS at pH 6.9 was added to the chi-

tosan working solution, which was then rapidly vortexed for 10 s and incubated at room temperature for 1 h before use. The chitosan-phMGFP nanoparticles were prepared using the same method. A quality check of the nanoparticles was performed by observing the migration of the nanoparticles by electrophoresis on a 0.8% agarose gel.

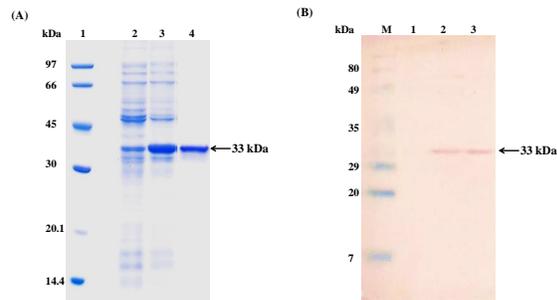
The chitosan-DNA nanoparticles were added to mashed commercial shrimp feed to prepare the experimental feed. Deionized water was added, and the sample was mixed until it exhibited a paste-like texture. The mixture was pressed through a sterile syringe and air-dried on a plastic sheet. The dried feed was flaked and stored at 4 °C until use.

### Effect of RPS3a nanoparticles on the *PAP* and *proPO* genes determined by RT-PCR

The shrimp were divided into 7 groups of three shrimp. Group 1 received feed mixed with PBS, and Groups 2–4 received feed mixed with chitosan-phMGFP nanoparticles at concentrations of 25, 50, and 75 µg/shrimp/day. Groups 5–7 received feed mixed with chitosan-S3a-phMGFP nanoparticles at concentrations of 25, 50, and 75 µg/shrimp/day. After feeding for 7 days, hemolymph was collected for total RNA extraction using TRIzol reagent according to the manufacturer's instructions (Invitrogen, USA). Then, 4 µg of the extracted RNA was reverse-transcribed into cDNA. The total reaction mixture of 25 µl containing 2 µl of random primers (100 ng/µl), 0.6 mM dNTP mix, 1.0 µl of AMV, and 5 U/µl reverse transcriptase (Promega, USA) was incubated at 48 °C. Then, 160 ng of cDNA was used to amplify the *PAP* and *proPO* genes, and the expression of the  $\beta$ -actin gene was used as an internal standard. Table S1 lists the primer pairs used to amplify the *PAP*, *proPO*, and  $\beta$ -actin genes.

### Effect of His-RPS3a on the protection of *L. vannamei* against WSSV

The shrimp were divided into three groups of 15 shrimp (the experiment was performed in triplicate,  $n = 3$ ). The shrimp in the first group received feed mixed with PBS. The second group was immunized with chitosan-phMGFP nanoparticles (75 µg/shrimp/day). The third group was immunized with chitosan-S3a-phMGFP nanoparticles (75 µg/shrimp/day). After feeding for 7 days, the shrimp were challenged with WSSV. The mortality rate in the shrimp groups was recorded 15 days after infection, and the relative percentage of survival (RPS) was calculated.



**Fig. 1** 12% SDS-PAGE gel. (A) Coomassie brilliant blue staining for evaluating the expression and purification of His-RPS3a. Lane 1: protein marker, lane 2: non-induced with IPTG, lane 3: induced with IPTG and lane 4: purified His-RPS3a. (B) Western blot analysis of His-RPS3a. Lane 1: protein marker, lane 2: non-induced with IPTG, lane 3: induced with IPTG and lane 4: purified His-RPS3a.

## RESULTS

### Expression and purification of the fusion protein

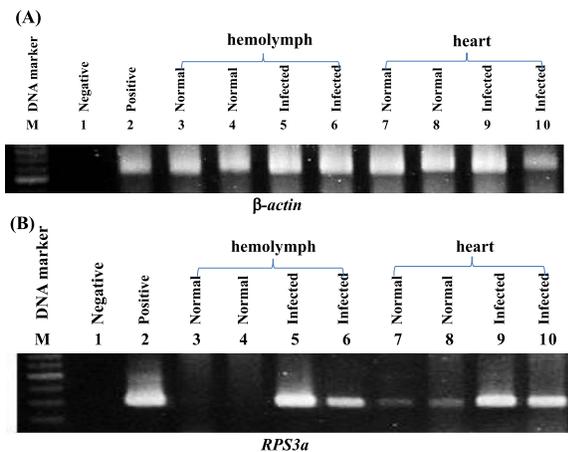
RPS3a was expressed as the His-RPS3a fusion protein and was primarily found in the soluble fraction. The molecular weight of His-RPS3a was approximately 33 kDa. The fusion protein can be obtained in a relatively pure form by purification with Ni-NTA resin (Fig. 1A) and western blotting (Fig. 1B).

### RPS3a expression in WSSV-infected shrimp

The heart and hemolymph were the target organs of viral infection, and hence the expression of the gene response to the virus was determined. RPS3a was semi-quantified in the heart and hemolymph by RT-PCR in both healthy and infected shrimp (*L. vannamei*). Results showed that the expression of RPS3a was increased in the heart and hemolymph of infected shrimp (Fig. 2).

### Expression of *PAP* and *proPO* in RPS3a protein-immunized shrimp

The activation of the *proPO* and *PAP* genes was detected by real-time PCR in the hemolymph at 48 h postinjection. Results showed that the maximum expression of the *PAP* gene increased by 4.1 times when His-RPS3a was injected at a concentration of 1 µg/shrimp (Fig. 3A), whereas a concentration >1 µg/shrimp inhibited the expression of *PAP*. The *proPO* gene expression was increased at a His-RPS3a concentration of >1 µg/shrimp. In addition, the *proPO* gene expression was increased by 5.3 times when the shrimp were injected with His-RPS3a at



**Fig. 2** Expression of the RPS3a gene from WSSV-infected *L. vannamei*. Lane M: 100-bp DNA marker, lane 1: negative control, lane 2: positive control, lanes 3–4, 5–6: RT-PCR products of the (A)  $\beta$ -actin (internal control) and (B) RPS3a genes in the hemolymph of normal and infected shrimp, respectively, lanes 7–8, 9–10: RT-PCR products of the (A)  $\beta$ -actin and (B) RPS3a genes in the heart of normal and infected shrimp, respectively.

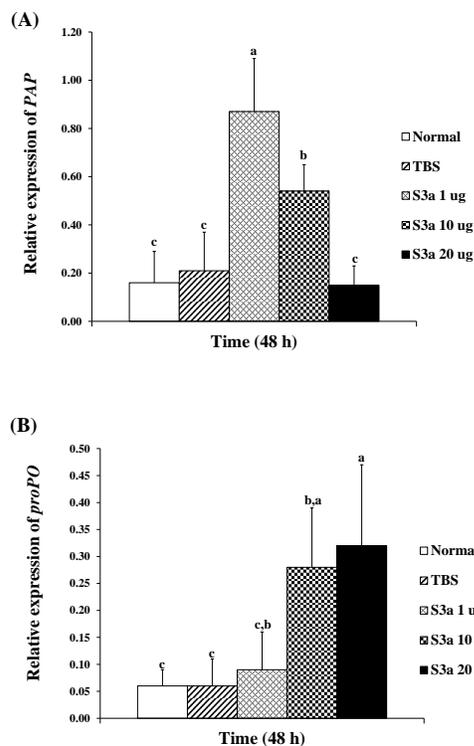
a concentration of 20  $\mu$ g/shrimp (Fig. 3B). These results indicate that His-RPS3a functions as an activator of the immune response in the white shrimp.

**Effect of His-RPS3a on the protection of *L. vannamei* against WSSV**

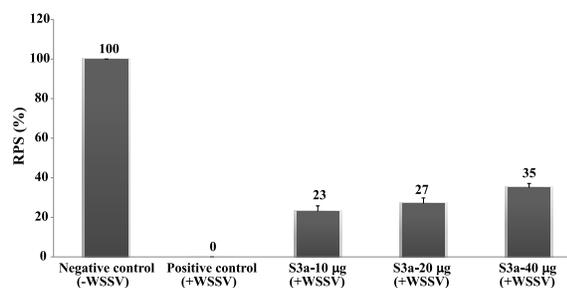
Shrimp were intramuscularly injected with His-RPS3a and then challenged with WSSV. Their mortality rate was recorded for 15 days after infection, and the results demonstrated that the shrimp in the groups injected with RPS3a died slowly after infection in a dose-dependent manner, whereas all the shrimp in the positive control group (challenged with WSSV) died within 11 days. The relative percentage of survival rate (RPS) was calculated according to a previously described formula. The relative percentages of survival (RPS) in the WSSV-challenged shrimp after injection with 10, 20, and 40  $\mu$ g of His-RPS3a protein/shrimp were 23%, 27%, and 35%, respectively (Fig. 4).

**Expression of the *PAP* and *proPO* genes in RPS3a nanoparticle-immunized shrimp**

RT-PCR was performed to detect the activation of the *proPO* and *PAP* genes in the hemolymph on the 3rd and 7th day after a 7-day oral immunization treatment with RPS3a-phMGFP at concentrations of 25, 50, and 75  $\mu$ g/shrimp/day. We observed that

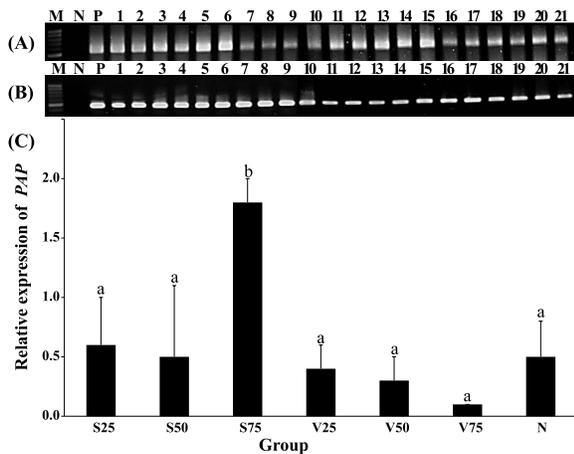


**Fig. 3** Relative expression of (A) *PAP* and (B) *proPO* genes in the hemolymph of *L. vannamei* injected with His-RPS3a.  $\beta$ -actin was used as an internal control ( $n = 5$ ). Different letters denote a significant difference in His-S3a groups compared with control groups using one-way ANOVA ( $p > 0.05$ ).



**Fig. 4** Relative percentage of survival of shrimp at 3 days after intramuscular injection with His-S3a protein. Positive and negative control groups were injected with TBS buffer. After 3 days, the positive control groups and His-RPS3a protein groups were injected with WSSV (diluted in PBS). The negative control groups were injected with PBS.

the *PAP* gene expression was higher in the group



**Fig. 5** Relative expression of (A) the  $\beta$ -actin and (B) *PAP* genes. (C) Ratio of the expression levels of *PAP* in the hemolymph at 7 days postoral immunization with chitosan-RPS3a-phMGFP nanoparticles (S) and chitosan-phMGFP nanoparticles (V). Feed was mixed with PBS for the control group. The  $\beta$ -actin gene was used as the internal control gene ( $n = 3$ ). Letters a and b denote a significant difference in chitosan-RPS3a-phMGFP groups compared with control groups using one-way ANOVA ( $p < 0.05$ ). Lane M: 100-bp DNA marker, lane N: negative control, lane P: positive control, feed mixed with RPS3a (lanes 1–3: 25  $\mu$ g, lanes 4–6: 50  $\mu$ g, lanes 7–9: 75  $\mu$ g), feed mixed with vector (lanes 10–12: 25  $\mu$ g, lanes 13–15: 50  $\mu$ g, lanes 16–18: 75  $\mu$ g), and lanes 19–21: normal feed.

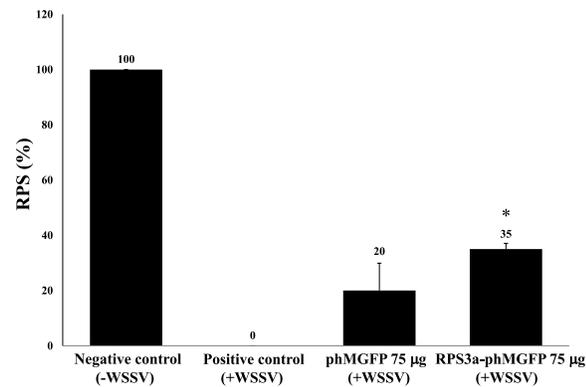
treated with 75  $\mu$ g/shrimp/day than that in the control group (Fig. 5).

#### Effect of RPS3a nanoparticles on the efficiency of protection against WSSV

The shrimp that were fed with feed containing PBS, chitosan-phMGFP nanoparticles, or chitosan-RPS3a-phMGFP nanoparticles for more than 7 days were challenged with WSSV. The relative percentage of survival (RPS) in the WSSV-challenged shrimp after feeding with RPS3a-phMGFP for 7 days was 35% (Fig. 6), indicating that RPS3a could delay the death of infected shrimp.

#### DISCUSSION

The immune responses of shrimp consist of humoral and cellular defenses [21]. Humoral defenses include melanization by activation of the *proPO*-activating system (*proPO* system) and clotting process [21, 22]. In addition,  $\beta$ -glucan, peptidoglycan (PG), and lipopolysaccharides (LPS) have been



**Fig. 6** The relative percentage of survival of shrimp after feeding for 7 days with chitosan RPS3a-phMGFP and chitosan-phMGFP nanoparticles. The positive and negative control groups were fed with normal feed. Then, the positive control and all nanoparticle-fed groups were challenged with WSSV. Significant differences between RPS3a-phMGFP and phMGFP were compared using the *t*-test ( $p < 0.05$ , \*).

found to activate inactive *proPO* from granulocyte to PO. The PO oxidizes phenols into quinones, which kill pathogens and are used for melanin production [22]. Cellular defenses such as phagocytosis are reactions performed by hemocytes [23]. Several studies have reported that the *PAP* gene activates phagocytic activity in shrimp. The *PAP* gene was first isolated from WSSV-infected *P. monodon*. It has been reported that the phagocytosis of shrimp hemocytes was increased after incubation with the GST-PAP protein [24]. Moreover, another study showed that the interaction between *PAP* and  $\alpha$ -2-macroglobulin may increase the entry of *PAP* into phagocytic cells and increase the survival rate of WSSV-infected shrimp [25]. Injection with the *PAP* gene was also found to significantly increase the percentage of phagocytosis and the phagocytic index [26]. In another investigation, oral administration of the chitosan-PAP-phMGFP gene was applied to determine the ability of the gene to induce shrimp immunity [27].

RPS3a was previously isolated from *F. merguensis* (GenBank accession no. HQ844972) [15]. In the present study, His-RPS3a was both injected and fed to activate phagocytosis. We suppose that melanization reaction could have occurred through the expression of the *PAP* and *proPO* genes. In addition, several studies have indicated that chitosan-DNA nanoparticles can protect fish and shrimp such as *Lates calcarifer* [20], *Cyprinus carpio* [28],

*P. monodon* [29], and *L. vannamei* [27] against pathogens. We prepared RPS3a-phMGFP in the form of chitosan-DNA nanoparticles and fed to the shrimp. Administration of RPS3a-phMGFP at 75 mg/shrimp/day resulted in only 35% of shrimp survival after WSSV infection, which was lower than that observed with other immunizations such as PAP and  $\beta$ -glucan [30]. However, it was significant in comparison with phMGFP vaccination. Although RPS3a could enhance the immune system through PAP and *proPO*, it may not be specifically involved in the host defense mechanism against WSSV infection. Recently, RPS3a has been reported to be required for LPS-triggered signaling during the induction of proinflammatory cytokines, including TNF- $\alpha$  and IL-6 [31]. Therefore, the increased RPS3a expression after WSSV infection in the present study may have occurred as it was required for WSSV to induce the production of proinflammatory cytokines.

#### Appendix A. Supplementary data

Supplementary data associated with this article can be found at <http://dx.doi.org/10.2306/scienceasia1513-1874.2020.S006>.

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#### REFERENCES

- Aoki T, Wang HC, Unajak S, Santos MD, Kondo H, Hirono I (2011) Microarray analyses of shrimp immune responses. *Mar Biotechnol* **13**, 629–638.
- Flegel TW (2006) Detection of major penaeid shrimp viruses in Asia, a historical perspective with emphasis on Thailand. *Aquaculture* **258**, 1–33.
- Liu H, Söderhäll K, Jiravanichpaisal P (2009) Antiviral immunity in crustaceans. *Fish Shellfish Immunol* **27**, 79–88.
- Lo CF, Chang YS, Peng SE, Kou GH (2003) Major viral diseases of *Penaeus monodon* in Taiwan. *J Fish Soc Taiwan* **30**, 1–13.
- Chang PS, Chen HC, Wand YC (1998) Detection of white spot syndrome associated baculovirus WSBV in experimentally infected wild shrimps, crabs, and lobsters by *in situ* hybridization. *Aquaculture* **164**, 233–242.
- Sarathi M, Ishaq Ahmed V, Venkatesan C, Balasubramanian G, Prabavathy J, Sahul Hameed AS (2007) Comparative study on immune response of *Fenneropenaeus indicus* to *Vibrio alginolyticus* and white spot syndrome virus. *Aquaculture* **271**, 8–20.
- Wongpanya R, Aoki T, Hirono I, Yasuie M, Tasanakajon A (2007) Analysis of gene expression in haemocytes of shrimp *Penaeus monodon* challenged with white spot syndrome virus by cDNA microarray. *ScienceAsia* **33**, 165–174.
- Lutsch G, Stahl J, Kargel HJ, Noll F, Bielka, H (1990) Immunoelectron microscopic studies on the location of ribosomal proteins on the surface of the 40S ribosomal subunit from rat liver. *Eur J Cell Biol* **51**, 140–150.
- Naora H, Nishida T, Shindo Y, Adachi M, Naora H (1995) Association of nbl gene expression and glucocorticoid-induced apoptosis in mouse thymus *in vivo*. *Immunology* **85**, 63–68.
- Cui K, Coutts M, Stahl J, Sytkowski AJ (2000) Novel interaction between the transcription factor CHOP (GADD153) and the ribosomal protein FTE/S3a modulates erythropoiesis. *J Biol Chem* **275**, 7591–7596.
- Metspalu A, Rebane A, Hoth S, Pooga M, Stahl J, Kruppa J (1992) Human ribosomal protein S3a: cloning of the cDNA and primary structure of the protein. *Gene (Amst)* **119**, 313–316.
- Song D, Sakamoto S, Taniguchi T (2002) Inhibition of poly (ADP-ribose) polymerase activity by Bcl-2 in association with the ribosomal protein S3a. *Biochemistry* **41**, 929–934.
- Song ZW, Xun BX, Ping XJ, Ying YZ, Ying Y, Ling HW, Bing WW (2010) Subcellular localization of *Bombyx mori* ribosomal protein S3a and effect of its over-expression on BmNPV infection. *Afr J Biotech* **9**, 2056–2061.
- Flegel TW (2007) Update on viral accommodation, a model for host-viral interaction in shrimp and other arthropods. *Dev Comp Immunol* **31**, 217–231.
- Navakanitworakul R, Deachamag P, Wonglapsuwan M, Chotigeat W (2012) The role of ribosomal protein S3a in ovarian development of *Fenneropenaeus merguensis* (De man). *Aquaculture* **338–341**, 208–215.
- Zhu F, Du H, Miao ZG, Quan HZ, Xu ZR (2009) Protection of *Procambarus clarkia* against white spot syndrome virus using inactivated WSSV. *Fish Shellfish Immunol* **26**, 685–690.
- Birnboim HC (1983). A rapid alkaline extraction method for the isolation of plasmid DNA. *Methods Enzymol* **100**, 243–255.
- Mao S, Shuai X, Unger F, Simona M, Bi D, Kissel T (2004) The depolymerization of chitosan: effects on physicochemical and biological properties. *Int J Pharm* **281**, 45–54.
- Zheng F, Shi XW, Yang GF, Gong LL, Yuan HY, Cui YJ, et al (2007) Chitosan nanoparticle as gene therapy vector via gastrointestinal mucosa administration: results of an *in vitro* and *in vivo* study. *Life Sci* **80**, 388–396.

20. Kumar SR, Ahmed VPI, Parameswaran V, Sudhakaran R, Babu VS, Hameed ASS (2008) Potential use of chitosan nanoparticles for oral delivery of DNA vaccine in Asian sea bass (*Lates calcarifer*) to protect from *Vibrio (Listonella) anguillarum*. *Fish Shellfish Immunol* **25**, 47–56.
21. Aguirre-Guzmán G, Sánchez-Martínez JG, Campa-Córdova AI, Luna-González A, Ascencio F (2009) Penaeid shrimp immune system. *J Vet Med* **39**, 205–215.
22. Jiravanichpaisal P, Söderhäll K (2000) The *proPO* and clotting system in crustaceans. *Aquaculture* **191**, 53–69.
23. Jiravanichpaisal P, Sricharoen S, Söderhäll I, Söderhäll K (2006) White spot syndrome virus (WSSV) interaction with crayfish haemocytes. *Fish Shellfish Immunol* **20**, 718–727.
24. Deachamag P, Intaraphad U, Phongdara A, Chotigeat W (2006) Expression of a Phagocytosis Activating Protein (*PAP*) gene in immunized black tiger shrimp. *Aquaculture* **255**, 165–172.
25. Chotigeat W, Deachamag P, Phongdara A (2007) Identification of a protein binding to the phagocytosis activating protein (*PAP*) in immunized black tiger shrimp. *Aquaculture* **271**, 112–120.
26. Khimmakthong U, Deachamag P, Phongdara A, Chotigeat W (2011) Stimulating the immune response of *Litopenaeus vannamei* using the phagocytosis activating protein (*PAP*) gene. *Fish Shellfish Immunol* **31**, 415–422.
27. Khimmakthong U, Kongmee P, Deachamag P, Leggat U, Chotigeat W (2013) Activation of an immune response in *Litopenaeus vannamei* by oral immunization with Phagocytosis Activating Protein (*PAP*) DNA. *Fish Shellfish Immunol* **34**, 929–938.
28. Wonglapsuwan M, Kongmee P, Suanyuk N, Chotigeat W (2016) Roles of phagocytosis activating protein (*PAP*) in *Aeromonas hydrophila* infected *Cyprinus carpio*. *Dev Comp Immunol* **59**, 25–33.
29. Rajeshkumar S, Venkatesan C, Sarathi M, Sarathbabu V, Thomas J, Basha KA, et al (2009) Oral delivery of DNA construct using chitosan nanoparticles to protect the shrimp from white spot syndrome virus (WSSV). *Fish Shellfish Immunol* **26**, 429–437.
30. Bai N, Gu M, Zhang W, Xu W, Mai K (2014) Effects of  $\beta$ -glucan derivatives on the immunity of white shrimp *Litopenaeus vannamei* and its resistance against white spot syndrome virus infection. *Aquaculture* **426–427**, 66–73.
31. Li YH, Wang J, Liu Y, Qiu L, Li Q, Li JZ, Hu HG, Hu ZL, et al (2018) Esculentoside A specifically binds to ribosomal protein S3a and impairs LPS-induced signaling in macrophages. *Int Immunopharmacol* **54**, 254–260.

**Appendix A. Supplementary data****Table S1** Primers used for the expression study.

Gene	Primer sequences (5'–3')
<i>β-actin</i>	F: 5'CAG ATC ATG TTY GAG ACC TTC 3'
	R: 5'GAT GTC CAC GTC RCA CTT CAT 3'
<i>RPS3a</i>	F: 5'AAG ATG GTG GAC ATC ATC ACC C 3'
	R: 5'TTA GAC ACT GGC TTG AAC TGG AGG 3'
<i>PAP</i>	F: 5'CAA TGT CCG TGC CAT GC 3'
	R: 5'CCG ACC AGC AGC TTT GTT 3'
<i>proPO</i>	F: 5'GTA CTG GCG GGA GGA CTA 3'
	R: 5'CCG TTG CGA TCG ACC ATG 3'