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 the zymogen 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-phenylethynyl) benzene, that inhibits PO [13]. The parasitic wasp *Microplitis demolitor* carries *M. demolitor* bracovirus (*MdBV*), which produces two proteins, Egf 1.0 and Egf1.5, 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, *Peneaus 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 (*Pm proPOAE2*) [19]. In turn, *Pm proPAE1* and 2 were previously proposed to be the terminal domain-serine proteinases that activate *Pm proPO1* and 2 [7, 24, 25]. However, no direct experimental data support these suggestions.

In this study, we report that knockdown of *Pm proPO1* and of *Pm proPO2* by RNA interference or WSSV infection disabled melanization and wound healing in *P. monodon*. In addition, we produced functionally active recombinant *Pm proPAE2*, *Pm proPO1*, and *Pm proPO2* to clearly demonstrate the anti-melanization mechanism of WSSV453. Our results indicate that WSSV453 interacts with *Pm proPAE2* and interferes with its activation to active *Pm proPAE2* and leads to the reduction of PO activity with both *Pm proPO1* and *Pm proPO2*.

**RESULTS**

**Melanization disabling in *Pm proPO1*, 2 silenced shrimp and in WSSV-infected shrimp**

The first goal of this study was to determine whether RNAi knockdown of *Pm proPO1*, 2 in *P. monodon* negatively affected melanization and wound healing. Shrimp treated with *Pm proPO1*, 2 dsRNA visually showed a reduction in melanization of a wound site compared to GFP-dsRNA-injected shrimp (Fig. 1a). This finding supported that *Pm proPO1* 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 *Pm proPAE2***

In our previous study, we hypothesized that WSSV453 suppresses shrimp melanization by inhibiting *Pm proPAE2* [19]. To directly examine this, we produced recombinant (r) proteins and used them in functional assays. *rPm proPAE2* and *rPm proPAE2* 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 *rPm proPAE2* followed by immunoblotting further showed processing to produce a 30 kDa band that corresponded to the predicted mass of active PAE2. Processing of *rPm proPAE2* was detectable at 6 h and complete processing was observed at 16 h (Fig. 2b). The serine proteinase activity of *rPm proPAE2* was assayed using the substrate B9385 (Boc-Val-Pro-Arg-7-amido-4-methylcoumarin hydrochloride; VPR). Results showed that *rPm proPAE2* exhibited VPRase activity while unactivated *rPm proPAE2* showed no activity (Fig. 2c). In the control group, incubation of factor Xa with culture medium or wild-type *rPm proPAE2* showed neither processing of *rPm proPAE2* nor serine proteinase activity (data not shown).

**Pm proPAE2 plays a role in *Pm proPO1*, 2 activation**

We next examined the role of *Pm proPAE2* in activation of *Pm proPO1*, 2. Incubation of activated *Pm proPAE2* with *Pm proPO1*, 2 (80 kDa) resulted in cleavage as shown by
the appearance of ~75 kDa bands that corresponded to PmPO1 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 PmPPAE2Xα, 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 rPmproPPAE2Xα 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 PmPPAE2Xα 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 PmproPPAE2.

Effect of WSSV453 on shrimp PPAE2 activity

We further elaborated on the preceding results by pre-incubating rPmproPPAE2Xα with increasing amounts of rWSSV453-NUS for 1 h before activating with factor Xa. This showed that rWSSV453-NUS dose-dependently reduced PmproPAE2 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 rPmproPAE2Xα 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 PmproPAE2 activity but might reduce PmproPAE2 activity by interfering with the activation of PmproPPAE2.
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 LvproPO and LvproPPAEs gene 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 PmproPPAE2Xa processes both PmproPO1 and 2 at the predicted site for activation of PmproPO1, 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
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 rPmpproPPAE2Xa, rPmpproPO1 or rPmpproPO2, before or after their activation. Results showed that PO inhibitory activity only occurs with rPmpproPPAE2Xa. We also show that incubating WSSV453 with PmpproPPAE2Xa before addition of factor Xa dose-dependently interferes with PmpproPPAE2Xa processing but WSSV453 has no effect on already activated PmPPAE2Xa. These results indicate that the order of interaction between WSSV453 and PmpproPPAE2 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 PmpproPPAE2, 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 in plasma might interact with PmPmproPPAE2, which results in reduced activation of PmproPOs. Supporting this interpretation, adding activated rPmPPAE2Xa to plasma from infected shrimp increased PO activity. The regulation of melanization in invertebrates generally is also mostly in plasma. For example, melanization inhibition proteins (MIPs) from the crayfish Pacifastacus leniusculus [32] and P. monodon [33] are secreted into plasma. Serine proteinase inhibitor proteins (serpins) in Manduca sexta and in Hyphantria cunea also function in plasma [34–36]. In conclusion, this study provides novel insights into the function of WSSV453 and its role as an inhibitor of melanization in shrimp.

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.

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 Na2HPO4, 1.8 mM KH2PO4, 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 × 10^9 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 PO1i T7-F/-R, PO1i- F/-R, PO2iT7-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 animal preparation and purification of intact WSSV viral particles

**METHODS**

**Animal preparation and purification of intact WSSV viral particles**

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 Na2HPO4, 1.8 mM KH2PO4, 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 × 10^9 genome copies of WSSV.
needle, while melanization of the wound site was assessed at 24 h. The effect of WSSV infection on injury-mediated melanization of shrimp was also assessed. Shrimp were injected with WSSV or PBS (control) as described above. The cuticle of treated shrimp was pierced at 3 hpi (early phase of infection) or 24 hpi (late phase of infection) followed by assessment of melanization at 24 h after the wounding event. All assays were performed in triplicate. Photos were taken using a Canon PowerShot SX510 HS camera.

**SDS-PAGE and immunoblot analysis**

Protein samples were run on SDS-PAGE gels, which were either stained with CBB or processed for immunoblotting. In brief, samples were mixed with 2x sample buffer, boiled for 5 min and then loaded on 15% SDS-PAGE gels. For immunoblotting, proteins were transferred to nitrocellulose (Amersham Hybond ECL Nitrocellulose Membrane; GE Healthcare) after electrophoresis. Membranes were blocked with 5% non-fat milk powder in PBS at room temperature for 1 h. After washing three times with PBS with 0.05% Tween 20 (PBS-T), membranes were probed with specific antibodies against WSSV453 (1:100), VP28 (1:3000), the V5 epitope (1:3000; Thermo), β-actin (1:3000; Thermo) or polyhistidine tags (1:3000; Millipore) at 4°C overnight. Primary antibodies against V5 epitope, β-actin and polyhistidine were detected with a goat anti-mouse IgG-HRP-conjugated secondary antibody (1:10 000; Jackson Immuno Research). The primary antibodies for VP28 and WSSV453 were detected with a goat anti-rabbit IgG-HRP-conjugated secondary antibody (1:10 000; Jackson Immuno Research). All blots were visualized using the SuperSignal West Pico Chemiluminescent Substrate (Thermo Fisher Scientific). The polyclonal antibody against VP28 was a kind gift from Dr. Paisarn Sithigorngul (Srinakharinwirot University, Thailand). The polyclonal antibody against WSSV453 was prepared against the peptide ‘TTSRASPSSVEYKRR’ (Genscript).

**Preparation of recombinant proteins**

rWSSV453, PmproPO1 and PmproPO2 were expressed in *Escherichia coli* while PmproPPAE2 was expressed in *Drosophila* S2 cells. Expression constructs and protein production for PmproPPAE2, mutant PmproPPAE2 (PmproPPAE2xa) and WSSV453-NUS were prepared as reported previously [19]. The expression constructs for PmproPO1 and PmproPO2 were generated by PCR amplification of the coding sequences for each using the primers (PmPO1Ndel-F: 5’CGCCATATGGCCATGGCCGCAA TGACCAGCAGCGT3’/PmPO1HindIII-R: 5’CCCAAGGC TTGTTAATTTCTCGCCAGAGA3’ and PmPO2Ndel- F: 5’CGCCATATGGCCATGGCCGCAA TGACCAGCAGCGT3’/PmPO2NotI-R: 5’TAAA CGGCCGCGG TTCAGCTTCTCTGCCAGAGA3’) and shrimp, *P. monodon*, cDNA as template. The purified PmproPO1, 2 products were digested with the corresponding restriction enzymes and ligated into pET22b (Novagen). Plasmids were transformed into competent *E. coli* JM109 cells and positive recombinant clones were analysed by nucleotide
sequencing. The selected recombinant plasmid (pET22b-
PmpproPO1, 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 16h 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 rPmpproPO1 and rPmpproPO2 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 rPmpproPPAE2**<sub>x</sub> **by factor Xa**

To investigate the role of PmpproPPAE2 in the shrimp PO activation cascade, the predicted activation site of PmpproPPAE2 was changed from NLNK to IEGR (PmpproPPAE2<sub>x</sub>) to allow its activation by bovine factor Xa [19]. The incubation time and concentration between factor Xa and rPmpproPPAE2<sub>x</sub> were optimized. Several concentrations (0.02, 0.1 and 0.5 µg) of factor Xa (New England Biolabs; P8010) were incubated with 0.1 µg of rPmpproPPAE2<sub>x</sub> at 1, 6 and 16 h at room temperature. Amidase activity of 0.1 µg rPmpproPPAE2<sub>x</sub> after activation by 0.5 µg factor Xa for 16h was assayed by adding to a 96-well plate containing protease buffer (100 mM Tris-HCl, 100 mM NaCl, 5 mM CaCl<sub>2</sub>, pH 7.8), followed by adding VPR (B9385) as substrate. Fluorescence signal was then read using 380 nm excitation and 460 nm emission at 1 h by the plate reader (SpectraMax M5).

**In vitro activation of rPmpproPO1, 2 by active rPmpproPPAE2**<sub>x</sub>

Activation of rPmpproPO1 and rPmpproPO2 by active rPmpproPPAE2<sub>x</sub> (factor Xa-activated rPmpproPPAE2<sub>x</sub>) was examined. To obtain active rPmpproPPAE2<sub>x</sub>, rPmpproPPAE2<sub>x</sub> (23 nM) was activated by 0.5 µg of factor Xa for 6h at room temperature. rPmpproPO1 (125 nM) or rPmpproPO2 (125 nM) were then incubated with active rPmpproPPAE2<sub>x</sub> (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. rPmpproPO1 and rPmpproPO2 activity were assessed after activation by incubating rPmpproPO1, rPmpproPO2 or BSA as a control (125 nM) with active rPmpproPPAE2 (23 nM) in a 96-well plate containing 10 mM Tris-HCl (pH 8.0) and 5 µM CuCl<sub>2</sub> (80 µl), for 30 min. L-DOPA (15 µl of 3.0 mg ml<sup>−1</sup>) was then added as substrate to determine PO activity. PO activity was observed by measuring absorbance at 490 nm at 1h 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 RNasy 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 CGATCGCCATGAAGTTGATGGAGTTCCTGAAAGAG/ WSSV453-R: CTTGTTTAACCTTGCCTCATTTCCTTG TTGTA) were used and the primer for 185 rRNA (18S-F: TATACCGTAGAGTTGAGCTGGA/18S-R: GGAGAG TAGTGACGAAAAAT) 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 lystate. Haemocyte lystate (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 rPmproPPAE2xa. rPmproPPAE2xa (23 nM) was activated by incubation with 0.5 µg factor Xa for 6 h while active proPOs were produced by incubating rPmproPOs (125 nM) with active rPmproPPAE2xa (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 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. rPmproPPAE2xa 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 with 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 25000 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. rPmproPPAE2xa 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.

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