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Recombinant VP28 Produced by Cell-Free Technique Confers Protection in Kuruma Shrimp (*Marsupenaeus japonicus*) Against White Spot Syndrome Virus

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Abstract

The protective efficacy of oral delivery and intramuscular (i.m.) injection of a recombinant protein, rVP28 derived from white spot syndrome virus (WSSV) and synthesized using wheat germ cell-free technology was investigated in kuruma shrimp (*Marsupenaeus japonicus*). Shrimps were administered with the unpurified rVP28 and challenged with WSSV. Expression of innate immune-related genes was examined in intestine, heart, and lymphoid organ at 1, 3 and 7 days after oral-administration. Shrimps received rVP28 protein through i.m. injection or oral route and then challenged with WSSV displayed higher survival (90 and 85.7%, respectively) compared to the respective control groups. A significant up-regulation ($P<0.01$) of innate immune-related genes, such as Rab7, penaeidin, lysozyme and crustin, was noticed in orally treated shrimps. Our results indicate that this recombinant protein could elevate immune responses and protection in kuruma shrimp and therefore would have potential utility against WSSV infection in shrimp aquaculture.

Keywords: *Marsupenaeus japonicus*, WSSV, VP28, Innate immune genes, cell-free technique.

Introduction

Shrimp is one of the most important species in aquaculture. During the last two decades, shrimp culture has been threatened worldwide by viral diseases. A highly pathogenic white spot syndrome virus (WSSV) is responsible for huge economic losses in the shrimp culture industry, and no effective treatment is currently available for it (Rajesh Kumar *et al.*, 2008). Cumulative mortalities in infected population may reach 100% within 2-10 days of onset of clinical signs (Lightner, 1996). WSSV infects penaeid shrimps, such as Chinese shrimp (*Fenneropenaeus chinensis*), kuruma shrimp (*Marsupenaeus japonicus*), black tiger shrimp (*Penaeus monodon*) and other crustaceans, including crayfishes, crabs and lobsters (Otta *et al.*, 1999; Supamattaya *et al.*, 1998). Due to the extreme virulence and wide host range including almost all crustaceans (Sahul Hameed *et al.*, 2003), it is difficult to contain the spread of this virus.

Invertebrates lack true adaptive immune system and depend on various innate immune responses (Kimbrell and Beutler, 2001). Innate immune responses are considered to be less sophisticated than adaptive immunity, however, they may rapidly and efficiently recognize and destroy non-self materials including pathogens (Lee and Söderhäll, 2002). Innate immune responses consist of cellular and humoral components. Hemocytes are responsible for most of the cellular responses, such as encapsulation, phagocytosis, melanization, cytotoxicity, cell-to-cell communication, clotting, and the pro-phenoloxidase activating system. Humoral response factors originate from granulocytes and include lectins, defensive enzymes, reactive oxygen intermediates, and the synthesis of a wide array of antimicrobial peptides (Destoumieux *et al.*, 2000; Kimbrell and Beutler, 2001; Lee and Söderhäll, 2002; Söderhäll, 1999). Treatment with microbial products like β-glucan or *Vibrio harveyi* vaccine stimulates the immune response in shrimp (Alabi *et al.*, 1999; Song and Hsieh, 1994). Moreover, vaccination using inactivated *Vibrio* spp. was shown to be effective against vibriosis in *P. monodon* and *M. japonicus* (Itami *et al.*, 1989; Teunissen *et al.*, 1998). Activation of innate immune responses primarily occurs with recognition of distinct pathogen-associated molecular patterns (PAMPs) by toll-like receptors (TLRs) (Medzhitov and Janeway, 1997). Additionally, TLRs are also activated by recognition of ‘‘self-antigens” such as heat shock proteins and uric acid that are normally present in cells (Akira *et al.*, 2001; Akira, 2003; Shi *et al.*, 2003). TLRs engage a variety of intracellular

adaptor molecules, leading to signal transduction events that regulate the expression of immune genes. Although the invertebrate immune system has been well studied in the context of antibacterial and antifungal responses, there is less information on cellular and molecular responses to viral pathogens (Robalino *et al.*, 2004).

To date, more than 40 structural proteins of WSSV have been discovered by genomic and proteomic methods (Sritunyalucksana *et al.*, 2012; Tsai *et al.*, 2004 and 2006). VP28, the most abundant exposed protein in the WSSV envelope, is considered as a major factor in systemic infections (van Hulten *et al.*, 2001). Furthermore, VP28 binds to the surface of shrimp cells (Yi *et al.*, 2004). In some researches, the use of recombinant VP28 proteins expressed in different host systems exhibited notable effects against WSSV (Du *et al.*, 2006; Jha *et al.*, 2006; Witteveldt *et al.*, 2004a), indicating their efficacy as promising vaccine candidates. A recent study on a DNA vaccine targeting VP28 suggested that continual expression of viral protein *in situ* can lead to sustained protection against WSSV infection. This group of vaccines is only effective when they are introduced into cells (Rout *et al.*, 2007).

Cell-free protein expression is a rapid and high-throughput technology used to express proteins from the genes of interest by involving cell-free lysate from an *Escherichia coli* cell, rabbit reticulocytes, or wheat germ as the protein translation machinery. Cell-free systems have been used extensively for studies on translational control and protein production (Laxminarayana *et al.*, 2002; Szamecz *et al.*, 2008) at mg per mL levels (Hillebrecht and Chong, 2008). It has several applications including proteomics, protein folding and amino acid functional studies. Added advantages of this technique include speedy and accurate synthesis of large purified proteins without contamination, *in vitro* evolution of proteins with specific biological properties, and incorporation of naturally modified amino acids into specific positions of a protein (Lamla *et al.*, 2002).

The objectives of the present study were to produce a recombinant WSSV envelope protein, VP28 using a cell-free technique, and elucidate the protection and immune responses in the recombinant V28 administered kuruma shrimps challenged with WSSV.

Materials and Methods

Experimental Shrimps

Healthy kuruma shrimps (mean body weight: 10 ± 1 g) without any overt disease symptoms were obtained from Matsumoto Fisheries (Miyazaki, Japan). Shrimps were maintained in an indoor system at 20°C with running artificial seawater and fed a commercial diet once daily. For checking of health status, randomly sampled shrimps were screened by polymerase chain reaction (PCR) using WSSV VP28 primers (Table 1). In addition to this, presence of any bacterial pathogens in shrimps was checked by culturing hemolymph and hepatopancreas smears on Marine Agar 2216 (Difco, Detroit, Michigan, USA) plates. The results showed existence of no pathogenic bacteria in shrimps.

Synthesis of Recombinant WSSV VP28 Protein Using Wheat Germ Extract

Recombinant VP28 (rVP28) was synthesized using the ENDEXT Wheat Germ Expression H Kit (CellFree Sciences, Ehime, Japan). The WSSV VP28 gene was amplified by PCR using the pEU-VP28 Fw and pEU-VP28 Rv primers (Table 1), which contain restriction enzyme sites for *Bam*HI and *Spe*I, respectively. PCR cycling conditions were: 1 cycle of 94°C for 3 min, 30 cycles of 94°C for 30 s, 55°C for 30 s and 72°C for 1 min, followed by 72°C for 5 min.

Table 1. Primers designed for construction of DNA vaccine and semi-quantitative gene expression analysis in this study.

Name	Sequence (5'→3')	Length (mer)	Accession No.	Purpose
pEU-VP28 Fw	GGATCCATGGATCTTTCTTTCAC	23	AJ551447	Synthesis of recombinant protein
pEU-VP28 Rv	ACTAGTTTACTCGGTCTCAGTGC	23		
WSSV VP28 Fw	TGGATCAGGCTACTTCAAGAT	21	AJ551447	WSSV screening
WSSV VP28 Rv	AAAGGTGGTACCACACACAAA	21		
Mj lysozyme Fw	TCCTAATCTAGTCTGCAGGGA	21	AB080238	Gene expression analysis
Mj lysozyme Rv	CTAGAATGGGTAGATGGA	18		
Mj penaeidin2 Fw	GCTGCACCCACTATAGTCTTT	21	AU175636	Gene expression analysis
Mj penaeidin2 Rv	CTACCATGGTGATGAAACAAA	21		
Mj Rab7 Fw	CTCGCAAGAAGATTCTCCTG	20	AB379643	Gene expression analysis
Mj Rab7 Rv	CTTCGTTGATACCGCCCTAT	20		
Mj crustin Fw	CACCTTCAGGGACCTTGAA	19	AB121740	Gene expression analysis
Mj crustin Rv	GTAGTCGTTGGAGCAGGTTA	20		
Mj β-actin Fw	ATGACACAGATCATGTTCGA	20	AB055975	Gene expression analysis
Mj β-actin Rv	GTAGCACAGCTTCTCCTTGA	20		

Fw= Forward; Rv= Reverse

The resulting PCR products were ligated into the pGEM-T Easy vector (Promega, Madison, WI, USA). Following transfection into *E. coli* TAM competent cells (Active Motif, Carlsbad, CA, USA), recombinants were identified through red-white color selection after growing on MacConkey agar (Sigma-Aldrich, St. Louis, MO, USA). Plasmid DNA samples were recovered from more than three clones using a plasmid mini-prep kit (Qiagen, Valencia, CA, USA) and sequenced using an ABI 377 Automated Sequencer (Applied Biosystems, Foster City, CA, USA). Sequenced plasmid DNA was digested with restriction enzymes, *Bam*HI and *Spe*I for 2 h at 37°C and ligated into the pEU-GST-E01-MCS vector (CellFree Sciences). Following transformation into *E. coli* TAM competent cells (Active Motif), recombinant plasmid with pEU-GST-VP28 and empty plasmid with pEU-GST construct (control) were identified *via* red-white color selection after growing on MacConkey agar (Sigma-Aldrich). Plasmid DNA was then purified using the QIAprep Spin miniprep kit (Qiagen). For *in vitro* transcription, 25 µL purified plasmid DNA (1.0 µg μL^{-1}) was added to 225 µL pre-mixed transcription buffer containing SP6 RNA polymerase (80 U μL^{-1}), 3.125 µL RNase inhibitor (80 U μL^{-1}), 25 µL of 25 mM dNTP mix, 50 µL of 5× transcription buffer and 143.75 µL nuclease free water; these reaction mixtures were gently mixed and incubated at 37°C for 6 h using a thermal cycler. The quality of the mRNA resulting from this transcription reaction was confirmed by agarose gel electrophoresis. Translation of mRNA using wheat germ extract was then conducted following the manufacturer's protocol (Cellfree Sciences). The two recombinant proteins, GST-VP28 and GST, were separately suspended in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) loading buffer (Bio-Rad Laboratories, Hercules, CA, USA), heated for 5 min at 100°C and loaded onto a 15% precast e-PAGEL gel (ATTO, Tokyo, Japan). After electrophoresis, the gel was stained with Coomassie G250 stain (Bio-Rad Laboratories).

Western Blot Analysis

Western blotting was performed using the Western breeze Chemiluminescent Immunodetection System (Invitrogen, San Diego, CA, USA) according to the manufacturer's instructions. Briefly, proteins were separated on a 15% polyacrylamide gel and transferred onto a nitrocellulose membrane (Invitrogen). The membrane was blocked with the Western breeze blocking solution for 30 min on a slow speed rotary shaker. After rinsing with water, the membrane was incubated with a primary antibody, rabbit anti-WSSV VP28 antibody (supplied by Takara Bio Inc., Tokyo, Japan) for 1 h and rinsed again with antibody washing solution and water. The washed membranes were incubated in secondary anti-rabbit-Ig/AP antibody solution (1:2000; Invitrogen) for 30 min. The membranes were again washed extensively, and then incubated in chromogenic substrate solution (BCIP/NBT) until purple bands developed.

Preparation of VP28 Containing Diet for Oral Treatment

Feed pellets were produced by Dr. Minoru Maeda (Kyushu Medical Co., Ltd., Fukuoka, Japan). The main protein sources (krill meal and squid meal) were finely ground and then mineral and vitamin premix were added. After all the ingredients were mixed, an adequate quantity of water (30% for 100 g of mixed ingredients), pollack liver oil and unpurified recombinant VP28 dissolved in phosphate-buffered saline (PBS) were added. The dough was placed in a 50-mL syringe and slowly pressed to form feed pellets. The moist pellets were placed in a dark room at 4°C and allowed to dry. The dried pellets were mixed with 3% of spreader to prevent dispersion of the feed particles into water. The amount of recombinant protein consumed from the diet was calculated based on the average body weight of the shrimp, feeding rate (5%) and feeding frequency.

Administration of VP28 Recombinant Protein and Artificial WSSV Challenge

VP28Rrecombinant Protein Injection and Oral Treatment

In the first experiment, groups of 25 shrimps were injected intramuscularly (i.m.) with 5 µg of wheat-GST-rVP28 (total protein including wheat protein; 701 µg) dissolved in 100 µL PBS. Shrimps in three control groups were injected with 100 µL PBS, wheat germ protein (total protein 701 µg), or wheat germ protein including GST (total protein 624 µg); all protein samples were dissolved in 100 µL PBS. In the second experiment, groups of 25 shrimps were fed with feed pellets containing rGST-VP28, feed pellets with wheat germ protein, or commercial diet (control) for 7 days (twice a day: morning and evening).

WSSV Challenge, Post Challenge Conditions and Recording Mortality

An artificial challenge with WSSV was carried out by immersion described previously by Kono *et al.* (2009) after 7 days post-injection. Heart and hepatopancreas were isolated under sterile conditions from WSSV-infected shrimps. Tissues were homogenized with PBS. DNA was extracted from homogenates using DNeasy tissue kit (Qiagen), according to the manufacturer's instructions. The copy numbers of WSSV-challenged stock were determined by quantitative real-time PCR (Kono *et al.*, 2009). Three groups of shrimps (n=25 per group) were immersed in 4 L of artificial sea water containing 5 mL of homogenate (1×10^{10} copies mL^{-1})

for 2 h at 20°C. After 7-days of feeding treatment, shrimps were also challenged by an immersion in WSSV-containing seawater as described. Dead shrimps were tested for virus infection by PCR, and it was confirmed that they were infected with WSSV. Mortality in injected and fed shrimps was recorded for 25 and 20 days post-challenge period, respectively. The relative percentage survival (RPS) in challenged shrimps was calculated according to Amend (1981).

Sampling of Organs from Orally Treated Shrimps

For expression analyses of innate immune-related genes, 10 shrimps were used in each group and all treatments had triplicates. Intestines, lymphoid organs and hearts from three individual shrimps in each group were collected at 1, 3 and 7 days after oral administration, and preserved for biochemical assay and reverse transcription-PCR (RT-PCR).

Total RNA Isolation and cDNA Synthesis

Total RNA was extracted from the intestine, lymphoid organ and heart of kuruma shrimps using ISOGEN (Nippon Gene, Osaka, Japan) in accordance with the manufacturer's instructions. The amount of nucleic acid in each total RNA sample was determined by measuring the absorbance at 260 nm using a NanoDrop spectrophotometer, ND-1000 (Thermo Scientific, Wilmington, DE, USA). The purity of each RNA sample was assessed by calculating the ratio of O.D. at 260 nm/O.D. at 280 nm. cDNA was synthesized from 1.0 µg of total RNA using a ReverTra Ace qPCR RT kit (Toyobo, Osaka, Japan) following the manufacturer's instructions and the resulting cDNA samples were used as templates for PCR.

Expression Analysis of Innate Immune-related Genes by Semi-quantitative PCR

PCR was conducted using the synthesized cDNA from RNA samples of intestine, heart and lymphoid organ with primer combinations shown in Table 1. To assess gene expression semi-quantitatively, kuruma shrimp innate immune-related genes and β-actin gene (as internal control) were amplified in a defined number of PCR cycles (25-35) using conditions as follows: 1 cycle of 94°C for 3 min, 25 or 35 cycles of 94°C for 30 s, 55°C for 30 s and 72°C for 45 s, followed by 72°C for 5 min. After amplification of a specific gene, the ratio of innate immune-related (35 cycles) signal to β-actin (25 cycles) signal was determined by densitometry using Science Lab99 Image Gauge software (Fujifilm, Tokyo, Japan). The expression analysis was conducted in triplicate. Assessment of statistical significance was made by one-way ANOVA, followed by a Tukey's test.

Results

Expression and Synthesis of Recombinant VP28 Protein

The VP28 gene was continuously expressed during the exponential and stationary growth phases after 18 h of incubation. In SDS-PAGE analysis of the culture supernatant samples, there was a common distinct band in lane 2, 3, 4 and 5 (Figure 1A). Therefore, the recombinant GST-VP28 protein was difficult to distinguish. However, the band indicated was further confirmed by Western blotting with anti-WSSV VP28 rabbit polyclonal antibody. Finally, the recombinant GST-VP28 was identified at a position equivalent to a molecular mass of 53 kDa (Figure 1B), whereas rGST (wheat control) was found to be 25 kDa.

Protective Efficacy of the Recombinant VP28 Protein

Protective efficacy of rVP28 was examined by challenging the injected and orally administered shrimp groups to WSSV. In the injected shrimps at 25

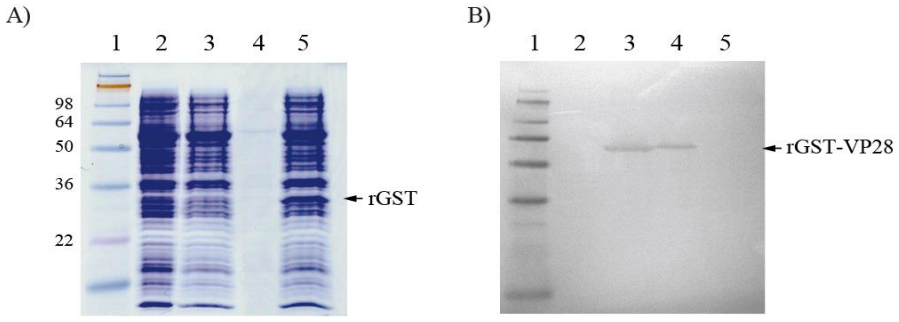

Figure 1. Expression of recombinant GST-VP28 in wheat germ extract. The translation products were separated on 15% SDS-PAGE reducing gels and visualized by Coomassie blue staining (A). Western blot analysis was conducted using primary antibody against WSSV VP28 (B). Lane 1, molecular weight (kDa) standard; lane 2, total wheat germ protein; lane 3, rGST-VP28 expressed in wheat germ extract; lane 4, precipitate after centrifugation of translation product; lane 5, rGST expressed in wheat germ extract.

days post-challenge, control groups injected with only wheat germ protein or PBS showed a survival of 45 and 20%, respectively. Vaccination with rGST-VP28 resulted in a significantly higher (P<0.01) RPS (94%) compared with the control groups (Figure 2). In the orally administered shrimps at 20 days post-challenge, positive effect of feeding rGST-VP28 was found, showing the RPS of 100% compared with commercial diet fed group (control) (Figure 3). The group fed with diet containing wheat germ had 26.3% survival. Randomly selected survivors from all groups were tested for WSSV using PCR and found negative. Therefore, a substantial increase in protection against WSSV was observed in the rVP28 injected and fed shrimps.

Innate Immune-related Gene Expressions in Shrimps

Expression profiles of four immune genes were examined in the lymphoid organ, heart and intestine of rGST-VP28 fed shrimps at 1, 3 and 7 days post-feeding. Antimicrobial peptide (AMP) and Rab7 mRNA transcripts in the lymphoid organ and heart were significantly higher (P<0.01) in treated shrimp than in commercial diet fed shrimp (control) at all-time points after administration (Figure 4A). In addition, no change in the expression level of Rab7 gene was observed in the intestine (Figure 4A). The expression of lysozyme and penaeidin genes in the

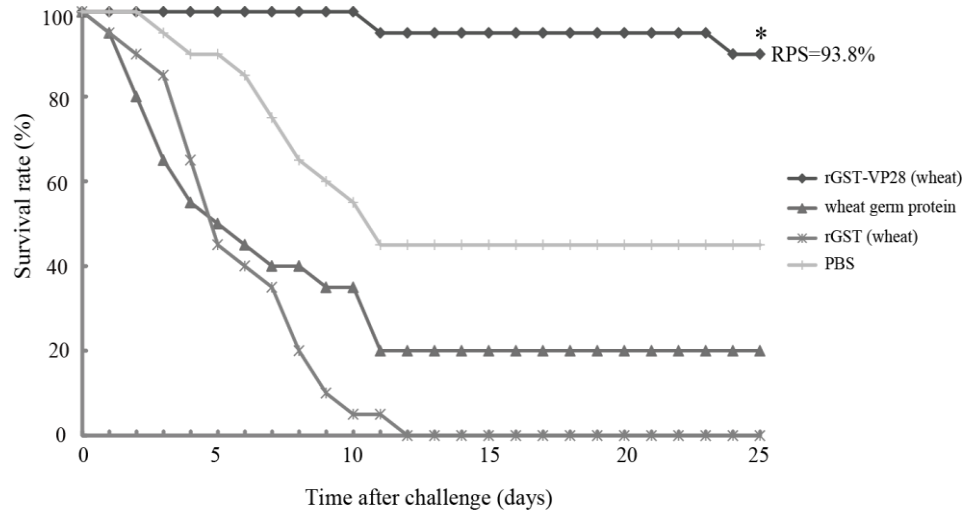

Figure 2. Survival (%) of shrimp from the experimental or control groups injected with rGST-VP28, wheat germ protein, rGST or PBS are plotted against the time after WSSV challenge. Asterisk indicates the significant difference (P<0.01) from the wheat germ protein and PBS injected groups. RPS; relative percentage survival.

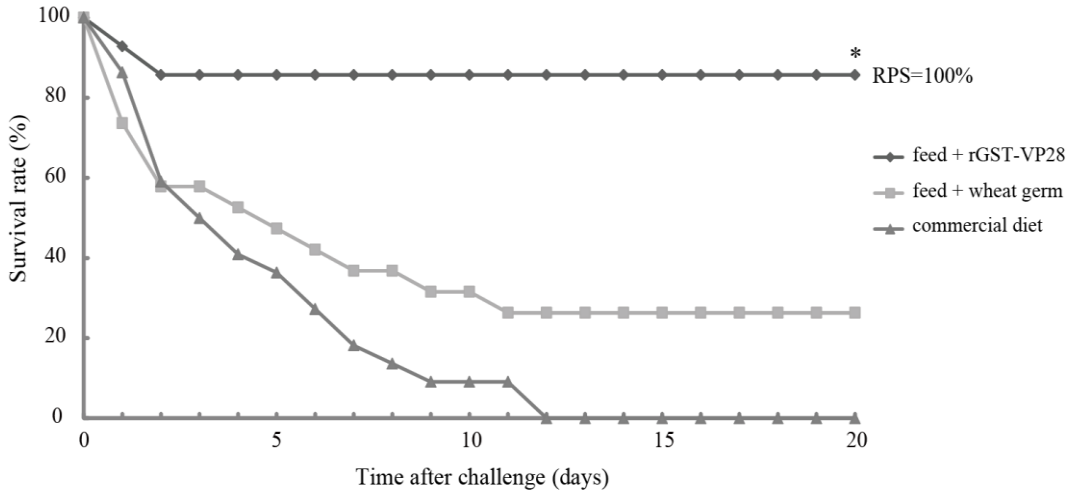

Figure 3. Survival (%) of shrimp from the experimental or control groups fed with diet containing rGST-VP28, wheat germ without rGST-VP28 and commercial diet. Asterisk indicates the significant difference (P<0.01) from wheat germ without rGST-VP28 and commercial diet fed groups. RPS; relative percentage survival.

lymphoid organ, heart and intestine was significantly increased (P<0.01) in orally treated shrimp than in control group (Figure 4B and C). However, crustin transcript level was only significantly elevated (P<0.01) in the intestine of rGST-VP28 fed shrimp at 3 day post-feeding (Figure 4C).

Discussion

Survivors of either natural or experimental WSSV infections exhibited a quasi-immune response upon re-challenge (Venegas *et al*., 2000; Wu *et al.*, 2002). Previous experiments have indicated that VP28 plays an important role in systemic WSSV infections in shrimp and it is possible to neutralize WSSV with anti-VP28 antibodies (van Hulten *et al.*, 2001). VP28-mediated protection against WSSV is maintained up to 3 weeks after vaccination; therefore, it is likely that residual VP28 stops WSSV infection by blocking acceptors needed by the virus to enter shrimp cells (Witteveldt *et al.*, 2004b). Here, we produced a recombinant envelope protein, the VP28 WSSV in a novel wheat germ cell-free system for oral administration to kuruma shrimp. Moreover, the delivery of the unpurified recombinant protein through i.m. injection conferred protection in shrimp.

Witteveldt *et al*. (2004a) injected shrimps with purified WSSV proteins (VP28 and VP29) and challenged the animals at 2 and 25 days post injection to assess the onset and duration of protection. Their results showed a significantly higher survival in shrimps immunized with the maltose binding protein (MBP)-VP28 fusion protein compared with the MBP alone (RPS of 44%, 2 days post injection). In contrast, our study revealed that i.m. injection of shrimp with unpurified rVP28 protein was effective against WSSV infection (RPS 93.8%). Therefore, this unpurified WSSV structural protein (rVP28) as an immune-enhancer conferred the protection. Moreover, unpurified rVP28 protein produced in wheat germ cell-free extract was safe and effective. However, the results also showed that injection of wheat germ protein or rGST alone caused lower survival than that of PBS-injection. This may be attributed to the higher stress exerted by rGST in shrimp, whereas the unpurified rGST-VP28 potentially contained this component at a very low amount which was unable to mount any negative effects on shrimp survival. As delivery of an immunostimulant through injection is neither a sustainable nor a practical strategy in shrimp aquaculture, further oral administration experiments would confirm whether the immune-enhancing

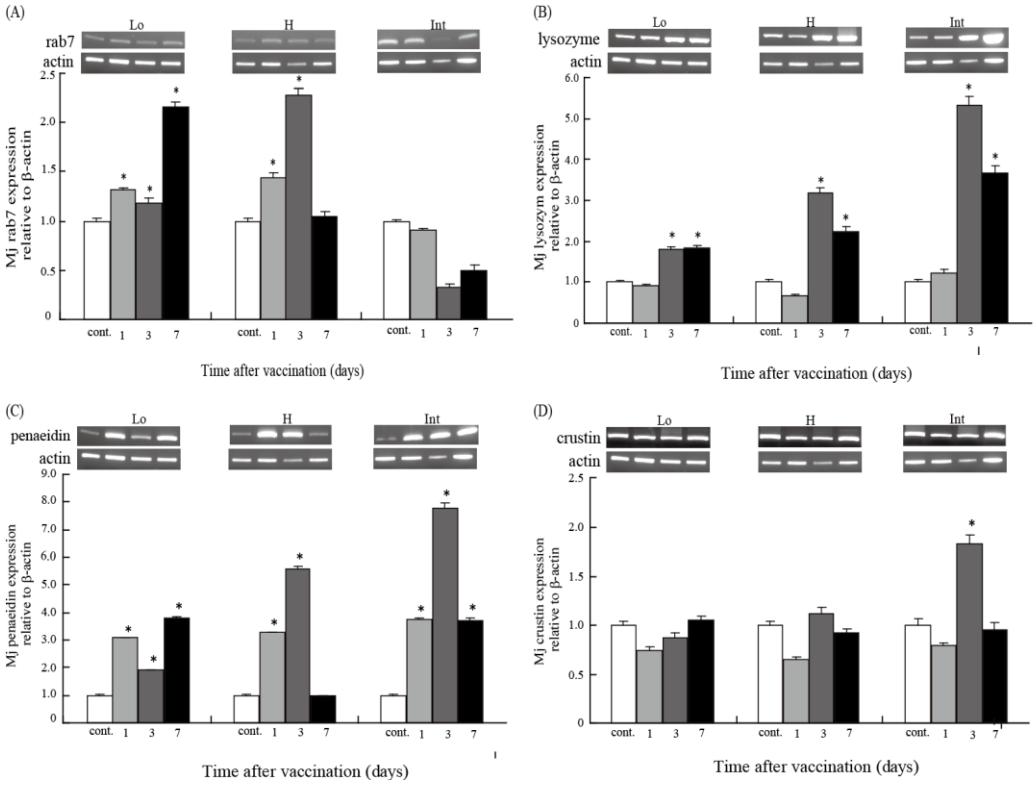

Figure 4. Effects of oral vaccination on the expression of kuruma shrimp (*M. japonicus*: Mj) innate immune-related genes in lymphoid organ (Lo), heart (H) and intestine (Int) at 1, 3 and 7 days post-vaccination. Semi-quantitative reverse transcription-PCR of innate immune-related genes was performed with mRNA extracted from lymphoid organ (Lo), heart (H) and intestine (Int) of vaccinated shrimp. Data represent innate immune-related gene PCR products after normalization against β-actin gene product. Data are presented as mean ± S.D. of triplicate samples. Asterisks indicate the significant difference (P<0.01) compared to the commercial diet fed group (cont.).

potential observed in injection study works similarly. Witteveldt *et al.* (2004b) investigated the potential of oral vaccination in *P. monodon* with subunit vaccines consisting of WSSV virion envelope proteins. Their results demonstrated that vaccination with VP28 resulted in significantly lower cumulative mortality after immersion challenge than vaccination with bacteria expressing the empty vectors; moreover, no protection was observed in vaccination with VP19. However, this protection was not long lasting with higher survival observed at 3 and 7 days post-oral vaccination (RPS, 64% and 77%, respectively). These results are in accordance with those of our study, which showed an increase in the protective immunity by immunization with the rVP28 against WSSV until 7 days post-administration in kuruma shrimp. Similarly, an effective oral application of VP28 protein was also demonstrated in *Litopenaeus vannamei* (Witteveldt *et al.*, 2006). Recently, an rVP28-bv coated on feed pellets conferred a slightly positive effect against WSSV to crayfish (Fu *et al.*, 2008). As administration of recombinant proteins orally presents substantial technical or theoretical challenges, oral delivery of DNA vaccine vectors would appear to be more complex (Johnson *et al.*, 2008). Similarly, the present work showed that the oral delivery of rVP28 protein resulted in significant protection against WSSV in the treated groups compared to the control groups (7 days post administration). In oral delivery of rVP28, shrimps were apparently healthy and exhibited higher survival, whereas lower or no survival with WSSV symptoms was noticed in wheat germ treated or control diet fed groups. Therefore, the robustness and ability of wheat germ lysate system for expression and synthesis of functionally active exogenous proteins are promising.

Expression analyses of antimicrobial peptide genes after oral immunization confirmed the protection provided against WSSV infection. Rab7 is a small GTPase protein of the Rab family involved in endocytosis pathway and virus entry into host cells and was shown to confer inhibition to WSSV and yellow head virus (YHV) replication in shrimp (Attasart *et al.*, 2009). In this study, an elevated expression of Rab7 gene in the lymphoid organ and heart in treated shrimp was consistent with a previous report that showed an increased protection against WSSV infection following injection of a recombinant Rab7 protein (Sritunyalucksana *et al.*, 2006). Recently, it was reported that intestinal expression of Rab7 gene was significantly higher in immunized shrimp than in control shrimp at all the time periods post treatment (Kono *et al.*, 2009). A Rab GTPase, MjRab, found lately in *M. japonicus* (Mj) plays a pivotal role in resistance to WSSV by forming a complex between the envelope protein VP466, β-actin and tropomycin, which increases phagocytosis (Fitzgerald-Bocarsly and Feng, 2007). MjRab was up-regulated during viral infection and silencing of this gene increased viral load following infection, directing the authors to postulate that it may serve as an intracellular viral recognition factor leading to a complex formation and increased phagocytosis (Fitzgerald-Bocarsly and Feng, 2007). The diversity, wide distribution and abundance of AMPs within penaeid shrimps and their multiple and complementary properties indicate that they have an essential role in the defense against various pathogens (Cuthbertson *et al.*, 2008; Destoumieux *et al.*, 1997; O'Leary and Gross, 2006). In this study, there was an increase in AMP gene expression following the oral delivery of rVP28. Expression of lysozyme and penaeidin genes was significantly increased in the intestine, heart and lymphoid organ after administration as compared with control. The expression level of crustin gene in the intestine was also significantly higher at 3 days post-administration. These results indicated that this unpurified rVP28 would increase immune responses and may become a tool to combat WSSV infections in kuruma shrimp. Consistent to our results, penaeidin, lysozyme and crustin gene expression was up-regulated upon DNA vaccination (Kono *et al.*, 2009).

In conclusion, our findings indicate that the shrimp immune system is able to recognize VP28 protein and may be stimulated with its administration. Moreover, the unpurified recombinant protein produced using cell-free technology was effective as an immune-enhancer when administered either orally or through injection. In addition, this method of recombinant protein synthesis is convenient, as it does not require purification. Further studies are needed to optimize this immunostimulant for practical application to control WSSV for the benefit of shrimp farming sector. For this recombinant protein against WSSV in kuruma shrimp, the dosage, duration and application time need further optimization to achieve the best protective efficacy in practical applications.

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