Suppression of Shrimp Melanization during White Spot Syndrome Virus Infection*

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Background: Melanization plays a major role in invertebrate defense.

Results: Suppression of shrimp melanization increased the WSSV susceptibility. The viral protein, WSSV453, interferes the proPO system via PmPPAE2 inhibition.

Conclusion: Shrimp melanization has an antiviral role. WSSV overcomes this by suppressing the host proPO proteinase cascade.

Significance: The regulation of shrimp melanization during WSSV infection was first demonstrated.

The melanization cascade, activated by the prophenoloxidase (proPO) system, plays a key role in the production of cytotoxic intermediates, as well as melanin products for microbial sequestration in invertebrates. Here, we show that the proPO system is an important component of the Penaeus monodon shrimp immune defense toward a major viral pathogen, white spot syndrome virus (WSSV). Gene silencing of PmpROPO(s) resulted in decreased cumulative shrimp mortality after WSSV infection, whereas incubation of WSSV with an in vitro melanization reaction prior to injection into shrimp significantly increased the shrimp survival rate. The hemolymph phenoloxidase (PO) activity of WSSV-infected shrimp was extremely reduced at days 2 and 3 post-injection compared with uninfected shrimp but was fully restored after the addition of exogenous trypsin, suggesting that WSSV probably inhibits the activity of some proteinases in the proPO cascade. Using yeast two-hybrid screening and co-immunoprecipitation assays, the viral protein WSSV453 was found to interact with the proPO-activating enzyme 2 (PmPPAE2) of P. monodon. Gene silencing of WSSV453 showed a significant increase of PO activity in WSSV-infected shrimp, whereas co-silencing of WSSV453 and PmPPAE2 did not, suggesting that silencing of WSSV453 partially restored the PO activity via PmPPAE2 in WSSV-infected shrimp. Moreover, the activation of PO activity in shrimp plasma by PmPPAE2 was significantly decreased by preincubation with recombinant WSSV453. These results suggest that the inhibition of the shrimp proPO system by WSSV partly occurs via the PmPPAE2-inhibiting activity of WSSV453.

White spot syndrome virus (WSSV),3 an enveloped and double-stranded circular DNA virus, is one of the major shrimp pathogens that causes a cumulative mortality of up to 100% within a week and has consistently affected shrimp farming worldwide and led to drastic losses in shrimp production (1–4). An insight into the molecular mechanisms underlying the shrimp-virus immune interactions is particularly essential for helping combat these viral infections and improving the cultured shrimp immunity.

The melanization cascade, activated by the prophenoloxidase (proPO) system, has been documented as an important immune mechanism in shrimp and other arthropods in their defense against pathogens (5–16). Most studies have shown that the melanization reaction is initiated by microbial elicitors, which then activate the proteolytic cascade that terminates with the activation of the zymogen proPO into the active phenoloxidase (PO) enzyme. The PO then oxidizes o-diphenols and tyrosine to quinones, which leads to the synthesis of melanin along with reactive oxygen and nitrogen intermediates as by-products (5, 8, 17–21). Melanization helps eliminate pathogens by using the cytotoxic intermediates that work along with the cellular responses via hemocyte attraction, inducing phago-

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3 The abbreviations used are: WSSV, white spot syndrome virus; dpi, days post injection; dsRNA, double-stranded RNA; Egf, epidermal growth factor-like motif; HLS, hemocyte lysate supernatant; hpi, hours post injection; PO, phenoloxidase; PmPPAE2, Ppae2 proteinase; PmPPAE2, proPO-activating enzyme; r, recombinant; SSS, sodium saline solution; SP, serine proteinase; trx, thioredoxin; X-α-gal, 5-bromo-4-chloro-3-indolyl-α-D-galactoside.
cytosis, particle encapsulation, and the formation of nodules (17–21).

In the shrimp *Penaeus monodon*, two proPO isoforms (*PmproPO1* and *PmproPO2*) have been characterized, and the activation ofzymogen *PmproPOs* into the active POs needs the proteolytic activity of the proPO-activating enzymes (*PmPPAE*) 1 and 2 (15, 22–24). The proPO system of *P. monodon* has been shown to play an important role in the defense against pathogenic bacterial and fungal infections (15, 22–25), but no reports currently indicate a role in the antiviral activity significantly decreased at 48 h post injection (hpi) (30). The mortality was recorded daily for 7 days, and the dose of the virus that caused 50% mortality (LD$_{50}$) within 3 days after injection was used for all subsequent viral infections of the shrimp.

**Effect of WSSV Infection on proPO(s)-silenced Shrimp**—The cumulative mortality of proPO(s)-silenced shrimp after WSSV infection was evaluated to examine the importance of the proPO system in the defense against WSSV. Double-stranded RNAs (dsRNAs) of *PmproPO1* and *PmproPO2* genes were generated in vitro using the T7 RiboMAX express large scale RNA production system (Promega) with the gene-specific primer pairs of T7PO1i-F/-R, PO1i-F/-R, T7PO2i-F/-R, and PO2i-F/-R (Table 1) as reported (22). Juvenile shrimp were intramuscularly injected with 25 μl of 150 μM NaCl (sodium saline solution; SSS) containing either (i) *PmproPO1* and *PmproPO2* dsRNA (5 μg each/g shrimp, wet body weight), (ii) GFP dsRNA at the same concentration, or (iii) SSS only. At 24 hpi, a second 25-μl injection as before but also containing WSSV (LD$_{50}$ dose) was administered. Thereafter, the number of dead shrimp was recorded daily over a 5-day period to ascertain the cumulative mortality. The experiments were performed in triplicate.

**Effect of the PO-generated Reactive Compounds on WSSV Infection**—The antiviral role of the reactive compounds produced by the shrimp proPO system was investigated in vitro by observing the survival rate of shrimp infected with *in vitro* melanization reaction-treated WSSV. The *in vitro* shrimp melanization reaction was prepared by incubating shrimp hemocyte lysate supernatant (HLS) (15 μg/g shrimp), prepared as previously described (25), with *Escherichia coli* O127:B8 LPS (Sigma; L4130) at 0.5 μg/g shrimp, laminarin (β-1,3-glucan) from *Laminaria digitata* (Sigma; L9634) at 0.5 μg/g shrimp, and dopamine (1 μM) in 10 mM Tris-HCl (pH 8.0) for 15 min. Then the WSSV stock solution was treated with this melanization reaction at room temperature for 1 h. Thereafter, five groups of shrimp (5 g) were injected with (i) the melanization reaction-treated WSSV or, for the four control groups, with WSSV incubated with either (ii) HLS or (iii) dopamine and with (iv) dopamine + HLS or (v) WSSV only. The number of surviving
shrimp in each group was recorded daily over a 10-day period. The experiments were performed in triplicate.

The quantification of WSSV replication in shrimp after injection with the different solutions was examined by semiquantiative two-stage RT-PCR with the gene-specific VP28 primer pair (Table 1). The hemolymph from three surviving shrimp in each group was collected at 5 days post injection (dpi), and the total RNA was extracted using an Illustra RNAspin mini kit (GE Healthcare). Then first strand cDNAs were reverse transcribed from the poly(A)-tailed mRNA using 180 μg of DNA-free total RNA sample and 0.25 g of oligo(dT)18 primer with the RevertAid first strand cDNA synthesis kit (Thermo Scientific), as per the manufacturer’s protocol. The transcript expression level of the WSSV VP28 and host EF1-α genes were then evaluated using the cDNA and the specific VP28 and EF1-α primers (Table 1). The PCRs were performed based on the Clontech Matchmaker GAL4 two-hybrid system to identify which WSSV proteins potentially interact with PmPPE2, a serine proteinase enzyme in the shrimp proPO system. To construct the PmpPPE2 bait vector, a mature peptide coding sequence of PmPPE2 (GenBankTM accession number FJ620685) was amplified using PmpPPE2NcoI-F and PmpPPE2NcoI-R that contain 5’- and 3’-end of the indicated sequence, whereas [T7s] indicates 5’-GATACCCTAAATCAGACTCTATAGG addition to the 5’-end of the indicated sequence, whereas [T7] indicates 5’-TAATACGACTCACTATAGG addition to the 5’-end of the indicated sequence.

TABLE 1

| Gene name | Accession number | Primer sequence (5’ → 3’) |
|-----------|-----------------|---------------------------|
| T7/VSSV453F | AA189321 | [T7] ATGAAGTTGATGGAAGTTCTAGTA |
| T7/VSSV453R | AA189321 | [T7] TTATCTGCCACTTCTTTTCTCTTGTG |
| T7/PO1i-F | AAFO99741 | [T7] CACCGTGGACGCTCCACTCT |
| T7/PO1i-R | AAFO99741 | [T7] GAGGACCTCTGGATGAACGTT |
| T7/PO2i-F | FJ025814 | [T7] CTCCTGTCCCTGGCCTGGCTCTT |
| T7/PO2i-R | FJ025814 | [T7] CCAGGGGATACGCTGCTG |
| T7/pmpPPE2i-F | FJ620685 | [T7] GGCGGCGTACGCTCCTTGTC |
| T7/pmpPPE2i-R | FJ620685 | [T7] ACCTCCTGGGGACACCTGTGTT |
| GFP/T7-F | U55761 | [T7] ATTAAGCTGACGCTGAGG |
| GFP/T7-R | U55761 | [T7s] TTTACTTTACAGCTCGTCCA |

| Semiquantiative RT-PCR analysis |
|-------------------------------|
| VSSV453-F | AA189321 | TAAAGCGGCTCCATGGAGTTGAGTTCCTGAGAAG |
| VSSV453-R | AA189321 | CTTTGTTTTAACTCCTGGCTACCTTTTCTGTGTA |
| VP28-F | AAAY40276.1 | TCACTCCTGGCTCCTGTTG |
| VP28-R | AAAY40276.1 | CACCAACAAAGGTGCCAC |
| PO1RT-F | AF099741 | GCTTCTCCTCCCTGCGTCG |
| PO1RT-R | AF099741 | GCGCAGGCTGGTTTGGAGC |
| PO2RT-F | FJ025814 | GCGAACGAGGGGCTGTGAG |
| PO2RT-R | FJ025814 | TCTCCTAGGGCTGGAGT |
| PPEA2R2T-F | FJ620685 | GCGGCGTACGCTCCTTGTC |
| PPEA2R2T-R | FJ620685 | ACCTCCTGGGGACACCTGTGTT |
| EF1α-F | – | GCCTGCTGACAGGCTGAGG |
| EF1α-R | – | GCTCCTGGGAATCGCTGCTGAGG |

| Cloning and recombinant protein expression |
|--------------------------------------------|
| PmpPPE2NcoI-F | FJ620685 | CTTGCTCGAATCGCTGAGG |
| PmpPPE2NcoI-R | FJ620685 | CCCTCGAGAATCCTGAGG |
| SPPmpPPE2NcoI-F | FJ620685 | ATGAAGACCGCTGCTAAGGTTGAGTTCCTGACG |
| SPPmpPPE2NcoI-R | FJ620685 | ATGAAGACCGCTGCTAAGGTTGAGTTCCTGACG |
| PmpPPE2SpeI-F | FJ620685 | GCTTCTAGGGCTGGAGT |
| PmpPPE2XhoI-F | FJ620685 | GCTTCTAGGGCTGGAGT |
| PmpPPE2i-F | FJ620685 | GCTTCTAGGGCTGGAGT |
| PmpPPE2i-R | FJ620685 | GCTTCTAGGGCTGGAGT |
| WSSV453pol-F | AA189321 | ATGAAGACCGCTGCTAAGGTTGAGTTCCTGACG |
| WSSV453pol-R | AA189321 | ATGAAGACCGCTGCTAAGGTTGAGTTCCTGACG |

TABLE 2

| Gene name | Accession number | Primer sequence (5’ → 3’) |
|-----------|-----------------|---------------------------|
| PmpPPE2 | FJ620685 | CTTGCTCGAATCGCTGAGG |
| PmpPPE2 | FJ620685 | CCCTCGAGAATCCTGAGG |
| SPPmpPPE2 | FJ620685 | ATGAAGACCGCTGCTAAGGTTGAGTTCCTGACG |
| SPPmpPPE2 | FJ620685 | ATGAAGACCGCTGCTAAGGTTGAGTTCCTGACG |
| PmpPPE2 | FJ620685 | GCTTCTAGGGCTGGAGT |
| PmpPPE2 | FJ620685 | GCTTCTAGGGCTGGAGT |
| WSSV453pol | AA189321 | ATGAAGACCGCTGCTAAGGTTGAGTTCCTGACG |
| WSSV453pol | AA189321 | ATGAAGACCGCTGCTAAGGTTGAGTTCCTGACG |

The shrimp hemolymph was withdrawn without using an anticoagulant from the shrimp ventral sinus at 1, 2, and 3 dpi. The PO activity in the hemolymph, expressed as the amount of dopachrome formation from the L-3,4-dihydroxyphenylalanine substrate, was measured as previously reported (22). Briefly, total hemolymph proteins (2 mg of protein) in 435 μl of Tris-HCl (10 mM, pH 8.0) were mixed with freshly prepared L-DOPA (3 mg/ml in water; Fluka). After incubation at room temperature for 30 min, 10% (v/v) acetic acid was added to the mixture, and the absorbance at 490 nm was monitored. The PO activity was recorded as ΔA 490/μg of total protein/min, and the protein concentration was determined by Bradford’s method. In addition, the exogenous trypsin activation of the WSSV-infected and PBS-injected shrimp hemolymph was observed by adding trypsin (20 μM) into both groups, and then the PO activity was determined as described above. All experiments were performed in triplicate and statistical analysis was performed using a one-way analysis of variance followed by Duncan’s test.

Yeast Two-hybrid Assay—Yeast two-hybrid screens were performed based on the Clontech Matchmaker GAL4 two-hybrid system to identify which WSSV proteins potentially interact with PmpPPE2, a serine protease enzyme in the shrimp proPO system. To construct the PmpPPE2 bait vector, a mature peptide coding sequence of PmpPPE2 (GenBankTM accession number FJ620685) was amplified using Pfu DNA polymerase (Promega) with the specific primers PmpPPE2NcoI-F/PmpPPE2XhoI-R that contain 5’-flanking NcoI and XhoI restriction enzyme sites, respectively (Table 1). PCR amplifica-
tion was conducted at 94 °C for 1 min, followed by 30 cycles of 30 s at 94 °C, 30 s at 55 °C, and 1 min at 72 °C and then a final 72 °C for 10 min. Amplified products were digested with NcoI and XhoI restriction nucleases. The digested fragments were purified and ligated into the Ncol/Sall sites of pGBKTK7 to yield the final PmPPAE2/pGBKTK7 construct that was cloned and subsequently sequenced to ensure the correct and in-frame insertion.

For the yeast two-hybrid screening, the PmPPAE2/pGBKTK7 construct obtained above was used as the bait to screen for interacting proteins (prey) from a WSSV DNA library fused with the AD domain of pGADT7 (34). Positive interactions were indicated by growth on the high stringency media lacking leucine, tryptophan, adenine, and histidine (SD/-L/-W/-A/-H) and by a blue color change caused by 5-bromo-4-chloro-3-indolyl-α-d-galactoside (X-α-gal; α-α-gal; Apollo Scientific) in the medium. Library plasmids from positive colonies were rescued in E. coli XL1-Blue cells and reconfirmed by yeast co-transformation. The positive prey plasmids were then subjected to DNA sequencing. The nucleotide sequences were compared with the deduced amino acid sequences against the GenBank™ database using the BLASTx program.

Cloning and Recombinant Protein Expression of PmPPAE2 and WSSV453—To investigate the biological role of the WSSV453 and PmPPAE2 proteins, recombinant (r)WSSV453 and rPmPPAE2 were expressed. First, the gene fragments encoding the respective serine proteinase (SP) domain of PmPPAE2 (SP-PmPPAE2) and the WSSV453 protein were amplified using Pfu DNA polymerase with the specific primer pairs SP/PmPPAE2NcoI-F/PmPPAE2NotI-R and WSSV453PciI-F/WSSV453NotI-R, respectively (Table 1). The purified SP-PmPPAE2 and WSSV453 PCR products were digested with the corresponding restriction enzymes (Table 1) and ligated into the pET28b and pET32 expression vectors (Novagen), respectively. Then the respective recombinant plasmid was transfected into E. coli JM1109, and positive recombinant clones were analyzed by nucleotide sequencing. The selected recombinant plasmid (pET28-SP-PmPPAE2 and pET32-WSSV453) was then transformed into E. coli Rosetta (DE3)-pLysS cells (Novagen) for recombinant protein expression and induced with 1 mM isopropyl-1-thio-D-galactopyranoside. At 6 h after induction, E. coli cells were harvested by centrifugation at 8,000 rpm for 15 min. The pellets were resuspended in 20 mM Tris-HCl (pH 8.0) and disrupted by an ultrasonic oscillator. The rSP-PmPPAE2 and rWSSV453, as a thioredoxin (trx) fusion protein (rWSSV453-trx), were each purified and refolded as described previously (35). In addition, the WSSV453 PCR product was digested with EcoRI and XhoI and cloned into the EcoRI/XhoI sites of the pET43.1a expression vector (Novagen) containing the NUS fusion protein. The selected recombinant plasmid (pET43.1a-WSSV453) was then transformed into E. coli Rosetta (DE3)-pLysS cells (Novagen), and the expression of rWSSV453, as a NUS fusion protein (rWSSV453-NUS), was induced as described above. The recombinant protein preparations were evaluated for purity by SDS-PAGE analysis and visualized by staining with Coomassie Blue. The concentration of the recombinant proteins was quantified by the Bradford assay.

In addition, the functionally active PmPPAE2 was produced. In this study, the activation site of PmPPAE2 was changed to allow activation by factor Xa. An expression construct of the full-length PmPPAE2 was generated by amplifying the coding sequence with the specific primer pairs PmPPAE2SpeI/PmPPAE2XbaI (Table 1) from shrimp, P. monodon, cDNA and cloning into the vector pIZT/V5-His (Invitrogen). The resulting construct (pIZT-PmproPPAE2) plasmid, after sequence confirmation, was used as the template to produce mutant plasmid according to PCR base mutagenesis. A mutation was introduced to change the codon at the predicted activation site of PmproPPAE2 from NLEK to IEGR (a cleavage site of bovine factor Xa) using specific primer pairs PmPPAE2-IEGR-F/R (Table 1). This construct was named PmproPPAE2xα. After DNA sequence verification, the plasmids were used to transfect into S2 cells using Cellfectin. At 48 h post-transfection, the cell culture medium was collected, and the cells were removed by centrifugation. The supernatant was then concentrated using a 10-kDa cutoff filter (Millipore). Proteins were analyzed by SDS-PAGE and visualized by staining with Coomassie Blue and immunoblotting. Then the proteolytic activation of rPmproPPAE2xα by factor Xa was confirmed (data not shown).

Co-immunoprecipitation—The binding of WSSV453 and PmPPAE2 proteins was confirmed using a Pierce™ co-immunoprecipitation kit according to the manufacturer’s instructions. Amino-link plus coupling resin and affinity-purified antibody of rSP-PmPPAE2 protein were incubated in a spin column at room temperature for 3 h and then washed in 1 M NaCl to remove the unbound antibody. The purified rWSSV453-trx protein (10 μl) or trx protein (10 μl) was incubated with rSP-PmPPAE2 protein (10 μl) in spin column containing affinity-purified antibody at 4 °C for 16 h. Bound proteins were eluted and analyzed by SDS-PAGE with Coomassie Blue staining and immunoblotting. For immunoblotting, proteins were transferred to a nitrocellulose membrane (Amersham Biosciences Hybrid ECL nitrocellulose membrane; GE Healthcare) after electrophoresis. Membranes were blocked by incubation in Tris-buffered solution (137 mM NaCl, 3 mM KCl, 25 mM Tris-HCl, pH 7.6) containing 0.05% (v/v) Tween 20 (TBST) and 1.5% (w/v) skimmed milk powder at room temperature for 2 h, then probed with mouse anti-His tag monoclonal antibody (1:3,000; GE Healthcare) in Tris-buffered solution, and washed twice in TBST. Primary antibodies were detected using alkaline phosphatase-conjugated rabbit anti-mouse IgG (1:10,000; Sigma) and visualized using incubation in bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium as the chromogenic substrate.

Determination of the Proteinase Activity in PmPPAE2-silenced and WSSV-infected Shrimp—The proteinase activity of PmPPAE2-silenced and WSSV-infected shrimp was compared so as to investigate the corresponding serine proteinase activity and thus whether WSSV infection suppresses the PO activation via inhibition of PmPPAE2 in the shrimp proPO system. Shrimp were divided into two groups. In the first group, shrimp were divided into three subgroups that were injected with PmPPAE2 dsRNA (experimental group) or with either GFP dsRNA or SSS (control groups). The PmPPAE2 dsRNA was generated in vitro using the T7 RiboMAX™ Express large scale RNA production system (Promega) with the T7PmPPAE2i-
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F/-R and PmPPAE2i-F/-R (Table 1) gene-specific primer pairs as reported (24). Juvenile shrimp were intramuscularly injected with PmPPAE2 dsRNA (2.5 μg/g shrimp, wet body weight) or control GFP dsRNA (same concentration) or SSS. At 24 hpi, shrimp were injected with dsRNA together with 1 μg/g shrimp wet body weight of each of LPS and laminarin and then reared for a further 48 h prior to determination of the protease activity. The second group of shrimp were divided into two subgroups and injected with either WSSV (experimental subgroup) or PBS (control subgroup) and then reared for a further 48 h prior to assay for protease activity. Each group was tested in triplicate using three shrimp per replication. Evaluation of the protease activity was performed by incubation of shrimp hemolymph protein (250 μg) in 100 μl of Tris-HCl (50 mM, pH 8.0) with three different kinds of protease substrates that have been reported previously to be suitable for the proteinases involved in the insect proPO system (36), namely 0.225 mM N-benzoyl-Phe-Val-Arg-p-nitroanilide hydrochloride (B-7632), 0.225 mM Boc-Phe-Ser-Arg-7-amido-4-methylcoumarin hydrochloride (B-9385), and 0.225 mM Boc-Val-Pro-Arg-7-amido-4-methylcoumarin hydrochloride (B-9385), (all from Sigma). Each reaction was incubated in a 96-well plate (costar) for 15 min and then measured at A405 for B-7632 or at A380–460 for B-6388 and B-9385. All experiments were performed in triplicate, and statistical analysis was performed using analysis of variance followed by Duncan’s test.

**Functional Analysis of WSSV453 by RNAi—In vivo WSSV453 gene silencing, as well as double silencing of WSSV453 and PmPPAE2 genes, during WSSV infection was performed.** The WSSV453 dsRNA and PmPPAE2 dsRNA were generated in vitro using the specific T7WSSV453i-F/-R and WSSV453i-F/-R primer pairs (Table 1). Juvenile shrimp were intramuscularly injected with 25 μl of SSS containing either (i) WSSV453 dsRNA (5 μg/g shrimp, wet body weight) or (ii) WSSV453 and PmPPAE2 dsRNA (5 μg each/g shrimp, wet body weight). Three control groups were injected with (iii) PmPPAE2 dsRNA or (iv) GFP dsRNA (same concentration) or (v) with SSS. Shrimp were then injected at 3 hpi with WSSV (LD50 dose) and reared for further 48 h before harvesting the hemolymph to determine the efficiency of the WSSV453 dsRNA gene knockdown. The hemocyte total RNA was extracted from the WSSV453 knockdown and WSSV453-PmPPAE2 knockdown and two the control shrimp groups (PmPPAE2 dsRNA and GFP dsRNA-injected) and analyzed by two stage semiquantitative RT-PCR using the WSSV453i-F/-R primers and PPAE2RTF/-R (Table 1) as described above, including the use of the EF1-α fragment as an internal control for cDNA template normalization and the VP28 primer to determine the effect of WSSV453 gene silencing on WSSV replication. In addition, the hemolymph of WSSV453 knockdown and WSSV453-PmPPAE2 knockdown shrimp and control shrimp were collected at 48 hpi with WSSV, and their PO activity was determined as described above.

**Effect of WSSV453 on Shrimp PO Activity—**The PO activity inhibitory activity of WSSV453 via inhibition of PmPPAE2 activity was evaluated. rPmPPAE2vxa (2.3 μM) were preincubated with rWSSV453-NUS (or NUS as control) (23 μM) in 25 μl of 20 mM Tris-HCl, 100 mM NaCl, 2 mM CaCl2 (pH 8.0) for 1 h, and each protein was incubated in separate wells of a 96-well plate (costar). Then factor Xa (0.25 μg) was added to activate PmPPAE2 activity and incubate for 1 h, and then the total plasma protein of shrimp (0.5 mg of protein) was added into each reaction and incubated for 1 h followed by adding tyrosine (50 μl of 1.5 mg/ml in water; Sigma) as substrate for determining PO activity, and 10 mM Tris–HCl (pH 8.0) up to 150 μl was added. The PO activity was observed by measuring the absorbance at 470 nm at 6 h using the plate reader (SpectraMax M5). All experiments were performed in replicate. Statistical analysis was performed by analysis of variance followed by Duncan’s test.

**RESULTS**

**Cumulative Mortality of proPOs-silenced Shrimp after WSSV Infection—**To investigate the potential important role of PmproPO1 and PmproPO2 in response to WSSV infection, suppression of both the PmproPO1 and PmproPO2 genes was performed by dsRNA-mediated gene silencing. The hemolymph from three surviving shrimp in each group were collected at 2 dpi to determine the efficiency of gene silencing of PmproPO1/2. Both PmproPO transcripts were effectively knocked down to an undetected level by the two-stage RT-PCR (Fig. 1A). Then the cumulative mortality of the PmproPO1/2 co-silenced shrimp after WSSV infection were ascertained and
compared with that of the GFP dsRNA-injected and SSS-injected control shrimp. The proPO1/2 co-silenced shrimp exhibited a higher mortality rate than the control groups during the 1–3-dpi period (Fig. 1B), reaching 75 and 95% mortality by 2 and 3 dpi compared with 33% and 63–78% for the control groups at 2 and 3 dpi, respectively. The result indicated that the shrimp proPO system is important in the shrimp defense against WSSV infection. When the proPO system was suppressed, shrimp were more susceptible to WSSV infection, especially at the early phase of WSSV infection. However, the cumulative mortality of the proPOs-silenced shrimp and control groups reached 100% at 4 and 4–5 dpi, respectively.

Antiviral Effect of the Reactive Compounds Generated by the proPO Cascade—The toxicity of the melanization reaction to WSSV and its ability to neutralize WSSV infection was investigated. The WSSV suspension was incubated with the melanization reaction (HLS and dopamine), prior to injection into the shrimp, and then the shrimp survival rate was monitored over 10 dpi, along with that for the control shrimp that were injected with WSSV incubated with either (i) HLS or (ii) dopamine and those injected with (iii) dopamine + HLS or (iv) WSSV only. A much higher shrimp survival rate was found in the group that was injected with the melanization reaction-treated WSSV compared with that in the other control groups (Fig. 2A). Indeed, the survival rate was maintained at over 80% up to the end of the assay (10 dpi), whereas the survival rate of the control shrimp declined to less than 40 and 20% at 4 and 5 dpi and to 0% at 6–9 dpi. In addition, VP28 transcripts were not detected in shrimp that were injected with the melanization reaction-treated WSSV, whereas they were detected in all the control groups (Fig. 2B). This result demonstrated that the melanization reaction products generated through the proPO system could neutralize the WSSV infections in shrimp.

**PO Activity of WSSV-infected Shrimp**—Because melanization in *P. monodon* appears to play an important role in the antiviral response, it was of interest that it has been reported in insects that some viruses are capable of suppressing the host melanization (31). To determine the effect of WSSV infection on the melanization cascade in *P. monodon*, the PO activity in the hemolymph from WSSV-infected shrimp was ascertained at 1, 2, and 3 dpi. The PO activity of WSSV-infected shrimp was dramatically (−6-fold) and significantly decreased at 2 and 3 dpi compared with that of the uninfected shrimp (Fig. 3A). Furthermore, the addition of trypsin (exogenous proteinase) into the PO reaction of the WSSV-infected and PBS-injected shrimp hemolymph restored the PO activity of the WSSV-infected shrimp, and no significant difference in the PO activity was then observed between the infected and uninfected shrimp (Fig. 3B). Thus, the shrimp PO activity might be suppressed by WSSV via the inhibition...
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Identification of Potential WSSV Proteins That Regulate the proPO Cascade during WSSV Infection Using the Yeast Two-Hybrid Assay—To identify WSSV proteins that might inhibit the proteinase activity in the shrimp proPO cascade, the yeast two-hybrid screening of a WSSV library for viral proteins that could interact with PmPPAE2 was performed. Only one positive clone was identified among the ~2 × 10^5 clones that were screened. The prey plasmid from the positive colony was then extracted. The interaction between PmPPAE2 and the isolated prey plasmid was confirmed by co-transformation into yeast and analysis in the two-hybrid system. Co-transformation of PmPPAE2/pGBK7 and empty pGADT7 plasmid served as the negative control. B and C, Western blot analysis of the co-immunoprecipitation of WSSV453 (as rWSSV453-trx) with PmPPAE2 (as rSP-PmPPAE2), the purified rSP-PmPPAE2 (10 μM) was incubated with WSSV453-trx protein (B, 10 μM) or trx protein (C, 10 μM) (control protein) and then subjected to co-immunoprecipitation (CO-IP) with anti-SP-PmPPAE2 agarose beads. The proteins were separated by SDS-PAGE and analyzed by Western blotting with anti-His antibody.

Interaction of rWSSV453 and rPmPPAE2 Protein by Co-immunoprecipitation—To confirm the interaction of WSSV453 and PmPPAE2 proteins, the rWSSV453 and the SP domain of PmPPAE2 (rSP-PmPPAE2) were separately expressed in E. coli. However, the production of a stable rWSSV453 could not be obtained because the recombinant protein degraded rapidly (data not shown). Therefore, the rWSSV453-thioredoxin fusion protein (rWSSV453-trx) was produced and used in the experiments. Co-immunoprecipitation, performed using an anti-His antibody according to the Western blotting analysis technique, revealed a positive binding between rWSSV453-trx and rSP-PmPPAE2 (Fig. 4B), as shown by the presence of both of these protein bands in the elution step compared with only the rSP-PmPPAE2 band in the control (trx) elution (Fig. 4C). These results suggested that WSSV453 binds to PmPPAE2.

Serine Proteinase Activity in WSSV-infected Shrimp and in PmPPAE2 Knockdown Shrimp—The proteinase activity of WSSV-infected shrimp was further examined in the hemolymph and compared with the control shrimp injected with PBS using different proteinase substrates (Fig. 5, A–C). The highest reduction in the proteinase activity in WSSV-infected shrimp compared with the control PBS-injected shrimp (68.6%) was found with the B-6388 proteinase substrate. This reduction corresponded to the decreased proteinase activity found in the PmPPAE2 knockdown shrimp (Fig. 5D). The results suggested that WSSV suppresses the proPO system via inhibition of the proteinase cascade and that PmPPAE2 is likely to be a target enzyme that is suppressed by the virus.

Functional Analysis of WSSV453 by RNAi—To investigate the function of WSSV453 in the suppression of the shrimp PO activity, WSSV453 or both WSSV453 and PmPPAE2 transcription were silenced by RNAi, and the PO activity of those groups after WSSV infection was determined. The efficiency of the gene knockdown was determined by semiquantitative RT-PCR analysis, which showed that the WSSV453 and PmPPAE2 transcript levels were significantly decreased in the WSSV453-silenced shrimp and PmPPAE2-silenced shrimp, respectively, whereas injection of the GFP dsRNA (control) had no significant effect on PmPPAE2 and WSSV453 transcription level (Fig. 6A). Moreover, the gene silencing of WSSV453, as well as PmPPAE2, had no effect on the replication of WSSV, as shown by the similar VP28 transcription levels in control shrimp (Fig. 6A). A significant increase in the total hemolymph PO activity was observed in the WSSV453 knockdown shrimp compared with the control group, but the double silencing of...
PmPPAE2 and WSSV453 did not increase the PO activity during WSSV infection. Thus, gene knockdown of WSSV453 could restore the PO activity dependent on PmPPAE2 in WSSV-infected shrimp, suggesting that WSSV453 might function in suppression of PO activity during WSSV infection via interaction with PmPPAE2. However, the increased PO activity in the WSSV453 knockdown shrimp was still below that in the control SSS-injected shrimp (Fig. 6B), suggesting that other unknown mechanism(s) or other WSSV protein(s) might also be involved in the suppression of the cascade during WSSV infection.

Effect of WSSV453 on Shrimp PO Activity—The recombinant protein of active PmproPPAE2Xa was produced with an estimated molecular mass of 43 kDa. The proteolytic activation of rPmproPPAE2Xa by factor Xa was confirmed (data not shown). In addition, rWSSV453 was expressed in E. coli as a fusion protein with a NUS tag to increase the protein solubility. The rWSSV453-NUS and rNUS (control) proteins have estimated molecular masses of 72 and 64 kDa, respectively. The rWSSV453-NUS was preincubated with PmproPPAE2Xa protein and followed by adding factor Xa and shrimp plasma, and then PO activity was measured by adding tyrosine as substrate. The results showed a significant increase of PO activity in shrimp plasma (~65%) by adding activated PmproPPAE2Xa protein (Fig. 7) compared with the control group (nonactivated plasma by PmPPAE2Xa), but the preincubation of PmproPPAE2Xa with rWSSV453-NUS protein showed the significant decrease (~40%) of PO activity when compared with preincubation with NUS as control protein (Fig. 7). This indicated that rWSSV453-NUS has the ability to inhibit PO activation probably via inhibition of PmPPAE2 activity in shrimp plasma.

DISCUSSION

Melanization, activated through the proPO-activating system, participates in invertebrate innate immune responses and appears to play a key role in the non-self-recognition system being responsible for parasite entrapment and microbe killing, as well as wound healing (8, 17, 37–40). The reactive intermediates produced during melanin synthesis, such as reactive oxygen and nitrogen intermediates and quinone-like substances, are toxic to some microorganisms and multicellular parasites and so restrain the invasion of these pathogens into the host body cavity (8, 17–21, 40). The complex melanization cascade involves several proPO-associated proteins that are fairly well characterized in many invertebrates (8, 38, 39). In P. monodon, two proPO isoforms (PmproPO1 and PmproPO2) and two proPO-activating enzymes (PmPPAE1 and PmPPAE2) have been identified and demonstrated to function in the shrimp proPO system and to play a crucial role in the defense against Vibrio harveyi and Fusarium solani infection (22–25). The melanization reaction products of shrimp also exhibited antimicrobial effects toward shrimp major bacterial and fungal pathogens (25). Here, we show the importance of the P. monodon melanization reaction in the defense against WSSV.

**FIGURE 5.** A–E, proteinase activity of WSSV-infected (A–C) and PmPPAE2 knockdown shrimp (D). The proteinase activity of the hemolymph from WSSV-infected and PmPPAE2 knockdown shrimp was assayed at 48 hpi with WSSV and dsRNA, respectively. Proteinase activity was assayed by incubation of the hemolymph protein (250 μg) with substrate B-7632 (A), B-9385 (B), or B-6388 (C and D). Each reaction was incubated in a 96-well plate for 15 min and then measured at A_{405} for B-7632 and at A_{380–460} for B-6388 and B-9385. The PBS-injected shrimp were used as a control group for WSSV-infected shrimp, and the GFP dsRNA- or SSS-injected shrimp were used as a control group for the PmPPAE2 knockdown shrimp. The data are shown as the means ± S.D. (error bars) and are derived from three independent experiments. Different letters (above each bar) indicate a significant difference (p < 0.05) in the mean proteinase activity. E, silencing efficiency of PmPPAE2 transcripts at 2 dpi was detected by RT-PCR.
infection. Additionally, *P. monodon* melanization reaction products exhibited an *in vitro* neutralization effect on WSSV infectivity, as shown by the reduced shrimp mortality and VP28 transcript levels when infected with WSSV preincubated with a melanization reaction prior to injection into the shrimp (Fig. 2A). This could reflect that the shrimp melanization cascade generates several highly reactive and toxic compounds (18–21) that are toxic to WSSV, because no viral VP28 transcript expression was observed in these shrimp (Fig. 2B). Likewise in *Manduca sexta*, the proPO activation and PO-generated reactive compounds, such as 5,6-dihydroxyindole, was reported to exhibit broad spectrum antibacterial, antifungal and antiviral activities (18, 19). Taken together, these results suggest that *P. monodon* melanization is an important immune response to virus infection.

In addition, WSSV infection affected the shrimp proPO system by inhibiting the PO activation. The PO activity of WSSV-infected shrimp had a reduced PO activity at 2 and 3 dpi compared with the uninfected shrimp (Fig. 3A). Interestingly, the exogenous addition of trypsin into the PO reaction of the hemolymph from WSSV-infected shrimp resulted in the full restoration of PO activity (Fig. 3B), suggesting that WSSV might block the PO activation via inhibition of a serine proteinase (trypsin-like serine proteinase) in the shrimp proPO cascade.

Similarly, in *Pacifastacus leniusculus*, it was found that WSSV inhibits the proPO system upstream of PO because no melanization was found in the granular cells of WSSV-infected crayfish, but the HLS PO activities of both sham- and WSSV-injected crayfish were the same, as were the proPO transcript levels detected by RT-PCR (41). In line with these results, it was previously reported that various kinds of pathogens suppress the host proPO system to obtain a successful infection. For example, the virulent bacterium *Photorhabdus* produces a small molecule antibiotic, (E)-1,3-dihydroxy-2-(isopropyl)-5-(2-phenylethenyl)benzene, to inhibit PO in the insect host *M. sexta*. Mutation of this gene to give a non-PO inhibitor isoform also resulted in the bacteria being nonvirulent, suggesting that PO activity is required for the elimination of this bacterium (42). In addition, the parasitic wasp *M. demolitor* that oviposits...
in M. sexta larvae also injects M. demolitor bracovirus, MdBV (31). This virus produces serine proteinase inhibitors (Egf1.0 and Egf1.5) that block the processing and the amidolytic activity of the host proPO activating proteinases (pro-PAP1 and 3), whereas Egf1.0 also binds to proPO and the serine proteinase homologs 1 and 2 (SPH1 and 2). Thus, Egf is important to prevent the host proPO activation and so the PO activity. If the Egf1.0 or Egf1.5 gene is inactive, the wasp eggs are melanized by measuring the total plasma protein of shrimp (0.5 mg protein) were added into each reaction and incubated for 1 h followed by adding tyrosine (50 μl of 1.5 mg/ml) as substrate to determine PO activity. The PO activity was determined by measuring the A670 nm at 6 h using the microplate reader. The PO activity (A670 nm) is shown as the mean ± S.D. (error bars) and is derived from replications. Different letters (above each bar) indicate a significant difference (p < 0.05) in the mean PO activity among the five samples.

Interestingly, using the yeast two-hybrid screening, we found that the WSSV protein WSSV453 (AAL89321.1) interacts with PmAEEA2 (Fig. 4A), one of the serine proteinase enzymes of the P. monodon proPO system. Co-immunoprecipitation confirmed that PmAEEA2 directly interacts with WSSV453 (Fig. 4B). WSSV453 is a small viral protein (estimated molecular mass of 11.92 kDa) but is of unknown function with no functional domains being predicted by SMART analysis. PmAEEA2 is a terminal clip-serine proteinase (clip-SP) that is implicated in the proPO activation cascade of shrimp converting the proPO to active PO. PmAEEA2 contains an N-terminal clip domain and a C-terminal trypsin-like SP domain that has a catalytic triad comprised of three catalytic residues (His, Asp, and Ser) in the SP domain (24). Because PmAEEA2 plays a very important role in proPO activation and WSSV453 shows a binding activity to PmAEEA2, we speculated that binding of WSSV453 with PmAEEA2 might somehow interfere with proPO activation in P. monodon. In the same way, the reduced proteinase activity in WSSV-infected shrimp at 48 hpi supports that the WSSV-inhibited PO activity was mediated via inhibition of proteinase activity (Fig. 5A-C). Moreover, using different proteinase-specific substrates the same pattern of reduced proteinase activity was found in both WSSV-infected and PmAEEA2-silenced shrimp, especially when using B-6388 as substrate (Fig. 5D). This implies that PmAEEA2 may be one of the inhibited proteinase enzyme targets during WSSV infection.

The increased PO activity in WSSV453-silenced shrimp after WSSV infection at 48 hpi but not in double silencing of WSSV453 and PmAEEA2 gene (Fig. 6) suggested that WSSV453 might be involved in the inhibition of proPO activation in the P. monodon hemolymph via PmAEEA2. Moreover, we found that PO activity of shrimp plasma was significantly activated after adding active PmAEEA2xa (activated by factor Xa), but the PO activity was significantly decreased when preincubating PmAEEA2xa with rWSSV453-NUS protein (Fig. 7). This again supports the ability of WSSV453 in interfering proPO activation in shrimp plasma via binding to PmAEEA2 protein. However, these results do not fully reveal how WSSV453 impaired proPO activation via PmAEEA2.

Generally, the proPO activating enzyme(s) in proPO systems in many species are regulated by serine proteinase inhibitors (serpins) (43, 44). The nucleotide sequence of WSSV453 shows no homology to other known serpins, suggesting that this viral protein may have a different mechanism for disabling the host melanization. Dengue virus, which is transmitted by the mosquitoes Aedes aegypti and Aedes albopictus, encodes for the NS1 protein that can interfere with the host coagulation and contributes to the hemorrhage in Dengue hemorrhagic fever. The secreted Dengue virus NS1 binds to thrombin and prothrombin, which play a very important role in the activation of coagulation, but NS1 did not decrease thrombin activity (45). Thus, rNS1 could inhibit prothrombin activation and prolong the activated partial thromboplastin time in Dengue hemorrhagic fever patients (45). By analogy, one possibility is that the binding of WSSV453 to PmAEEA2 might interfere with the PmAEEA2 activation and so prevent PmAEEA2 from activating proPO to PO.

In summary, the importance of the proPO system of P. monodon in the defense against WSSV infection was established, as was the fact that WSSV could suppress the host proPO system. In addition, the possible interaction of PmAEEA2 of the proPO system with the WSSV453 protein might result in the suppression of the host PO activity. These findings provide novel insights into the molecular events of virus-host interactions directed by melanization in shrimp and reveal a novel regulatory function of WSSV453. Nevertheless, the biological function of WSSV453 in the shrimp proPO system remains to be fully elucidated.

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