1 **Anti-lipopolysaccharide factor isoform 3 from *Penaeus monodon* (ALFPm3) exhibits**
2 **antiviral activity by interacting with WSSV structural proteins**

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1 **Abstract**

2 In innate immunity, antimicrobial peptides (AMPs) play a vital role in combating
3 microbial pathogens. Among the AMPs identified in *Penaeus monodon*, only anti-
4 lipopolysaccharide factor isoform 3 (ALF*Pm*3) has been reported to exhibit activity against
5 white spot syndrome virus (WSSV). However, the mechanism(s) involved are still not clear.
6 In the present study, ALF*Pm*3-interacting proteins were screened for from a WSSV library
7 using the yeast two-hybrid screening system, revealing the five potential ALF*Pm*3-interacting
8 proteins of WSSV186, WSSV189, WSSV395, WSSV458 and WSSV471. Temporal
9 transcriptional analysis in WSSV-infected *P. monodon* revealed that all five of these WSSV
10 gene transcripts were expressed in the late phase of infection (24 h and 48 h post-infection).
11 Of these, WSSV189 that was previously identified as a structural protein, was selected for
12 further analysis and was shown to be an enveloped protein by Western blot and
13 immunoelectron microscopy analyses. The *in vitro* pull-down assay using recombinant
14 WSSV189 (rWSSV189) protein as bait confirmed the interaction between ALF*Pm*3 and
15 WSSV189 proteins. Moreover, pre-incubation of rWSSV189 protein with rALF*Pm*3 protein
16 interfered with the latter's neutralization effect on WSSV *in vivo*, as shown by the increased
17 cumulative mortality of shrimp injected with WSSV following prior treatment with pre-
18 incubated rWSSV189 and rALF*Pm*3 proteins compared to that in shrimp pre-treated with
19 rALF*Pm*3 protein. Thus, ALF*Pm*3 likely performs its anti-WSSV action by binding to the
20 envelope protein WSSV189 and possibly other WSSV structural proteins.

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24 **Keywords:** Anti-lipopolysaccharide factor; *Penaeus monodon*; WSSV; Antiviral activity;
25 Yeast two-hybrid screening

1. Introduction

White spot syndrome virus (WSSV) is a large enveloped virus causing a severe infectious disease in shrimp as well as in other crustaceans. The WSSV infection in shrimp causes a 100% mortality rate within 7–10 d and results in enormous economic losses in the shrimp farming industry (Flegel, 1997). Thus, several preventive and curative measures have been developed, such as vaccination (Rout et al., 2007; Satoh et al., 2008), immunostimulants (Chotigeat et al., 2004; Balasubramanian et al., 2008), direct neutralization by antiviral proteins (Dupuy et al., 2004; Tharntada et al., 2009) and RNAi (Ongvarrasopone et al., 2008), although they have not been successfully implemented in shrimp farms. In addition, the innate immunity in shrimp has been studied intensively in order to understand the response of shrimp to viral infections, including to WSSV (Liu et al., 2009).

Antimicrobial peptides (AMPs) are effector molecules that play an important role in the innate immune system and function as the first line of defense against invading microorganisms (Hancock et al., 2006). AMPs are active against a large spectrum of microorganisms: bacteria, virus, yeast, parasites and fungi, and even against tumor cells. Some AMPs have been shown to have antimicrobial activity and directly kill pathogens, although others appear to participate in immunoregulatory mechanisms by modulating signal transduction and cytokine production and/or release (Brown and Hancock, 2006; Nagaoka et al., 2012). In response to viral infection, target cells can produce several antiviral factors to control viral invasion, including AMPs that are typically induced in the early innate immune response. AMPs may directly act on viral virions or indirectly suppress viral replication (Mulder et al., 2013). In mammals, it has been reported that lactoferrin, an iron binding glycoprotein, exhibits antimicrobial activity and is involved in the antiviral mechanism. Lactoferrin prevents viral infection of the host cell either by direct binding to virus particles or by binding to the viral receptor or co-receptor molecules of host cells (van der Strate et al.,

2001). Indolicidin, an AMP, inactivates HIV-1 by damaging the virion membrane (Robinson et al., 1998), whilst α -defensin peptides are able to inhibit the assembly of polyomavirus particles (Dugan et al., 2008).

Anti-lipopolysaccharide factor (ALF) is an AMP that additionally contains the LPS-binding domain and has been found in many crustaceans, including shrimp. In *Penaeus monodon*, transcripts of the most abundant isoform (ALFPm3) are upregulated upon *Vibrio harveyi* or WSSV infection revealing its potential role(s) in the shrimp immune response (Somboonwiwat et al., 2008; Ponprateep et al., 2012). Recombinant ALFPm3 (rALFPm3) protein has been found to exhibit antimicrobial activity against both Gram-negative and Gram-positive bacteria as well as fungi (Somboonwiwat et al., 2005), whilst the antimicrobial action of ALFPm3 against Gram-negative bacteria has been implied to involve bacterial membrane disruption (Jaree et al., 2012). However, ALFPm3 is the only shrimp AMP that is currently reported to exhibit an anti-WSSV activity, where it can efficiently neutralize bacterial pathogens and also protect *P. monodon* from WSSV infection (Tharntada et al., 2009). The antiviral activity of ALF derivatives has also been reported, where the cyclic synthetic fragment of ALF exhibited antiviral activity against nervous necrosis virus (NNV), a fish non-enveloped viral pathogen, by agglutinating the NNV virions (Chia et al., 2010). In addition, a synthetic ALF based on the LPS-binding domain of *Limulus* ALF blocked the viral entry of various human pathogenic viruses, such as HIV-1, HCV and HSV1 and 2, by binding to the docking molecule on the host cell surface (Krepstakies et al., 2012; Hoffmann et al., 2014). However, the antiviral mechanism(s) of ALF against WSSV are not understood.

In this study, potential ALFPm3-interacting proteins of the WSSV were identified using yeast two-hybrid screening coupled with *in vitro* pull-down assay in order to investigate how the ALFPm3 acts on the WSSV. The effect of ALFPm3 binding to interacting WSSV proteins was demonstrated by testing the WSSV-neutralizing activity of

1 the rALFPm3 compared to that pre-incubated with the selected WSSV protein. The direct
2 binding between ALFPm3 and WSSV proteins implied a potential rational for how ALFPm3
3 neutralizes WSSV.

4 5 **2. Materials and Methods**

6 **2.1. Construction of the ALFPm3 bait vector**

7 The DNA sequence corresponding to a mature peptide of ALFPm3 was amplified by
8 PCR from a pBluescript SK plasmid containing the ALFPm3 gene using a specific primer
9 pair; ALFPm3F and ALFPm3R (Table 1), in which the *Eco*RI and *Bam*HI recognition sites,
10 respectively, were included at their 5' ends. The PCR product was cut with *Eco*RI and
11 *Bam*HI and then cloned in-frame into the pGBKT7 bait vector, digested with the same
12 restriction enzymes. The recombinant plasmid was then isolated and subjected to nucleotide
13 sequencing to verify the correct in frame insert sequence. The bait vector, which encoded the
14 Gal4 DNA binding domain fused with ALFPm3 (named pGBKT7-ALFPm3) was tested for
15 the autoactivation of the reporter gene and toxicity before use in the two-hybrid screening.

16 17 **2.2. Yeast two-hybrid screening**

18 Yeast two-hybrid library construction and screening were performed with the
19 Matchmaker Library Construction and Screening Kit (BD Biosciences). *Saccharomyces*
20 *cerevisiae* AH109 was co-transformed with pGBKT7-ALFPm3 (bait) and the prey pGADT7-
21 WSSV ORF library (Sangsuriya et al., 2014), and transformants were selected on a synthetic
22 defined (SD) medium lacking leucine and tryptophan (SD/-Leu/-Trp). Interaction between
23 the bait and prey fusion proteins was indicated by the growth and blue color change on the
24 double dropout SD/-Leu/-Trp media containing X-alpha-Gal (DDO/X). In order to ascertain
25 that the positive clones contained the interacting proteins, the positive blue colonies were

1 subsequently grown on a higher stringency quadruple dropout medium lacking leucine,
2 tryptophan, adenine and histidine (SD/-Leu/-Trp/-Ade/-His) but containing X-alpha-Gal
3 (QDO/X). Prey plasmids were then isolated from those clones that had all four yeast reporter
4 genes [*ADE2*, *HIS3*, *MEL1* (encodes for α -galactosidase) and *lacZ* (encodes for β -
5 galactosidase)] activated, transformed into *E. coli* XL1-Blue to recover the plasmid, and
6 subjected to DNA sequencing. The obtained sequences were searched against the NCBI
7 GenBank database using BLASTX to identify the clones. To confirm the screening results,
8 recovered prey plasmids were co-transformed with the pGBKT7-ALFPm3 plasmid into *S.*
9 *cerevisiae* AH109 and plated onto QDO/X medium according to the manufacturer's
10 instructions (BD Biosciences).

11

12 **2.3. Temporal analysis of WSSV gene transcription by RT-PCR**

13 **2.3.1. Animals and WSSV infection**

14 Two hundred and fifty *P. monodon* (average weight 6 grams) were purchased from a
15 local shrimp farm in Suratthani Province, Thailand. The shrimp were acclimatized in the
16 laboratory aquaria at 28 ± 4 °C and a salinity of 15 ppt for at least 1 week before use in each
17 experiment.

18 The WSSV stock used for experimental infection was prepared according to the
19 method of Xie et al. (2005). Shrimp were infected with WSSV and left until moribund, when
20 the gills were dissected and then homogenized in TNE buffer (50 mM Tris-HCl, 0.1 M NaCl,
21 1 mM EDTA, pH 7.5) containing 1 mM phenylmethylsulfonyl fluoride (PMSF). After
22 centrifugation at $5,000 \times g$ for 5 min at 4 °C, the supernatant was filtered through a 0.45 μ m
23 filter membrane and centrifuged at $30,000 \times g$ for 30 min. The supernatant was discarded and
24 the pellet was resuspended in TM buffer (50 mM Tris-HCl, 10 mM MgCl₂, pH 7.5). The
25 WSSV suspension was diluted in 0.9% (w/v) NaCl at a 1:10,000 dilution and 100 μ L was

used for injection into each shrimp. Infected shrimp were sampled at different time-points. The WSSV titre used was empirically determined to kill all the infected shrimp in about 5 d (data not shown).

2.3.2. Semi-quantification of WSSV gene expression by RT-PCR

At 0, 3, 6, 12, 24 and 48 h post-injection (hpi), the gills were individually collected and the total RNA was extracted by grinding with a pestle in 1 mL of ice-cold TriReagent® and further performed according to the manufacture's instructions (Molecular Research Center). The total RNA (1 µg) was reverse-transcribed into cDNA using the RevertAid™ First Strand cDNA Synthesis Kit (Fermentas). The specific WSSV genes were amplified using gene specific primers (Table 1). The *P. monodon* elongation factor-1α (*EF-1α*) gene was generally used as an internal control whilst the WSSV late gene *VP28* was used as a positive control. The PCR thermal cycling was performed at 94 °C for 1 min followed by 40 cycles at 94 °C for 30 sec, 58 °C or 60 °C for 30 sec and 72 °C for 30 sec, and followed by a final 72 °C for 7–10 min. The PCR products were analyzed by 1.5% (w/v) agarose gel electrophoresis.

2.4. Recombinant expression of WSSV protein and antibody production

The WSSV proteins that potentially interacted with ALFPm3 were identified by the yeast two-hybrid assay. To produce these recombinant ALFPm3-interacting WSSV proteins, specific PCR primers were designed with 5' extensions containing a *NcoI* restriction site at the 5'-end of the forward primer and a (His)₆ tag and *BamHI* restriction site at the 5'-end of the reverse primer (Table 1). The plasmid containing each respective WSSV gene was used as the PCR template for amplification using each gene specific primer pair and Phusion™ High-Fidelity DNA polymerase (Finnzymes). The PCR thermal cycling was performed at 98 °C for 30 sec followed by 30 cycles of 98 °C for 10 sec, 60 °C for 30 sec and 72 °C for 30

1 sec, before a final 72 °C for 10 min. The purified DNA amplicons containing the *NcoI* and
2 *BamHI* restriction sites were then cloned into pET-19b (Novagen) that had previously been
3 cut with the same restriction enzymes.

4 The pET-19b/WSSV plasmid was transformed into the *Escherichia coli* strain BL21-
5 CodonPlus(DE3)-RIL expression host. The transformant was cultured to an OD₆₀₀ of about
6 0.4, and then induced by adding IPTG to a final concentration of 1 mM and cultured for a
7 further for 2 h. The cells were harvested by centrifugation at 8000 × g for 15 min and
8 resuspended in phosphate-buffered saline (PBS; 137 mM NaCl, 2.7 mM, 10 mM Na₂HPO₄, 2
9 mM KH₂PO₄, pH 7.4). After sonication, the inclusion bodies were collected and solubilized
10 in 20 mM Tris-HCl, pH 7.4 containing 6 M urea, and subsequently dialyzed against 20 mM
11 sodium carbonate buffer pH 10.0. The recombinant WSSV protein (rWSSV) was purified
12 using a Ni-Sepharose 6 Fast Flow bead column (GE Healthcare), eluted in 20 mM sodium
13 carbonate, 0.5 M NaCl, pH 10.0 buffer containing 100 mM imidazole. The eluate was then
14 dialyzed against 0.1 × PBS, pH 7.4. The purification of rWSSV was traced by SDS-PAGE
15 and Western blot analysis using an anti-His antibody (millipore) as the primary antibody and
16 alkaline phosphatase (AP) conjugated goat anti-mouse IgG antibody as the secondary
17 antibody. The protein concentration was determined using the Bradford method.

18 Two WSSV proteins, WSSV189 (GenBank accession no. NP_477656) and
19 WSSV471 (GenBank accession no. NP_477934) were successfully over-produced and 500
20 µg of each purified protein was used to immunize separate mice in order to generate
21 polyclonal antiserum at the Biomedical Technology Research Unit, Chiangmai University,
22 Thailand. Unexpectedly, the polyclonal antibody obtained with WSSV471 immunization did
23 not recognize WSSV471 protein.

24 25 **2.5. Expression and purification of the recombinant ALFPm3 protein**

1 The recombinant ALFPm3 (rALFPm3) was produced in the yeast *Pichia pastoris* and
2 purified by HiTrap SP Sepharose™ Fast Flow column (GE Healthcare) as described
3 previously (Somboonwiwat et al., 2005). The purified protein was dialyzed overnight against
4 sterile deionised water at 4 °C to remove the salt. Before use, the antimicrobial activity was
5 assayed against *E. coli* 363 using liquid broth assay to confirm the activity of the purified
6 rALFPm3.

8 **2.6. ALFPm3 and WSSV protein interaction using an in vitro pull-down assay**

9 The pull-down experiment was performed according to the instructions for the
10 ProFound™ Pull-down PolyHis Protein (Thermo Scientific). The interaction between the
11 rWSSV189-(His)₆ and rALFPm3 was examined by incubating the rWSSV189 protein with a
12 cobalt chelate resin for 1.50 h at 4 °C with gentle rocking. Then, the beads were extensively
13 washed 12 times with 600 µl of wash solution (1 × TBS: ProFound™ Lysis Buffer (1:1), and
14 40 mM imidazole, pH 7.2). The rWSSV189-coated beads were further incubated with 15 µg
15 of rALFPm3 for another 3.5 h at 4 °C with gentle rocking. After incubation, the beads were
16 extensively washed as above. The bound protein complex was then eluted with 1 × TBS:
17 ProFound™ Lysis Buffer (1:1), and 290 mM imidazole, pH 7.2. The proteins were resolved
18 on 15% SDS-PAGE and detected with silver staining.

19 For the control, rWSSV189 alone as a bait control, or rALFPm3 alone as a prey
20 control was incubated with the cobalt chelate resin. After incubation, the bait and prey
21 proteins were then extensively washed and eluted as above.

23 **2.7. Detection of native WSSV189 in shrimp tissues**

24 Hemolymph was withdrawn from shrimp using a 1 ml sterile syringe preloaded with
25 an anticoagulant (0.5 ml of MAS solution: 27 mM sodium citrate, 336 mM NaCl, 115 mM
26 glucose and 9 mM EDTA, pH 7.0) and the gills were dissected from the WSSV-infected *P.*

1 *monodon* at 0, 6, 24 and 48 hpi. Hemocytes were collected by centrifugation, homogenized in
2 1 × PBS and centrifuged at 10,000 × g for 10 min at 4 °C to separate the hemocyte lysate
3 supernatant (HLS). Gills were homogenized in 1 x PBS and 200 mM PMSF buffer. The
4 protein content was determined by the Bradford method. The HLS and gill lysate were
5 separated by 15% SDS-PAGE and analyzed for the presence and relative amount of ALFPm3
6 and WSSV189 proteins by Western blot analysis. A purified rabbit anti-ALFPm3 polyclonal
7 antibody (1:1,000) and a purified mouse anti-rWSSV189 antibody (1:1,500) were used as the
8 primary antibodies to detect native proteins in Western blot analysis, respectively, followed
9 by a goat anti-rabbit IgG-AP or a goat anti-mouse IgG-AP as the secondary antibody for
10 ALFPm3 or WSSV189 detection, respectively. The NBT/BCIP chromogenic substrate was
11 then applied and developed for protein detection.

12

13 **2.8. *Effect of rWSSV189 binding on WSSV-neutralizing activity of ALFPm3***

14 Shrimp were divided into seven groups of 10 shrimp each (each of 3–5 g body
15 weight). For the WSSV-infected control (group 1), a 50 µl suspension of 10⁻⁹ diluted purified
16 WSSV was adjusted to 150 µl with TN buffer (20 mM Tris/HCl, 400 mM NaCl, pH 7.4) and
17 injected into the second abdominal segment of the shrimp. This dosage resulted in a 100%
18 shrimp mortality within 5 d. For the injection control (group 2), 150 µl of TN buffer only was
19 injected into each shrimp. For the latter groups (groups 3-7), the same amount of WSSV as
20 above was used to study the effect of rWSSV189 on the WSSV neutralizing activity of
21 rALFPm3. The amount of rALFPm3 used to neutralize WSSV before being injected into
22 shrimp was 280 µM. At this dosage, the cumulative mortality was 70% lower than the WSSV
23 injection only. The rWSSV189 at a concentration much lower or at least equal if compared to
24 that of rALFPm3, in this case at 1:4 (rWSSV189:rALFPm3) by mole, was used to test the
25 effect of rWSSV189 on the WSSV neutralizing activity of rALFPm3. The third group was

1 injected with 150 μ l mixture of WSSV that had been pre-incubated with 280 μ M rALFPm3
2 in TN buffer for 30 min at room temperature. The fourth group was injected with a 150 μ l
3 mixture of WSSV that had been pre-incubated with 77 μ M rWSSV189 in TN buffer for 30
4 min. Group 5 shrimp were injected with 280 μ M rALFPm3 that had first been incubated with
5 77 μ M rWSSV189 for 10 min and then incubated with WSSV for another 30 min. The
6 glutathione-S-transferase (GST) protein, whose size is almost equal to rWSSV189, was used
7 as a control protein, where groups six and seven were the same treatment as groups four and
8 five, respectively, but rWSSV189 was replaced by the GST protein. The cumulative mortality
9 of shrimp was observed every 12 h post WSSV infection for the duration of 8 d. The
10 experiment was performed in triplicate.

11

12 **2.9. Localization of WSSV189 by immunoelectron microscopy (IEM)**

13 Aliquots (10 μ l) of the purified WSSV virion suspension prepared from *Procambarus*
14 *clarkii* as described by Xie et al. (2005) were adsorbed to Formvar-supported, carbon-coated
15 nickel grids (150 mesh) for 5 min at room temperature, and then the excess solution was
16 removed. The grids were blocked with blocking buffer (5% (w/v) bovine serum albumin, 5%
17 (v/v) normal goat serum, 0.1% (w/v) cold-water fish skin gelatin (Aurion), 10 mM phosphate
18 buffer, 150 mM NaCl, pH 7.4) for 30 min, incubated with incubation buffer (0.1% (w/v)
19 Aurion BSA-c, 15 mM NaN₃, 10 mM phosphate buffer, 150 mM NaCl, pH 7.4) for 5 min,
20 and then incubated for 2 h at room temperature with the purified mouse anti-WSSV antibody
21 diluted 1:40 in incubation buffer. As a control, an additional grid containing WSSV virions
22 was incubated with a dilution of pre-immune mouse serum. After three washes with
23 incubation buffer, the grids were incubated for 1 h at room temperature with a goat anti-
24 mouse secondary antibody conjugated with 18-nm-diameter gold particles (1:100 dilution in
25 incubation buffer). The grids were then washed three times with incubation buffer and then

three times with distilled water to remove the excess salt, and finally stained with 1% (w/v) phosphotungstic acid (pH 7.2) for 3 min. Specimens were examined by transmission electron microscopy (TEM) using a Hitachi (Science & Technology - H-7650) instrument.

3. Results

3.1. Identification of ALFPm3 binding proteins

With regards to the evidence of ALF possessing anti-WSSV activity (Tharntada et al., 2009; Liu et al., 2006), it was of interest to study how ALF performs its activity against WSSV. A yeast two-hybrid assay was employed to identify potential WSSV proteins that could interact with ALFPm3. The gene coding for the mature ALFPm3 was cloned into the pGBKT7 vector and the resulting recombinant plasmid was used as a bait vector for screening for ALFPm3-interacting proteins in the WSSV ORF library. Subsequent screening and confirmation by co-transformation yielded five independent clones that grew and activated reporter genes, as indicated by the blue colonies on the QDO/X plate (Fig. 1). The positive WSSV ORF clones were then subjected to DNA sequencing and the obtained sequences were analyzed by searching against the GenBank database using BLASTX. The five positive WSSV ORF clones showed a 100% sequence match to WSSV186 (accession no. NP_477653), WSSV189 (accession no. NP_477656), WSSV395 (accession no. NP_477861), WSSV458 (accession no. NP_477921) and WSSV471 (accession no. NP_477934). Although, their biological functions in WSSV are not known, previous studies indicated that WSSV186 (WSV131), WSSV189 (WSV134) and WSSV395 (WSV339) are structural proteins (Li et al., 2007). However, there is no information available for WSSV458.

3.2. Temporal analysis of WSSV gene transcription

To understand the involvement of these five potential ALF $Pm3$ -interacting WSSV proteins in WSSV-infected shrimp, their expression levels in shrimp gills at various time points after WSSV infection were determined using semi-quantitative RT-PCR analysis. The analysis showed that the *WSSV186*, *WSSV189*, *WSSV471*, *WSSV395* and *WSSV458* transcripts were detected at 24 and 48 hpi. The major WSSV structural protein gene *VP28*, previously identified as a late gene (Zhang et al., 2002), was, however, detected from 12 hpi and so was used as a positive control. Thus, these five WSSV genes were expressed at the late stage of WSSV infection (Fig. 2).

3.3. Recombinant WSSV protein production

The full-length nucleotide sequences of the five potential ALF $Pm3$ -interacting WSSV genes were amplified and cloned in-frame into the pET-19b expression vector and the respective recombinant plasmids (pET-19b/WSSVs) were then transformed into the *E. coli* strain BL21-CodonPlus(DE3)-RIL. Each recombinant clone was grown and the recombinant protein, containing a (His)₆ tag at the C-terminus, was then produced by induction with 1 mM IPTG. The whole cells, collected at 2 h after IPTG induction, were tested for the presence of overproduced proteins by SDS-PAGE. Only two of the five WSSV proteins were successfully produced, the ~26 kDa rWSSV189 and the ~20 kDa rWSSV471 (data not shown). These two rWSSV proteins were then further purified by Ni Sepharose 6 Fast Flow bead column chromatography and analyzed with SDS-PAGE and Western blot analyses using an antibody specific to the (His)₆ tag. The purified proteins of rWSSV189 (Fig.3) and rWSSV471 (data not shown) were then used as an antigen for mouse immunization in order to produce the respective specific anti-WSSV189 or anti-WSSV471 polyclonal antibodies. For an unknown reason, only the antiserum against rWSSV189 could be used to detect the WSSV protein, whereas the anti-WSSV471 antiserum was not usable. The specificity of the

1 anti-WSSV189 antibody was verified by Western blotting performed on the purified
2 rWSSV189. Hence, only the rWSSV189 was further used in this study.

3 4 **3.4. In vitro pull-down assay**

5 The crude preparation of the rWSSV189-(His)₆ protein and the purified rALFPm3
6 protein were used in the *in vitro* pull-down assay to confirm the interaction between
7 rWSSV189 and rALFPm3. Thus, the rWSSV189-(His)₆ protein was incubated with the
8 cobalt chelate resin and then the resin was washed to remove the excess (unbound) protein
9 until no rWSSV189 protein was detected in the washing, as determined by SDS-PAGE
10 analysis. Then the prey protein (rALFPm3) was added and allowed to bind to the WSSV, and
11 the resin was then washed to remove excess (unbound) prey protein until no further protein
12 was detected in the washing as above. The protein complex was eluted and resolved by SDS-
13 PAGE followed by silver staining. The cobalt chelate bead effectively pulled-down the
14 protein complex since both rWSSV189 (~26 kDa) and rALFPm3 (~11 kDa) were detected
15 (Fig. 4). Thus, the results indicated that the rWSSV189 protein could specifically bind to
16 rALFPm3 *in vitro*.

17 18 **3.5. WSSV189 protein is expressed in WSSV-infected shrimp tissues**

19 Having detected the transcripts of WSSV genes that are presumably the targets of
20 ALFPm3 binding, the expression of the protein in the WSSV-infected shrimp was evaluated
21 by collecting their hemocytes and gills at 0, 6, 24 and 48 hpi and preparing the HLS and gill
22 lysate, respectively. The proteins in the HLS and gill lysate were separated by SDS-PAGE
23 resolution, transferred to the nitrocellulose membrane and the presence of WSSV189 and
24 ALFPm3 were then detected by Western blot analysis with the anti-WSSV189 polyclonal
25 antiserum and the purified anti-ALFPm3 polyclonal antibody, respectively. The

immunostained bands of WSSV189 and ALFPm3 at the expected sizes of about 24 kDa and 11 kDa, respectively, were observed (Fig. 5), where the level of WSSV189 gradually increased at 6, 24 and 48 hpi in both the hemocytes and gills. As expected, the ALFPm3 protein was observed mainly in hemocytes at 6 to 48 hpi and some was detected at 24 hpi in gills and this presumably was ALFPm3 produced in the infiltrating hemocyte.

3.6. Effect of rWSSV189 binding on WSSV-neutralizing activity of rALFPm3

Previously, rALFPm3 has been shown to inhibit the replication of WSSV in *P. monodon* shrimp (Tharntada et al., 2009). If WSSV189 is one of the WSSV proteins that are the targets of ALFPm3 neutralization, then it is possible that rWSSV189 could intervene with and protect the virus from the action of ALFPm3. Accordingly, whether rWSSV189 could interfere with the WSSV neutralizing activity of rALFPm3 when they were pre-incubated together before being injected along with WSSV into shrimp was evaluated in terms of the cumulative mortality of the shrimp (Fig. 6). Shrimp injected with only TN buffer (control) did not die during the observation period of 8 d. In contrast, shrimp injected with only WSSV had a 100% mortality rate within 5 d. Infection with the same level of WSSV that had been pre-incubated with rWSSV189 also killed the shrimp to the same extent as WSSV alone, indicating that the exogenous rWSSV189 had no effect on the WSSV infectivity. The prior incubation of WSSV with the exogenous protein GST, alone or together with rALFPm3, also did not affect the WSSV infectivity or ability or the rALFPm3 activity, at least in terms of the observed shrimp cumulative mortality.

As expected, rALFPm3 was able to neutralize the WSSV causing a slowdown in the mortality rate. For example, a 100% mortality rate was not observed in 8 d compared to within 5 d with WSSV alone. However, the addition of rWSSV189 reduced the neutralizing activity of rALFPm3, such that a 100% shrimp mortality rate was observed in 7 d. The

1 decreased apparent WSSV neutralizing activity of rALFPm3 was presumably due to the
2 binding of rWSSV189 to the rALFPm3 and blocking the access to the WSSV.

3 4 **3.7. Localization of WSSV189 on WSSV virion**

5 To better understand the target WSSV protein of ALFPm3, WSSV189 was further
6 characterized. As reported previously WSSV189 was the structural protein, and so the WSSV
7 virion was fractionated into the envelope and nucleocapsid protein fractions, and each was
8 then subjected to Western blot analysis. The polyclonal antibody specific to WSSV189, used
9 to detect the WSSV189 in both fractions (Fig. 7A), revealed the presence of WSSV189 in the
10 envelope fraction but not in the nucleocapsid fraction, as defined by the control VP28
11 envelope protein. Moreover, the evidence from the IEM analysis supported that WSSV189 is
12 an envelope protein. A gold-labeled secondary antibody together with a purified anti-
13 WSSV189 polyclonal antibody was used to detect WSSV189 on the WSSV virion. As
14 compared to the control, where the gold particles were undetectable, positive signals were
15 observed on the envelope of the virion, but not on the nucleocapsid surface (Fig. 7B). Taken
16 together, these results identified WSSV189 as a likely WSSV envelope protein.

17 18 **4. Discussion**

19 AMPs can possess an antiviral activity as well as an antibacterial activity. The
20 antiviral properties of several AMP families against both enveloped and non-enveloped
21 viruses have been characterized (Findlay et al., 2013), including for ALFPm3 that is an
22 antimicrobial peptide from *P. monodon* that exhibits an anti-WSSV activity. The interaction
23 between WSSV-binding proteins and viral proteins has been reviewed recently
24 (Sritunyalucksana et al. 2013). Several shrimp secreted and cell-surface proteins have been
25 shown to be able to bind to WSSV proteins and play roles in WSSV infection. The

1 interaction between VP26, a major tegument protein of WSSV, with shrimp cytoskeletal
2 protein β -actin has been shown to play an important role in WSSV infection. (Xie and Yang
3 2005; Liu et al. 2011). Moreover, binding of VP26 protein with 3 kDa WSSV-binding protein
4 (WBP) resulted in the reduction of the viral replication (Youtong et al. 2011). Like other
5 shrimp proteins, such as *PmRab7* (Sritunyalucksana et al., 2006) and C-type lectin (Zhao et
6 al., 2009), the antiviral activity of ALF*Pm3* is probably due to its interaction with WSSV. In
7 this study, screening of the ALF*Pm3*-interacting proteins from the WSSV ORF library using
8 the yeast two-hybrid technique revealed that five WSSV proteins (WSSV186, WSSV189,
9 WSSV395, WSSV458 and WSSV471) were found to likely interact with ALF*Pm3*. However,
10 the biological functions of these candidate ALF*Pm3* binding proteins have not been reported
11 (annotated). Among the five ALF*Pm3*-binding proteins detected, only two recombinant
12 proteins (rWSSV189 and rWSSV471) were successfully produced and were then used for
13 polyclonal antibody production. For an unfavorable reason, a specific polyclonal antibody
14 was only obtained against WSSV189. Accordingly, this study was restricted to rWSSV189,
15 although attempts to produce and use sufficient quantities of high purity rWSSV471 protein
16 for antibody production are underway.

17 Only the structural localization has been reported for some WSSV proteins. A
18 shotgun proteomic study of WSSV (China isolate) revealed that WSSV186 (WSV131) and
19 WSSV189 (WSV134) are WSSV structural proteins (Li et al., 2007). Recently, WSSV395
20 and WSSV471 were identified as hub proteins interacting with over 20 WSSV proteins
21 (Sangsuriya et al., 2014). Previously, WSSV395 (WSV339), or VP39B, was determined by
22 Western blot and IEM analyses to be an envelope protein (Tsai et al., 2004), whilst here
23 WSSV189 was identified as being located at the envelope of the WSSV virion by the same
24 approach and so a likely structural protein. Interactions between structural proteins are
25 common in the envelope viruses, and they might form complexes that have specific roles in

1 host-viral interactions or the infectivity of viruses (Xie and Yang., 2006; Chen et al., 2007). It
2 can be hypothesized that these five ALF*Pm3*-interacting proteins might interact with each
3 other forming a complex that is the target molecule of ALF*Pm3*. However the interaction
4 between the rest of ALF*Pm3*-interacting WSSV proteins with ALF*Pm3* will be further
5 verified.

6 Previously, a DNA microarray analysis of the transcriptional profiling of WSSV
7 genes in hepatopancreas of WSSV-infected crayfish revealed that *WSSV189* and *WSSV471*
8 might be early expressed genes (Lan et al., 2006), whilst *WSSV395* gene expression in the
9 hepatopancreas of WSSV-infected crayfish increased at the late-stage post-WSSV infection
10 (Zhu et al., 2006). In contrast, a microarray analysis of WSSV genes expressed in the gills of
11 WSSV-infected *P. monodon* showed that the *WSSV189* was an immediate-early gene
12 expressed at 2 hpi (Wang et al., 2004). As shown here, *WSSV186*, *WSSV395*, *WSSV458* and
13 *WSSV471* are reported to be late genes expressed at 24 hpi (Wang et al., 2004) in the WSSV-
14 infected *P. monodon* gills. Generally, the analysis of gene expression by microarray needs
15 confirmation by either RT-PCR or quantitative RT-PCR (Liu et al., 2005; Ciofani et al.,
16 2014). It should be noted that the results of microarray analysis for our genes of interest
17 reported by Lan et al., 2006 and Wang et al., 2004, were not confirmed by RT-PCR or
18 quantitative RT-PCR. In this study, the mRNA expression of these five WSSV genes was
19 analyzed by semi-quantitative RT-PCR. The disparity of *WSSV189* gene expression might be
20 due to the different host and tissue examined and perhaps the ability of WSSV infectivity. It
21 is known that WSSV genes such as *VP24*, *VP95*, *WSSV010*, *VP26*, *VP15*, *VP124*, *VP466*,
22 *VP31*, *VP39*, *VP19* and *VP28*, are expressed at the late stage and their protein products play
23 important roles in virus infection and morphogenesis (Sánchez-Paz, 2010). Therefore, it can
24 be hypothesized that the five ALF*Pm3*-interacting WSSV proteins are late viral proteins and

1 may be proteins necessary for virion assembly. However, further experiments should be
2 conducted to verify this hypothesis.

3 From our study, the native WSSV189 protein detected in HLS and gill lysate of
4 WSSV-infected shrimp implied its importance for WSSV infection in shrimp. The ALFPm3
5 protein was observed mainly in hemocytes at 6 to 48 hpi as reported previously
6 (Somboonwiwat et al., 2008). From the evidence that the ALFPm3 producing hemocytes are
7 infiltrated in gills of *V. harveyi*-infected shrimp (Somboonwiwat et al., 2008), our result
8 implied that the ALFPm3 protein detected in the gills of 24 h WSSV-infected shrimp was of
9 ALFPm3 producing infiltrating hemocyte.

10 AMPs exhibit antiviral activity by either directly acting on the viral virion or
11 indirectly suppressing viral replication (Mulder et al., 2013). By using an *in vitro* pull-down
12 assay, rWSSV189 was shown to interact with rALFPm3 *in vitro* suggesting that ALFPm3
13 directly inhibits WSSV by binding to the WSSV189 envelope protein. To test the
14 significance of this interaction, rWSSV189 was tested to see if it could prevent rALFPm3
15 from neutralizing the WSSV-induced mortality in shrimp (Tharntada et al., 2009). As
16 expected, pre-incubation of rALFPm3 with rWSSV189 decreased the *in vivo* neutralizing
17 activity of rALFPm3 against the WSSV-mediated shrimp mortality. Accordingly, ALFPm3
18 might directly acts on the WSSV virion and, as a result, prevents WSSV infection in shrimp.

19 Currently, it is not known how the shrimp ALFPm3 interacts with WSSV protein(s).
20 Considering the ALFPm3 structure, the LPS binding site is the cluster of positively charged
21 amino acid residues, whilst the primary structure of WSSV189 protein contains a series of
22 aspartate and glutamate negatively charged residues (Asp¹⁰³-Ile¹⁰⁴-Glu¹⁰⁵-Asp¹⁰⁶-Asp¹⁰⁷).
23 Hence, rALFPm3 probably binds directly to the WSSV by charge interaction. Further
24 experiments should be done to clarify the anti-WSSV nature of ALFPm3.

1 In summary, the yeast two-hybrid assay was employed and five ALF*Pm*3-interacting
2 proteins from WSSV were identified. They were expressed at the late phase of infection as
3 determined by RT-PCR. Of these, WSSV189 was successfully characterized as a WSSV
4 envelope protein. However, the other four ALF*Pm*3-interacting proteins are awaiting further
5 characterization. The interaction between rWSSV189 and rALF*Pm*3 was further confirmed
6 by an *in vitro* pull-down assay. Collectively, the WSSV-neutralizing activity of ALF*Pm*3
7 may be mediated via its direct binding to the WSSV189 envelope protein and possibly other
8 WSSV structural proteins.

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1 **Figure Legends**

2 **Fig. 1.** Confirmation of the interaction between ALFP $m3$ and WSSV proteins by co-
3 transformation. The *S. cerevisiae* AH109 cells were co-transformed with pGBKT7-ALFP $m3$
4 bait vector and empty pGADT7 (Empty-AD); pGBKT7-ALFP $m3$ bait vector and WSSV
5 ORF prey vectors of WSSV186, WSSV189, WSSV395, WSSV458 and WSSV471.
6 Transformed cells were selected on SD/-Leu/-Trp/-Ade/-His containing X-alpha-Gal
7 (QDO/X) plates.

8
9 **Fig. 2.** Temporal transcription analysis of the WSSV genes from gill of WSSV-infected *P.*
10 *monodon* by RT-PCR. Total RNA was extracted from gill of 3 individual WSSV-challenged
11 shrimp at 0, 3, 6, 12, 24 and 48 hpi, respectively. The cDNA derived from reverse
12 transcription reaction was used as a template for WSSV186, WSSV189, WSSV471,
13 WSSV395 and WSSV458 gene amplification by PCR. VP28 and EF-1 α transcripts were
14 amplified as positive control and internal control, respectively. The PCR products were
15 analyzed by agarose gel electrophoresis. Lane Neg is negative control for PCR reaction.

16
17 **Fig. 3.** Analysis of recombinant WSSV189 protein. The purified rWSSV189 protein was
18 analyzed by 15% SDS-PAGE and Western blot analysis detected using anti-His antibody as a
19 primary antibody and AP-conjugated goat anti-mouse IgG antibody as a secondary antibody.

20
21 **Fig. 4.** *In vitro* pull-down assay between rWSSV189 and rALFP $m3$ proteins. The rWSSV189
22 was immobilized on the cobalt chelate resin. The rALFP $m3$ was added. The excess proteins
23 were extensively washed out and the bound protein complex was eluted and analyzed by 15%
24 SDS-PAGE followed by silver staining. Lane rWSSV189-His: bait control (only rWSSV189
25 added to the resin); Lane ALFP $m3$: prey control (only rALFP $m3$ added to the resin) and Lane

1 rWSSV189-His+ALFPm3: elution of bait-prey complex (both rWSSV189 and rALFPm3
2 added) from the beads with 290 mM imidazole.

3
4 **Fig. 5.** Detection of WSSV189 and ALFPm3 in hemocyte and gill of WSSV-challenged
5 shrimp at 0, 6, 24 and 48 hpi. One hundred micrograms of hemocyte lysate supernatant at 0,
6 6, 24 and 48 hpi and two hundred micrograms of gill supernatant at 0, 6, 24 and 48 hpi were
7 separated on 15% SDS-PAGE and analyzed by Western blot using polyclonal antiserum
8 specific to WSSV189 and polyclonal antibody specific to ALFPm3, respectively. The
9 expected bands of about 24 kDa and 11 kDa are for WSSV189 and ALFPm3, respectively.

10
11 **Fig. 6.** The effect of rWSSV189 on the neutralizing activity of rALFPm3. Seven groups of
12 shrimp were challenged with TN buffer (■), WSSV pre-incubated with rWSSV189 (▲),
13 WSSV only (●), WSSV pre-incubated with a mixture of rALFPm3 and rWSSV189 (×), and
14 WSSV pre-incubated with rALFPm3 (◆). The cumulative mortality of WSSV-infected
15 shrimp was observed for 8 d. The experiment was performed in triplicate. The values shown
16 here in the graph are means of % cumulative mortality ± SD.

17
18 **Fig. 7.** Localization of WSSV189 on WSSV virion. The WSSV virion was fractionated by
19 Triton X-100 treatment into enveloped and nucleocapsid protein fractions. Both fractions
20 were separated on 12.5% SDS-PAGE and analyzed by Coomassie staining and Western blot
21 analysis using the purified polyclonal antibody specific to WSSV189 (A). Moreover
22 immunoelectron microscopy was performed using a purified anti-WSSV189 antibody as a
23 primary antibody and a gold-labeled secondary antibody to detect WSSV189 on the WSSV
24 virion. As compared to the control (pre-immune serum) where the gold particle was
25 undetectable, the positive signals were observed on the envelope of the virion as indicated by

1 black arrows, but not on the nucleocapsid surface (B). E and N indicate intact virion and
2 nucleocapsid, respectively.

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Table. 1 Primer pairs used for PCR amplification

Gene	Primer	Sequence*	PCR product	Task
ALFPm3	ALFPm3F	5'-ATACTAG <u>GAATT</u> CCAAGGGTGGGAGGCTGTGGCA-3'	318 bp	Cloning and RT-PCR
	ALFPm3R	5'-TATTATGGATCCCTATGAGCTGAGCCACTGGTTGGCCT-3'		
WSSV186	WSSV186F	5'-GGACCCCATGGGCAGTAGCTACCTAGATCTTTTA-3'	1,455 bp	Cloning and RT-PCR
	WSSV186R	5'-GGTCCAGGATCCCTAATGATGATGATGATGATGTAGAACAGTGTTATTTCTTC-3'		
WSSV189	WSSV189F	5'-GGACCCCATGGGCGAATGGATAAACC AACGGACA-3'	699 bp	Cloning and RT-PCR
	WSSV189R	5'-GGTCCAGGATCCTTAATGATGATGATGATGATGTTGGATAAAGTAGTTTAAAT-3'		
WSSV395	WSSV395F	5'-GGACTCCATGGGCTCGTCTAACGGA GATGAG-3'	852 bp	Cloning and RT-PCR
	WSSV395R	5'-GGTCTAGGATCCCTAATGATGATGATGATGATGATAAAACAAACAGATTGAAA-3'		
WSSV458	WSSV458F	5'-GGACCCCATGGGCTTCCAGAAATGGT TTGAATC-3'	552 bp	Cloning and RT-PCR
	WSSV458R	5'-GGTCCAGGATCCTTAATGATGATGATGATGATGTTTGTGTTGATAATACAATTTT-3'		
WSSV471	WSSV471F	5'-GGACCCCATGGGCGAGGACCTAAAA TCCACTATC-3'	480 bp	Cloning and RT-PCR
	WSSV471R	5'-GGTCCAGGATCCTTAATGATGATGATGATGATGATGTCATTGTTTGTATACA-3'		
EF-1 α	EF-F	5'-GGTGCTGGACAAGCTGAAGGC-3'	145 bp	Internal control for RT-PCR
	EF-R	5'-CGTTCCGGTGATCATGTTCTTGATG-3'		
VP28	VP28-F	5'-ATCGCCATGCTATTTATTGTGATT-3'	600 bp	Positive control for RT-PCR
	VP28-R	5'-TAGCGGATCCCTCGGTCTCAGTGC-3'		

* The restriction sites are underlined

Figure1

Figure2

Figure3

Figure4

Figure5

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Figure 7

A)

B)