Anti-melanization mechanism of the white spot syndrome viral protein, WSSV453, via interaction with shrimp proPO-activating enzyme, PmproPPAE2

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Abstract

Inhibition of the host melanization reaction, activated by the prophenoloxidase activating (proPO) system, is one of the crucial evasion strategies of pathogens. Recently, the shrimp pathogen, white spot syndrome virus (WSSV), was found to inhibit melanization in the shrimp Penaeus monodon. The viral protein WSSV453 was previously shown to interact with PO-activating enzyme 2 (PmproPPAE2) and reported to be involved in suppressing the shrimp melanization response after WSSV infection. Here, we characterized how WSSV453 inhibits melanization. WSSV453 is a non-structural viral protein, which was first detected in shrimp haemocytes at 6 hours post-infection (hpi) by WSSV and in shrimp plasma at 24 hpi. We produced recombinant proteins for three components of the P. monodon proPO system: PmproPPAE2, PmproPO1 and PmproPO2. Functional assays showed that active PmproPPAE2 processed PmproPO1 and 2 to produce functional PO. Incubation of WSSV453 with PmproPPAE2 dose-dependently reduced PmproPPAE2 activity toward PmproPO1 or PmproPO2. In contrast, WSSV453 had no effect on activated PmproPPAE2. The addition of active PmproPPAE2 to WSSV-infected shrimp plasma at day 2 post-infection also rescued PO activity. Taken together, these results indicate that the anti-melanization activity of WSSV is due to WSSV453, which interacts with PmproPPAE2 and interferes with its activation to active PmproPPAE2.

INTRODUCTION

The inhibition of immune defences is one strategy pathogens use to successfully infect hosts [1]. In arthropods, melanization is an important innate immune response with functions in pathogen killing, wound healing and cuticle sclerotization [2–4]. Melanization is mediated by the phenoloxidase (PO) cascade, which can be activated by pathogen-associated molecular patterns (PAMPs). This leads to the proteolysis of multiple serine proteinases in the PO cascade. The terminal serine proteinase is called prophenoloxidase-activating proteinase or enzyme (PAP or PPAE), which is responsible for the cleavage of thezymogen prophenoloxidase (proPO) to active PO [4–6]. In the shrimp Penaeus monodon, two proPOs (PmproPO1 and 2) were identified and characterized [4]. However, direct evidence showing the relationship between PmproPO1 and 2 is unclear. PmproPO1 shows an amino acid sequence similarity (81%) to PmproPO2. Suppression of either PmproPO1 or 2 or both genes resulted in a strong PO activity reduction and a very high mortality after Vibrio harveyi challenge. Thus, two POs might cooperatively function in the proPO-activating system and probably act as a heteromer [7]. Nevertheless, the role of PmproPO1 and PmproPO2 in shrimp melanization has not yet been clarified. In Drosophila, proPOs have been shown to be involved in melanization [8].

PO uses o-diphenols and tyrosine as substrates to produce quinones, which are converted to melanin that binds to wound sites or the surface of pathogens [5, 6]. Reactive oxygen and nitrogen intermediates produced during melanization exhibit antibacterial, antifungal and antiviral activities. Melanization can also promote encapsulation, phagocytosis and the formation of nodules [9–12]. Not surprisingly, bacterial and viral pathogens have evolved diverse strategies for overcoming host melanization responses. For example, the pathogenic bacterium Photorhabdus produces a small molecule antibiotic, (E)-1,3-dihydroxy-2-(isopropyl)-5-
(2-phenylethyl)benzene, that inhibits PO [13]. The parasitic wasp Microplitis demolitor carries M. demolitor bracovirus (MdBV), which produces two proteins, Egfl.0 and Egfl.5, that competitively inhibit PAPs [14–16].

White spot syndrome virus (WSSV) is a serious shrimp pathogen that causes high mortality and drastic losses in commercial shrimp production worldwide [17, 18]. The antiviral role of the shrimp melanization response toward WSSV was recently reported in the black tiger shrimp, Penaeus monodon [19]. However, WSSV overcomes this by disabling the melanization response of P. monodon and several other species of crustaceans. Previous studies report that the proPO genes of Litopenaeus vannamei and the crayfish Procambarus clarkii were down-regulated after WSSV infection while PO activity was also significantly reduced in L. vannamei at 48 h post-infection (hpi) [20–22]. Disabled melanization in WSSV-infected haemocytes was also reported for the freshwater crayfish Pacifastacus leniusculus [23]. However, the molecular mechanism by which WSSV inhibits host melanization is unclear. Recently, we proposed that WSSV inhibits melanization in P. monodon by disabling the activity of proteinases in the PO cascade [19]. The viral protein WSSV453 was implicated in inhibiting melanization by binding to P. monodon proPO-activating enzyme 2 (PmPPAE2) [19]. In turn, PmPPAE1 and 2 were previously proposed to be the terminal clip domain-serine proteinases that activate PmproPO1 and 2 [7, 24, 25]. However, no direct experimental data support these suggestions.

In this study, we report that knockdown of PmproPO1 and of PmproPO2 by RNA interference or WSSV infection disabled melanization and wound healing in P. monodon. In addition, we produced functionally active recombinant PmPPAE2, PmproPO1 and PmproPO2 to clearly demonstrate the anti-melanization mechanism of WSSV453. Our results indicate that WSSV453 interacts with PmPPAE2 and interferes with its activation to active PmPPAE2 and leads to the reduction of PO activity with both PmproPO1 and PmproPO2.

**RESULTS**

**Melanization disabling in PmproPO1, 2 silenced shrimp and in WSSV-infected shrimp**

The first goal of this study was to determine whether RNAi knockdown of PmproPO1, 2 in P. monodon negatively affected melanization and wound healing. Shrimp treated with PmproPO1, 2 dsRNA visually showed a reduction in melanization of a wound site compared to GFP-dsRNA-injected shrimp (Fig. 1a). This finding supported that PmproPO1 and 2 contribute to melanization of wound sites. We then examined whether WSSV infection had a similar effect. The results indicated that melanization around a wound produced at 3 hpi was similar to PBS-treated control shrimp, whereas no melanization was observed around a wound produced at 24 hpi (Fig. 1b). This result indicated that melanization of wound sites in P. monodon was disabled by 24 hpi with WSSV.

**Production and activation of recombinant PmproPPAE2**

In our previous study, we hypothesized that WSSV453 suppresses shrimp melanization by inhibiting PmPPAE2 [19]. To directly examine this, we produced recombinant (r) proteins and used them in functional assays. rPmPPAE2 and rPmproPPAE2Xa were successfully expressed as zymogens in Drosophila S2 cells that were secreted into the medium (Fig. 2a). Each had a molecular mass of 43 kDa, which was also similar to the predicted molecular mass of 42.85 kDa. Incubation of factor Xa with rPmproPPAE2Xa followed by immunoblotting further showed processing to produce a 30 kDa band that corresponded to the predicted mass of active PPAE2. Processing of rPmproPPAE2Xa was detectable at 6 h and complete processing was observed at 16 h (Fig. 2b). The serine proteinase activity of rPmproPPAE2Xa was assayed using the substrate B9385 (Boc-Val-Pro-Arg-7-amido-4-methylcoumarin hydrochloride; VPR). Results showed that rPmproPPAE2Xa exhibited VPRase activity while unactivated rPmproPPAE2Xa showed no activity (Fig. 2c). In the control group, incubation of factor Xa with culture medium or wild-type rPmproPPAE2 showed neither processing of rPmproPPAE2 nor serine proteinase activity (data not shown).

**PmPPAE2 plays a role in PmproPO1, 2 activation**

We next examined the role of PmPPAE2 in activation of PmproPO1, 2. Incubation of activated PmPPAE2Xa with PmproPO1, 2 (80 kDa) resulted in cleavage as shown by
the appearance of ~75 kDa bands that corresponded to *Pm*PO1 and 2 (Fig. 3a, b) [7]. In contrast no cleavage was observed in control assays containing BSA (Fig. 3c). *PmproPO1* and *PmproPO2* also exhibited high PO activity after activation by *PmPPAE2*<sub>x</sub> but very low activity when incubated with only BSA (Fig. 3c). Moreover, when wild-type *rPmproPPAE2* was incubated with factor Xa and *PmproPO1*, 2 proteins, no activation of *PmproPOs* was observed (data not shown). These results indicated that *PmPPAE2* activates *PmproPO1*, 2 to produce functional POs.

**Characterization of WSSV453 gene/protein in WSSV-infected shrimp**

Time course studies indicated that WSSV453 expression was first detectable at 6 hpi (Fig. 4a). Transcript abundance also qualitatively increased by 48 h (Fig. 4a). Sampling of tissues at 48 hpi indicated that WSSV453 was highly expressed in haemocytes and stomach and lower expression was observed in heart, gill, intestine and hepatopancreas (Fig. 4a). Immunoblotting confirmed that WSSV453 protein was present in haemocytes from 6 to 48 hpi and in plasma at 24 and 48 hpi (Fig. 4b). Since WSSV453 has been previously reported as a protein of unknown function [26], we investigated whether it is a structural or non-structural protein. Purified WSSV particles were prepared and subjected to SDS-PAGE and immunoblot analysis using anti-VP28 and anti-WSSV453. WSSV453 was not detected in virions, whereas VP28, a known structural component of WSSV virions [27], was detected (Fig. 4c). These results supported that WSSV453 is a non-structural viral protein.

**Effect of WSSV453 on shrimp PO cascade**

Recombinant WSSV453 was produced as a fusion protein rWSSV453-NUS and purified as previously reported [19]. Pre-incubation of rWSSV453-NUS with *rPmproPPAE2*<sub>x</sub> before activation by factor Xa resulted in a significant decrease in PO activity of *rPmproPO1* and *rPmproPO2* protein when compared to control treatments [rNUS, phenylthiourea (PTU)] (Fig. 5a). In contrast, pre-incubation of rWSSV453-NUS with *PmPPAE2*<sub>x</sub> that had already been processed by factor Xa as well as with zymogen or active *rPmPO1* and *rPmPO2* did not reduce PO activity (Fig. 5b–d). This outcome indicated that WSSV453 could reduce PO activity by interacting with *PmproPPAE2* but not already activated *PmPPAE2*.

**Effect of WSSV453 on shrimp PPAE2 activity**

We further elaborated on the preceding results by pre-incubating *rPmproPPAE2*<sub>x</sub> with increasing amounts of rWSSV453-NUS for 1 h before activating with factor Xa. This showed that rWSSV453-NUS dose-dependently reduced *PmPPAE2* activity toward a VPR (B9385) substrate whereas rNUS and BSA did not (Fig. 6a). On the other hand, adding increasing amounts of rWSSV453-NUS to already active *rPmPPAE2*<sub>x</sub> had no effect on activity when compared to the same controls (Fig. 6b). rWSSV453-NUS also showed no inhibitory activity toward factor Xa (Fig. 6c). This suggested that WSSV453 did not directly inhibit *PmPPAE2* activity but might reduce *PmPPAE2* activity by interfering with the activation of *PmproPPAE2*. 

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PO activity of shrimp haemocytes and plasma was changed after WSSV infection

PO activity of WSSV-infected shrimp haemolymph was separately evaluated in haemocytes and plasma at 2 days post-infection (dpi). Results showed that WSSV had no effect on PO activity in haemocytes (Fig. 7a) but reduced PO activity in plasma (Fig. 7b). Adding active rPmPPAE2Xa to haemocytes dramatically increased PO activity in non-infected cells but only slightly increased activity in WSSV-infected cells (Fig. 7c). Interestingly, adding active rPmPPAE2Xa restored PO activity in WSSV-infected plasma (Fig. 7d). This outcome suggested that disabling shrimp melanization via inhibition of PmPPAE2 by WSSV might occur in plasma rather than haemocytes.

**DISCUSSION**

The PO cascade plays an important role in immune defence and wound healing in arthropods. Melanin accumulation around pathogens or wound sites is often observed [2–5]. The antimicrobial and antiviral effects of compounds generated during melanin synthesis have also been demonstrated [9–12]. It is also known that some pathogens have evolved strategies to suppress melanization [13, 14]. In the case of WSSV, previous studies showed that infection resulted in down-regulation of LvpPO and LvpPPAE genes expression in L. vannamei [20, 22] and reduced melanization of haemocytes in the crayfish *Pacifastacus leniusculus* [23]. However, proPO transcript abundance remained similar in infected *Pacifastacus leniusculus*, which suggested that WSSV might inhibit the proPO system upstream of PO or simply consumed the native substrate of the PO enzyme [23]. In contrast, studies of *Penaeus stylirostris* showed that WSSV infection upregulated expression of lipopolysaccharide and β-1,3-glucan binding protein (LGBP) but also reduced melanization, which suggested that WSSV may activate expression of an inhibitor(s) that blocks the activity of serine protease in the PO cascade or PO itself [28].

Previous results with *P. monodon* showed that PmPPAE2 interacted with a WSSV protein named WSSV453 [19]. Co-immunoprecipitation experiments showed positive binding of WSSV453 with PmPPAE2 protein. Furthermore, the RNAi-mediated gene silencing of WSSV453 and PmPPAE2 suggested that WSSV453 might be involved in suppression of PO activity during WSSV infection via interaction with PmPPAE2 [19]. We began this study by showing that RNAi knockdown of proPO1 and 2 had a similar effect on melanization of wound sites as WSSV infection. Previously, PmproPPAE1 and 2 were characterized as the clip domain-serine proteinases that probably process shrimp proPO [7, 24, 25]. However, we still lacked direct evidence showing that PmPPAE1 and 2 have this activity. We therefore produced in this study the mutant PmproPPAE2 (PmproPPAE2Xa) that allowed its activation by bovine factor Xa. We showed that active rPmPPAE2Xa processes both rPmproPO1 and 2 at the predicted site for activation of rPmPO1, 2 (~75 kDa) [7]. This result confirmed that PmPPAE2 processes both PmproPO1 and PmproPO2 and leads to melanization.

WSSV453 has no functional domains predicted by the SMART analysis program that provide insights into activity. We therefore focused on functional assays in this study. We first show that WSSV453 protein is not detectable in WSSV particles. This indicates that WSSV453 is likely to be a non-structural viral protein, which is also consistent with other data in the literature [27, 29]. Previously, one life cycle of WSSV was reported to be around 24 hpi and the time course of WSSV protein synthesis is different for the
different viral proteins [17]. In this study, WSSV453 expression was detected at 6 hpi in virus-infected haemocytes, which corresponded with a previous report that also detected WSSV453 at an early intermediate stage of WSSV infection [30]. Protein expression of WSSV453 in infected shrimp is detected in shrimp haemocytes at 6 hpi and in shrimp plasma at 24 and 48 hpi. Based on the ORF of WSSV453 (GenBank accession number AAL89321.1), the protein has no signal peptide, so how it is released into shrimp plasma remains unclear.

To determine with which step of the PO cascade WSSV453 interferes, we incubated rWSSV453-NUS with rPmproPPAE2Xα, rPmproPO1 or rPmproPO2, before or after their activation. Results showed that PO inhibitory activity only occurs with rPmproPPAE2Xα. We also show that incubating WSSV453 with PmproPPAE2Xα before addition of factor Xa dose-dependently interferes with PmproPPAE2Xα processing but WSSV453 has no effect on already activated PmPPAE2Xα. These results indicate that the order of interaction between WSSV453 and PmproPPAE2 processing is a key factor in reducing PmPPAE2 activity. Moreover, it suggests that rWSSV453-NUS does not inhibit PmPPAE2 enzymatic activity but instead binds to PmproPPAE2, which affects both processing and activation of proPOs leading to a reduction in shrimp melanization.

In comparison, polydnavirus proteins named Egf1.0 and 1.5 from Microplitis demolitor Bracovirus (MdBV) disable melanization of host insect haemolymph by blocking the processing and amidolytic activity of insect proPO-activating proteinases (pro-PAP1 and 3). Egf1.0 also binds to serine proteinase homologues 1 and 2 (SPH1 and 2) [14–16]. Egf1.0 and Egf1.5 contain cysteine-rich motifs similar to the trypsin inhibitor-like domain of small serine proteinase inhibitors (serpins), but this motif or other serine proteinase inhibitor domains are absent in WSSV453. This suggests that WSSV453 interacts differently with PPAEs/PAPs than Egf proteins. Potentially similar to WSSV453, a viral protein from Dengue Virus named NS1 was recently shown to inhibit protrombin activation by interfering with activation without any specific inhibitor domain [31].

To assess where inhibition of melanization by WSSV occurs, PO activity was separately investigated in haemocytes and plasma of WSSV-infected shrimp. These results showed that at 2 dpi PO activity of WSSV-infected shrimp plasma was reduced whereas activity in haemocytes was not. This supports the suggestion that WSSV453 interacts differently with PPAEs/PAPs than WSSV453 that WSSV453 interacts differently with PPAEs/PAPs than WSSV453.

Fig. 4. Characterization of WSSV453 gene and protein expression in WSSV-infected P. monodon. (a) Reverse transcriptase PCR analysis of WSSV453 in haemolymph collected 0–48 hpi and in hepatopancreas, intestine, stomach, heart, gill and haemocyte samples collected at 48 h p.i. Template for each sample was first-strand cDNA synthesized from total RNA. Gene-specific primers for WSSV453 and the 18S ribosomal gene (18S rRNA) (as an internal control) were then used. PCR was performed and analysed on 1.5 % agarose gels stained with ethidium bromide. (b) WSSV453 protein in haemocyte lysate (30 µg) and plasma (200 µg) of WSSV-infected shrimp at 0, 6, 24 and 48 hpi. Samples were subjected to SDS-PAGE and immunoblotting using anti-WSSV453 antibody and anti-β-actin antibody as control. (c) Purified WSSV particles were subjected to SDS-PAGE and visualized by staining with CBB and immunoblotting using an anti-VP28 antibody and anti-WSSV453 antibody, respectively.
METHODS

Animal preparation and purification of intact WSSV viral particles

Specific pathogen-free *P. monodon* were obtained from the Shrimp Genetic Improvement Center, BIOTEC, Thailand. The specific pathogen-free shrimp were screened to be free of major shrimp pathogens including WSSV by PCR diagnosis. Shrimp were kept in laboratory tanks with diluted natural seawater with a salinity of approximately 20 ppt and reared for 7 days before processing. WSSV was isolated as described previously with slight modifications [37]. Briefly, 5 g of gills from WSSV-infected crayfish, *Procambarus clarkii*, were collected and homogenized in 36 ml of TNE buffer (50 mM Tris-HCl, 400 mM NaCl, 5 mM EDTA, pH 8.5) containing a complete protease inhibitor (using complete mini EDTA-free; Roche). After centrifugation at 3500 g for 5 min at 4 °C, the preparation was filtered through a nylon net (400 mesh). The supernatant was centrifuged at 30 000 g for 30 min at 4 °C; the supernatant was then carefully discarded, and the lower white pellet was suspended in 1 ml PBS buffer (140 mM NaCl, 2.7 mM KCl, 10 mM Na$_2$HPO$_4$, 1.8 mM KH$_2$PO$_4$, pH 7.4). Viral genome copy number was determined by real-time quantitative PCR (qPCR) [38]. All shrimp used in experiments weighted ~10 g and were infected with $1 \times 10^5$ genome copies of WSSV.

**Effect of PmproPOs gene silencing and WSSV infection on shrimp melanization**

PmproPO1 and 2 genes were knocked down by injection of complementary dsRNA. In brief, *PmproPO1* and *PmproPO2* dsRNA were generated using the T7 RiboMAX Express Large Scale RNA Production System (Promega) with the gene-specific primer pairs P01i T7-F/-R, P01i- F/-R, PO2iF7-F/-R and PO2i-F/-R [7]. Juvenile shrimp were intramuscularly injected with 25 µl of 150 mM NaCl (sodium saline solution; SSS) containing either (i) *PmproPO1* and *PmproPO2* dsRNA (5 µg per gram of shrimp, wet body weight), or (ii) GFP dsRNA as control (at the same concentration). At 24 hpi, a second 25 µl injection was administered as before. The cuticle of treated shrimp was pierced 24 h after the second injection using a sterile...
Fig. 6. WSSV453 interferes with PmproPPAE2 subactivity. (a) rWSSV453-NUS, rNUS or BSA was pre-incubated with PmproPPAE2 x 1 for 1 h at a molar ratio of 1–16:1 followed by addition of factor Xa and VPR as a substrate to determine PmproPPAE2 activity. Results are presented as a percentage relative to when no added protein was present (100%). (b) PmproPPAE2 x 1 already activated by pretreatment with factor Xa was incubated with rWSSV453-NUS as described in (a). (c) Effect of rWSSV453-NUS, rNUS or BSA on factor Xa activity using EAR as a substrate. Data shown in (a) to (c) are means±SD from three independently replicated experiments.
sequencing. The selected recombinant plasmid (pET22b-PmpPO1, 2) was transformed into E. coli BL21 (DE3) pLysS cells (Novagen) and induced with 0.1 mM IPTG. Transformed E. coli were cultured at 16 °C for 16 h and harvested by centrifugation at 8000 rpm for 15 min. The pellets were resuspended in 20 mM Tris-HCl (pH 8.0) and disrupted by an ultrasonic oscillator. The rPmpPO1 and rPmpPO2 were found in the soluble fraction and purified by Ni-NTA affinity chromatography according to the manufacturer’s protocol for soluble proteins (Qiagen). The concentration of the recombinant proteins was quantified by a Bradford assay while purity and size were assessed by SDS-PAGE and staining with CBB.

**In vitro activation of rPmpPO1, 2 by active rPmPPAE2**

Activation of rPmpPO1 and rPmpPO2 by active rPmPPAE2 (factor Xa-activated rPmPPAE2Xa) was examined. To obtain active rPmPPAE2Xa, rPmPPAE2 (23 nM) was activated by 0.5 µg of factor Xa for 6 h at room temperature. rPmpPO1 (125 nM) or rPmpPO2 (125 nM) were then incubated with active rPmPPAE2Xa (23 nM) for 30 min. proPO cleavage, indicative of activation, was analysed by SDS-PAGE and staining with CBB or immunoblotting using a His-tag primary antibody as described above. rPmpPO1 and rPmpPO2 activity were accessed after activation by incubating rPmpPO1, rPmpPO2 or BSA as a control (125 nM) in a 96-well plate containing 10 mM Tris-HCl (pH 8.0) and 5 µM CuCl₂ (80 µl), for 30 min. L-DOPA (15 µl of 3.0 mg ml⁻¹) was then added as substrate to determine PO activity. PO activity was observed by measuring absorbance at 490 nm at 1 h using a plate reader (SpectraMax M5). Each assay was performed in triplicate and statistical analysis was performed using a one-way ANOVA followed by a post-hoc Duncan’s test.

**WSSV453 gene and protein characterization**

Healthy WSSV-susceptible shrimp (size ~10 g) were infected with WSSV, and haemolymph was collected from an abdominal segment at 0, 3, 6, 12, 24 and 48 hpi using a 1 ml sterile syringe without anticoagulant buffer. Shrimp tissues, including hepatopancreas, stomach, intestine, heart, haemocytes and gill, were collected at 48 hpi. Total RNA was isolated from each sample using the RNAeasy Mini Kit (Qiagen) followed by cDNA synthesis using the RevertAid first strand cDNA synthesis kit (Thermo Scientific). Gene-specific primers for WSSV453 (WSSV453-F: TAAAG CGATGCCCATGAAGGTTGATGGAGTCTGTGAAAGAG/ WSSV453-R: CTTGTTTAACCTGCTCCATTTTCCTGTTGTA) were used and the primer for 18S rRNA (18 S-F: TATACGCTAGTGTCGCTAG/18S-R: GGGGAGG TAGTTGACGAAAAAT) was used as an internal control. PCR products were analysed on 1.5 % agarose gels stained with ethidium bromide.

To detect WSSV453 protein in WSSV-infected shrimp, haemolymph was collected at 0, 6, 24 and 48 hpi with a 22 gauge needle, fitted to a 1 ml syringe that contained 0.5 ml of anticoagulant consisting of 140 mM NaCl, 30 mM citric acid, 110 mM glucose and 30 mM sodium citrate, pH 5.6. Then, shrimp haemolymph was centrifuged at 800 g for 10 min at 4 °C. The supernatant was collected as plasma and...
the haemocyte pellet was dissolved in 0.2 ml of PBS buffer by sonication for 10 s as haemocyte lysate. Haemocyte lysate (30 µg) and plasma sample (200 µg) were then analysed by SDS-PAGE and immunoblotting using anti-WSSV453 antibody and anti-β-actin as control as described above. Puriﬁed WSSV (10 µg) was prepared as described above and analysed by SDS-PAGE and visualized by staining with CBB and immunoblotting using the anti-WSSV453 antibody or anti-VP28 antibody as a control.

Effect of rWSSV453-NUS on shrimp PO cascade
To understand the inhibition mechanism of WSSV453 on the shrimp proPO system, rWSSV453-NUS (230 nM) was pre-incubated with proPO-associated proteins in four separate reactions: rWSSV453-NUS with rPmproPPAE2Xa before (A) or after (B) activation by factor Xa, and pre-incubation of rWSSV453-NUS with rPmproPO1 or rPmproPO2 before (C) or after (D) activation by active rPmPPAE2Xa. rPmproPPAE2Xa (23 nM) was activated by incubation with 0.5 µg factor Xa for 6 h while active rPmPOs were produced by incubating rPmproPOs (125 nM) with active rPmPPAE2Xa (23 nM) for 1 h at room temperature. Each reaction was incubated in a 96-well plate containing 10 mM Tris-HCl (pH 8.0) and 5 µM CuCl2 (80 µl). L-DOPA (15 µl of 3.0 mg ml⁻¹) was then added as a substrate for determining PO activity by measuring absorbance at 490 nm at 1 h using the plate reader (SpectraMax M5). All experiments were performed in triplicate and statistical analysis was performed using a one-way ANOVA followed by Duncan’s test.

Functional study of WSSV453 on PmproPPAE2 and processed PmPPAE2
rPmproPPAE2Xa (2.3 nM) was pre-incubated with rWSSV453-NUS at different molar ratios (0, 2.3, 4.6, 9.2, 18.4 and 36.8 nM) for 1 h before adding 0.05 µg factor Xa. rPmproPPAE2Xa (2.3 nM) was also activated by adding 0.05 µg factor Xa for 6 h followed by addition of rWSSV453-NUS (or rNUS or BSA as controls) at different molar ratios (0, 2.3, 4.6, 9.2, 18.4 and 36.8 nM) for 1 h. The reaction mixtures were incubated in a 96-well plate containing proteinase buffer (80 µl). The substrate VPR (50 µM) was then added to PL and HLS samples followed by measurement of fluorescence at 490 nm for 16 h. rPmPPAE2Xa was then added to PL and HLS samples followed by measurement of PO activity as described above. All experiments were performed in triplicate and statistical analysis was performed using a one-way ANOVA followed by Duncan’s test.

Effect of WSSV infection on PO activity of shrimp plasma and haemocytes
Healthy WSSV-susceptible shrimp (size ~10 g) were injected with WSSV or PBS followed by collection of haemolymph from an abdominal segment at 2 dpi as described above. Haemocyte lysate supernatants and plasma were prepared by collecting haemolymph from a 3 ml syringe and 22 gauge needle that contained ice-cold 10 mM Tris-HCl, pH 8.0. Samples were immediately centrifuged at 1000 g for 10 min at 4°C. The supernatant was collected as plasma (PL) and the haemocyte pellet was washed twice with PBS and homogenized in 0.2 ml of ice-cold homogenizer buffer (10 mM sodium cacodylate and 5 mM CaCl2; pH 7.0) and then centrifuged at 25 000 g for 10 min at 4°C. The supernatant was collected as haemocyte lysate supernatant (HLS). PO activity of shrimp PL (200 µg) and HLS (20 µg) were determined by adding samples to a 96-well plate containing 10 mM Tris-HCl (pH 8.0; 80 µl) and L-DOPA (15 µl of 3.0 mg ml⁻¹) followed by measurement of absorbance at 490 nm at 30 min using the plate reader (SpectraMax M5). To compare PO activation in infected and normal shrimp, active rPmproPPAE2Xa was prepared by incubating rPmproPPAE2Xa (115 nM) with 1 µg factor Xa for 16 h. rPmPPAE2Xa was then added to PL and HLS samples followed by measurement of PO activity as described above. All experiments were performed in triplicate and statistical analysis was performed using a one-way ANOVA followed by Duncan’s test.

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Conflicts of interest
The authors declare that there are no conﬂicts of interest.

References
7. Amparyup P, Charoensapsri W, Tassanakajon A. Two propheno- loxidases are important for the survival of Vibrio harveyi.


