**Melanization reaction products of shrimp display antimicrobial properties against their
major bacterial and fungal pathogens**

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22 ABSTRACT

23 Melanization is a rapid defense mechanism in invertebrates. The substrate specificity of
24 phenoloxidas (POs) and the role of melanization reaction products were investigated in the
25 black tiger shrimp, *Penaeus monodon*. Two *Pm*POs (*Pmpro*PO1 and *Pmpro*PO2) were found
26 to display a substrate specificity towards monophenols and diphenols, and exhibit relatively
27 weak activity against 5,6-dihydroxyindole (DHI). Systemic infection of the *Pmpro*PO1/2 co-
28 silenced shrimp with the fungus, *Fusarium solani*, led to a significantly increased mortality,
29 suggesting an important role of *Pmpro*POs in shrimp's defense against fungal infection.
30 Using L-DOPA, dopamine or DHI as a substrate, the melanization reaction products
31 exhibited *in vitro* antimicrobial activities towards Gram-negative bacteria (*Vibrio harveyi* and
32 *V. parahaemolyticus*) and Gram-positive bacteria (*Bacillus subtilis*), whereas the lower effect
33 was detected against the fungus (*F. solani*). SEM analysis revealed the morphological
34 changes and damage of cell membranes of *V. harveyi* and *F. solani* after treatment with
35 shrimp melanization reaction products. Together, these findings demonstrate the crucial
36 functions of the proPO system and the importance of melanization reaction products in the
37 shrimp's immune defense.

38

39 *Keywords:* Shrimp, Melanization reaction products, Phenoloxidase, *Vibrio harveyi*, *Fusarium*
40 *solani*

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42 **1. Introduction**

43 Phenoloxidase (PO)-mediated melanization is a conserved non-self defense response
44 involved in the synthesis of a melanin coat, and several cytotoxic effectors, such as reactive
45 intermediates of oxygen or nitrogen molecules, to combat foreign microbes in invertebrates
46 (Amparyup et al., 2013a; Beutler, 2004; Cerenius and Söderhäll, 2004; Cerenius et al., 2008;
47 Janeway and Medzhitov, 2002; Kanost and Gorman, 2008). Uncontrolled or systemic
48 melanogenesis is thought to be deleterious to the host cells because excess or prolonged
49 levels of the cytotoxic substances can lead to host tissue damage and cell death. Melanin
50 synthesis should, therefore, be very site-specific and tightly regulated to minimize the
51 damage of an aberrant process (Cerenius et al., 2008; Nappi and Christensen, 2005).

52 The melanization cascade is initiated by the specific recognition the pathogen-
53 associated molecular patterns, including peptidoglycan from Gram-positive bacteria,
54 lipopolysaccharide (LPS) from Gram-negative bacteria and β -1,3-glucan from fungi, by host
55 pattern recognition proteins. This is followed by the subsequent sequential activation of the
56 clip-domain serine proteinases (clip-SPs) and their homologues, which in turn activates the
57 terminal clip-SP, proPO-activating enzymes (PPAEs), and leads to the specific conversion of
58 the proPO precursor to the functionally active PO (Söderhäll et al., 2013). Active PO then
59 oxidizes the mono- and di-phenolic substances to quinone precursors that are required for the
60 synthesis of melanin at the wound site or around the foreign invaders (Cerenius and
61 Söderhäll, 2004; Nappi and Christensen, 2005).

62 PO is a multimeric type III copper protein that contains two copper atoms co-
63 ordinated by six histidine residues and participates in multiple steps of melanin formation
64 (Nappi and Christensen, 2005; Sugumaran, 2002). Arthropod POs have been classified into
65 two types based on their substrate specificity (Barrett and Trevella, 1989; Decker et al.,
66 2007). The first type, the tyrosinase-POs catalyze the *o*-hydroxylation of monophenols

(monophenolase or cresolase activity) and the subsequent oxidation of *o*-diphenols to the reactive intermediate *o*-quinones (*o*-diphenolase or catecholase activity), whilst the second type, the catecholoxidase-POs contain only a diphenolase activity and so catalyze only the oxidation of diphenolic substrates. During melanization, PO converts tyrosine to L-3,4-dihydroxyphenylalanine (L-DOPA) by its monophenolase activity. DOPA in turn is oxidized to dopaquinone by the diphenolase activity of PO or to dopamine by DOPA decarboxylase (DDC). Dopaquinone is converted to dopachrome non-enzymatically and further decarboxylated to form 5,6-dihydroxyindole (DHI) using the dopachrome conversion enzyme (Bai et al., 1996; Shelby et al., 2000; Sugumaran, 1996). Ultimately, dopamine and its oxidation product (DHI) are converted to melanin either by PO activity or via a series of intermediate steps involving both enzymatic and non-enzymatic reactions (Fang et al., 2002; Shelby et al., 2000; Sritunyalucksana and Söderhäll, 2000).

In invertebrates, the melanization reaction, proPO activation and DHI production play critical roles in the defense against microbial infections (Amparyup et al., 2009, 2013a, 2013b; Binggeli et al., 2014; Cerenius et al., 2008, 2010; Charoensapsri et al., 2009, 2011; Eleftherianos et al., 2007; Fagutao et al., 2009; Kan et al., 2008; Liu et al., 2007; Nappi and Christensen, 2005; Yassine et al., 2012; Zhao et al., 2007, 2011). Several reactive compounds generated during melanization have been reported to be toxic to pathogens. In *Manduca sexta*, the melanization reaction products generated from dopamine and DHI are much more efficiently aggregated and killed bacterial cells than that of L-DOPA (Zhao et al., 2007). In *Tenebrio molitor*, the melanization complex assembled from PO and serine proteinase homologue (SPH) 1 has been reported to possess strong bactericidal effect and induced melanin synthesis deposition on the surface of bacteria (Kan et al., 2008). In addition, DHI and its spontaneous oxidation products also shown to exhibit a strong toxicity against various fungi, baculoviruses, bacteriophages and parasitic wasps (Zhao et al., 2007, 2011). In the

92 crustacean *Pacifastacus leniusculus*, it is also evident that the melanization reaction products
93 generated during an *in vitro* PO activation exhibited an antibacterial effect against selected
94 bacteria in which a strong antibacterial activity was observed when dopamine was used as
95 substrate, as compared with L-DOPA, and this effect was abolished by the PO inhibitor,
96 phenylthiourea (PTU) (Cerenius et al., 2010). These findings collectively indicated that the
97 potential roles of reactive intermediates generated during melanization include their toxicity
98 towards, and killing of, invading microorganisms (Cerenius et al., 2010; Kan et al., 2008;
99 Zhao et al., 2007, 2011).

100 In the black tiger shrimp, *Penaeus monodon*, several genes implicated in the proPO
101 system, including two proPOs (*PmproPO1* and *PmproPO2*), two PPAEs (*PmPPAE1* and
102 *PmPPAE2*), a LPS- and β -1,3-glucan binding protein (*PmLGBP*) and a clip-SP (*PmClipSP2*),
103 have been identified and their important functions in the shrimp's defense against *Vibrio*
104 *harveyi* infection have been characterized (Amparyup et al., 2009, 2012, 2013b;
105 Charoensapsri et al., 2009, 2011). In this study, the substrate specificity of the two *PmproPO*
106 proteins in *P. monodon* shrimp was investigated using RNAi-mediated gene silencing of the
107 *PmproPO1* and *PmproPO2* genes (and so likely represents the substrate specificity of *PmPO1*
108 and *PmPO2*). *Fusarium solani* is one of the fungal pathogens caused disease problem in
109 shrimp aquaculture. Here, the crucial role of the two *PmproPOs* in the host defense against
110 infection with the filamentous fungus *F. solani* was subsequently elucidated. The *in vitro*
111 potential effects of the reactive compounds generated from the available phenolic substrates,
112 L-DOPA, dopamine and DHI, during the melanization process in contributing to the killing
113 of selected model pathogenic Gram-negative bacteria, Gram-positive bacteria and a fungus
114 was demonstrated.

115

116 2. Materials and methods

2.1. Animals

Specific pathogen-free (SPF) *P. monodon* shrimp (~5 g wet weight) were obtained from the Shrimp Genetic Improvement Center, BIOTEC, Thailand. Prior to the experiments, all shrimp were acclimatized in aerated seawater (20 ppt salinity) under laboratory conditions for at least 7 d.

2.2. Chemicals

Dopamine, L-tyrosine, LPS from *Escherichia coli* 0111:B4 and laminarin (β -glucan) from *Laminaria digitata* were purchased from Sigma-Aldrich, L-DOPA was from Fluka and DHI was from Santa Cruz Biotechnology. All general chemicals, unless otherwise noted, were of analytical grade and purchased from local agencies.

2.3. Microbial cell preparation

Vibrio harveyi isolate 639 and *V. parahaemolyticus* was cultured in aerated tryptic soy broth at 30 °C as previously described (Charoensapsri et al., 2009). The mid-exponential phase bacteria (optical density at 600 nm (OD₆₀₀) of 0.6) were harvested by centrifugation at 1000 × g for 10 min at 4 °C, and then washed twice and resuspended in sterile 150 mM NaCl solution to an OD₆₀₀ of 0.06.

A single colony of *Bacillus subtilis* was picked from Luria-Bertani (LB) agar plate and grown overnight in LB broth at 30 °C with constant shaking. After re-inoculated into fresh LB medium, bacterial cells were cultivated with shaking until they reached exponential log phase. Cells were subsequently harvested by centrifugation, washed twice and resuspended at an OD₆₀₀ of 0.06 as described above.

Pure cultures of *F. solani* were grown on potato dextrose agar (PDA) at 25 °C in the dark for 10 d. Conidia (spores) were harvested from the agar surface by flooding with a

sterile 150 mM NaCl solution. The suspension was filtered through a sterilized cheese cloth before being centrifuged at $500 \times g$ for 10 min. After washing twice and re-suspending in 150 mM NaCl, the suspension of fungal conidia was counted under a microscope, diluted to the appropriate concentration and used for all experiments.

2.4. Total RNA isolation, cDNA synthesis and semi-quantitative (sq)-RT-PCR analysis

Total RNA was extracted using the TRI Reagent® (Molecular Research Center) following the manufacturer's protocol. First-strand cDNA was synthesized from the DNase I-treated total RNA according to the manufacturer's instructions provided by the RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific). The semi-quantitative (sq)-RT-PCR was carried out as described previously (Amparyup et al., 2009) using the gene specific primers listed in Table 1. A fragment of elongation factor 1- α (EF1- α) gene was amplified and used as an internal reference control (Table 1). Each amplified product was analyzed by agarose gel electrophoresis followed by ethidium bromide staining and UV-transillumination.

2.5. Double-stranded RNA (dsRNA) preparation and in vivo gene silencing

DsRNAs corresponding to the *PmproPO1* and *PmproPO2* transcripts and the control GFP gene were synthesized *in vitro* by PCR amplification (primers in Table 1) using the T7 RiboMAX™ Express Large Scale RNA Production Systems (Promega) as described previously (Amparyup et al., 2009). *In vivo* *PmproPO1* and *PmproPO2* gene silenced shrimp, plus the GFP dsRNA- and 150 mM NaCl-injected shrimp controls, were created as described (Amparyup et al., 2009). Hemolymph was collected from the knockdown and control shrimp at 48 h after the second dsRNA(s) injection. The efficiency and specificity of gene knockdown was subsequently verified by means of sq-RT-PCR (Amparyup et al., 2009).

2.6. Substrate specificity of *PmproPO1* and *PmproPO2* (*PmPO1* and *PmPO2*)

Substrate specificity analysis of *PmproPO1* and *PmproPO2* was achieved by examining the ability of the *PmproPO1* and *PmproPO2* gene-silenced shrimp to hydrolyze various PO substrates. At 48 h after the second dsRNA injection (or 150 mM NaCl only for the control), hemolymph was collected from the gene silenced and control shrimp and 400 µg of this was separately mixed with the PO substrates of: the monophenol L-tyrosine, the diphenols L-DOPA or dopamine, and the catechol compound DHI, all in 10 mM Tris-HCl buffer (pH 8.0). The reaction mixture was incubated at room temperature (RT) for 30 min before an equal volume of 10% (v/v) acetic acid was added. The increase in the absorbance at 490 nm (A_{490}) was subsequently monitored using a SpectraMax M5 microplate reader (Molecular Devices). The protein concentration was determined by the Bradford assay (Bio-Rad). The addition of reaction buffer instead of shrimp hemolymph was used as the negative control. The activity of the PO enzyme was defined as $\Delta A_{490}/\text{mg total protein}/\text{min}$. The experiment was performed in triplicate with three samples from three pooled shrimp/group.

2.7. Cumulative mortality of *PmproPO1/2* co-silenced shrimp upon infection with *F. solani*

To assess the essential role of the proPO system in the immune response against the pathogenic fungus *F. solani*, SPF *P. monodon* shrimp were co-injected with *PmproPO1* and *PmproPO2* dsRNAs (2.5 µg/g shrimp, wet body weight). Control shrimp were injected with GFP dsRNA or just 150 mM NaCl. At 24 h after the first injection (hpi), each respective group of shrimp was re-injected again with the same amount/type of dsRNA as the first time but also containing 7×10^5 conidia of the filamentous fungus *F. solani*. Shrimp were then examined daily and the mortality was recorded, from which the percent cumulative mortality was evaluated. The experiment was performed in triplicate, with each replicate containing 9–10 shrimp/group.

2.8. Preparation of shrimp hemocyte lysate supernatant (SHLS)

Hemolymph (500 μ L) was individually withdrawn from the ventral sinus cavity of healthy *P. monodon* shrimp using a 1-mL syringe containing 500 μ L ice-cold anticoagulant solution (0.45 M NaCl, 0.1 M glucose, 26 mM citric acid, 30 mM trisodium citrate, 10 mM EDTA; pH 4.6). Hemocytes were immediately collected by centrifugation at $800 \times g$ for 10 min at 4 °C and the obtained hemocyte pellet was immediately washed twice and homogenized in 100 μ L cacodylate buffer (10 mM sodium cacodylate, 10 mM CaCl_2 ; pH 7.0) before being centrifuged at $25000 \times g$ for 20 min at 4 °C. The shrimp hemocyte lysate supernatant (SHLS) was harvested, adjusted to a protein content of 1 mg/mL and subsequently used as a source of the PO enzyme for preparation of the active melanization reaction products.

2.9. *In vitro* antibacterial and antifungal activities of shrimp melanization reaction products

Melanization reaction products were prepared using L-DOPA, dopamine or DHI as the substrate. Twenty-five μ g of SHLS was pre-incubated with either 25 μ L of L-DOPA (2 mM), dopamine (2 mM) or DHI (2mM) at RT for at least 10 min before being incubated with 25 μ L of one of the Gram-negative bacteria (*V. harveyi* and *V. parahaemolyticus*), Gram-positive bacteria (*B. subtilis*) or the filamentous fungus (*F. solani*) at RT for 1 h with constant shaking. The treated microbial cells were serially diluted and dotted onto LB or PDA plates (for bacterial or fungal counts, respectively, as appropriate). The number of bacterial colonies or fungal hyphal body as colony forming units (CFUs) was then counted after incubation at 30 °C for 18–48 h. SHLS pre-incubated with 0.1mM PTU before being mixed with PO substrates and microbial cells were included as control. Microbial cells incubated with SHLS or substrates alone were included as control groups, and no significant differences between the mean colony counts, except for DHI due to its cytotoxicity (Zhao et al., 2007, 2011), were observed (data not shown).

2.10. Scanning electron microscopy (SEM) analysis

The cytotoxicity of the PO substrates and the effect of melanization reaction products on the pathogenic bacteria *V. harveyi* and the fungus *F. solani* were evaluated in terms of any changed cellular morphology using scanning electron microscopy (SEM). *V. harveyi* or *F. solani* (1×10^7 CFUs) were incubated alone with L-DOPA, dopamine or DHI (2 mM or 20 mM), or incubated with the shrimp melanization reaction products prepared from SHLS using L-DOPA, dopamine or DHI at a 2 mM final concentration as the substrate at RT for 1 h. Cells were then fixed with 2.5% (w/v) glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) for 2 h at RT. Preparation of the microbial cells for SEM analysis was carried out as described previously (Amparyup et al., 2013b). Samples were observed by SEM under a JEOL model JSM-5410LV scanning electron microscope.

2.11. Data analysis

Numerical data is shown as the mean $\pm$ one standard error of the mean (1 SE) (as error bars in figures) and the statistical significance of the differences between means was tested for using a one-way ANOVA followed by Duncan's test, excepting significance at the $P < 0.05$ level.

3. Results

3.1. *PmproPO1* and *PmproPO2* both exhibit substrate specificity toward monophenol and diphenols

PO catalyzes the key step of melanin biosynthesis by oxidation of mono- and diphenol substrates. In order to assess the substrate specificity of the two known POs in *P. monodon* shrimp, the gene transcripts of their precursors (*PmproPO1* and *PmproPO2*) were

independently silenced by dsRNA-mediated gene knockdown. As shown in Fig. 1A, sq-RT-PCR analysis revealed that *PmproPO1*- and *PmproPO2*- dsRNAs significantly suppressed the expression of *PmproPO1* and *PmproPO2* transcripts, respectively, and no RNAi effect was observed among the two transcripts as compared with the control GFP dsRNA- and NaCl-injected shrimp, suggesting that these gene knockdown experiments were effective and sequence specific. Thus, the subsequent ability of the knockdown shrimp to oxidize a range of phenolic substrates was then evaluated. Using L-tyrosine as a monophenolic substrate the PO activity of *PmproPO1*- and *PmproPO2*-silenced shrimp were found to be significantly and equally decreased to around a 59% residual activity, some 1.7- to 1.8-fold lower than that of the control shrimp injected with GFP dsRNA or NaCl alone (Fig. 1B). The ability of two shrimp POs to oxidize diphenolic substrates was assessed using L-DOPA or dopamine as the substrate, where a significant decrease in the PO activity was observed in both the *PmproPO1*- and *PmproPO2*-silenced shrimp (Fig. 1C and D). With L-DOPA as the substrate a residual hemolymph PO activity of 5.5% and 7.4% was observed in the *PmproPO1*- and *PmproPO2*-silenced shrimp, respectively (Fig. 1C). Using dopamine as the substrate, a similar residual hemolymph PO activity of 8.5% and 10.3% was detected in the *PmproPO1*- and *PmproPO2*-silenced shrimp, respectively (Fig. 1D). It has previously been reported that DHI is an intermediary product that is essential for melanin formation and exhibits a broad-spectrum antimicrobial activity (Zhao et al., 2007, 2011). In this study, the substrate specificity of the two *P. monodon* POs against DHI was evaluated as above and a 61.4% residual PO activity was observed in the *PmproPO1*-silenced shrimp and this was not significantly different to the numerically smaller residual PO activity of 45.7% in the *PmproPO2*-silenced shrimp, indicating that DHI is a relatively poor substrate for shrimp *PmPOs* (Fig. 1E). Taken together, these findings suggest that *PmproPO1* and *PmproPO2* are tyrosinase-POs that exhibit substrate specificity towards monophenol and diphenol substrates.

3.2. Melanization is essential for the defense against *F. solani* infection

Melanization is suggested to be an effective immune mechanism that plays important roles in protecting shrimp from bacterial infections (Amparyup et al., 2013a). A previous study demonstrated that gene silencing of *PmproPO1* and *PmproPO2* transcripts significantly increased the susceptibility of *P. monodon* to infection with the Gram-negative pathogenic bacteria *V. harveyi* (Amparyup et al., 2009). However, the potential role of the shrimp proPO system in the defense against pathogenic fungal infections remains unclear. Here, *in vivo* RNAi-mediated silencing of the two *PmproPO* gene transcripts in *P. monodon* was followed by challenge with *F. solani*. Interestingly, a significant increase in the cumulative mortality of the *PmproPO1/2* co-silenced shrimp was observed after *F. solani* challenge (Fig. 2), reaching 49% and 93% after 2 and 5 dpi with *F. solani* compared to 7 - 14%, and 4 - 33% for the two groups of NaCl- and GFP dsRNA- injected control shrimp, respectively. This indicates that suppression of the two *PmproPOs* transcripts led to an enhanced shrimp susceptibility to fungal infection, and so suggests the important roles of the melanization cascade of shrimp in the defense against *F. solani* infection.

3.3. *In vitro* antibacterial and antifungal effects of shrimp melanization reaction products

L-DOPA and dopamine are the available substrates for shrimp *PmPOs* (as above), whereas DHI has previously shown to exhibit strong antimicrobial activities *in vitro* (Zhao et al., 2007, 2011). Analysis of the *in vitro* antimicrobial effects of the melanization reaction products prepared from *P. monodon* SHLS using L-DOPA, dopamine or DHI as the substrate efficiently diminished the colony counts of *V. harveyi*, *V. parahaemolyticus* and *B. subtilis*, whereas less effects were observed against the fungus *F. solani* (Fig. 3A-D). A 60–100% decrease in the bacterial cell numbers was found for the Gram-negative *V. harveyi* and *V. parahaemolyticus* following treatment with the shrimp melanization reaction products (Fig.

3A and Fig. 3B, respectively), whilst the Gram-positive *B. subtilis* showed an 80–100% decrease in viable cell numbers after such treatment (Fig. 3C). However, only 20%, 29% and 82% decrease in the number of hyphal body colonies were detected in *F. solani* following treatment with the melanization reaction products using L-DOPA, dopamine and DHI as substrate, respectively (Fig. 3D). In addition, 0.1 mM PTU were found to abolish the effect of melanization on microbial cell viability (Fig. 3A-D). Taken together, these data suggested that the melanization reaction products produced from shrimp using L-DOPA, dopamine or DHI as the substrate are responsible for the *in vitro* antibacterial activities against *V. harveyi*, *V. parahaemolyticus* and *B. subtilis*, but less efficient against the fungus *F. solani*.

3.4. *In vitro* effect of the melanization reaction products on the morphology of *V. harveyi*

SEM analysis was performed to unveil the cytotoxic effect of the phenolic substrates (L-DOPA, dopamine or DHI) as well as their derived melanization reaction products on the bacterial cells in terms of any altered cellular morphology. Analysis of the cytotoxic of L-DOPA on the *V. harveyi* cells showed that L-DOPA at 2 mM or 20 mM did not cause any detectable increased morphological damage to cells compared to the control group (Fig. 4). Dopamine at 2mM did not affect the bacterial cells, however, some minor cellular damages were observed after treatment with 20 mM dopamine (Fig. 4). Treatment with 2mM DHI, a higher proportion of *V. harveyi* cells was damaged and to a greater extent, including rough-surfaced cells, the presence of dimples at each end of the bacterial cell and the aggregation of treated cells. Moreover, severe damage with segmentation of bacterial cells into small pieces and cell clumping was observed after *V. harveyi* cells were treated with 20 mM DHI (Fig. 4).

The effect of melanization reaction products on the morphology of *V. harveyi* cells was then accessed by incubated the bacterial cells with shrimp melanization reaction products prepared using 2 mM of L-DOPA, dopamine or DHI as the substrate and the ultrastructure of the bacterial cells was then elucidated. SEM analysis revealed that the melanization reaction

products prepared from SHLS with each of the three selected substrates caused cell corrugated and numerous disruptions to the cell membranes of *V. harveyi* (Fig. 4). In addition, bacterial aggregation was observed in *V. harveyi* treated with the melanization reaction products prepared using SHLS with dopamine or DHI as the substrate. In addition, SEM images revealed that the extensive damage to *V. harveyi* cells caused by treatment with the melanization reaction products could be abolished by 0.1 mM PTU (Fig. 4). In the case of the control groups, some minor changes in the bacterial cell length, a decrease in cell compactness and the presence of some corrugated cells were observed in the SHLS-treated control cells, but no damage was detected on the surface of the bacterial cells. These findings suggest that the melanization reaction products generated by *P. monodon* proPO system exhibit an *in vitro* antibacterial activity against *V. harveyi*.

3.5. *In vitro* effect of the melanization reaction products on the morphology of *F. solani*

To further examine the cytotoxicity of three phenolic substrates (L-DOPA, dopamine and DHI) on the *F. Solani* cells, the fungus conidia were incubated with 2 mM or 20 mM of each substrates and morphology of cells were then observed under the SEM. As shown in Fig. 5, no adverse effect on the morphology of *F. solani* cells was detected after incubated with 2 mM of L-DOPA and dopamine. However, when the concentration was increase ten times, the distinct cell morphologies were evident (Fig. 5), with the presence of burst cells and clearly visible membrane pores after treatment with 20 mM L-DOPA, and the decomposition and rupture of cell membranes after treatment with 20 mM dopamine. In the case of DHI, an extensive effect in the fungal cell morphology including the presence of fungal cell debris, as observed after treatment with 2 mM DHI, and a higher frequency of more severely damaged and ruptured cells with a preponderance of cell debris after treatment with 20 mM DHI was visualized (Fig. 5).

The cytotoxic effect of the shrimp melanization reaction products against *F. solani* was then evaluated using SEM analysis. Fungal conidia were treated with shrimp melanization reaction products prepared using 2 mM L-DOPA, dopamine or DHI as the substrate. Interestingly, it was found that severe damage and membrane rupture was observed on the surface of fungal cells after treatment with the melanization reaction products prepared from three substrates, whilst the fungal cell aggregate was observed after treatment with the melanization products generated from the dopamine or DHI (Fig. 5). For the control group, some corrugated cells were observed after cells were treated alone with SHLS, however, no cell damage was detected. These results suggested a potentially important function of the shrimp melanization reaction products in destroying the fungal pathogen *F. solani in vitro*.

4. Discussion

Melanin biosynthesis in invertebrates is achieved by the activation cascade of the proPO system (Amparyup et al., 2013a; Cerenius and Söderhäll, 2004; Cerenius et al., 2008; Kanost and Gorman, 2008). In *P. monodon*, several proPO system associated genes, including two proPOs (*PmproPO1* and *PmproPO2*), two PPAEs (*PmPPAE1* and *PmPPAE2*) and a clip-SP (*PmClipSP2*), have been identified and characterized as important components that not only play key roles in the shrimp proPO system, but are also crucial for the shrimp's immune defense against infection with the pathogenic bacteria *V. harveyi* (Amparyup et al., 2009, 2013b; Charoensapsri et al., 2009, 2011). However, the substrate specificity of *P. monodon* POs as well as the toxicity of their oxidative products have not been clarified.

Here, RNA interference (RNAi)-mediated gene silencing of *PmproPO1* and *PmproPO2* genes was employed to elucidate the substrate specificity of two shrimp POs. The PO activity of the *PmproPO1*- and/or *PmproPO2*-silenced shrimp was assayed using L-

tyrosine as a monophenol substrate, L-DOPA or dopamine as the diphenol substrates, and additionally the catechol compound, DHI. The results indicated that both *PmPO1* and *PmPO2* likely exhibit substrate specificity toward diphenols (L-DOPA and dopamine) followed by monophenol (tyrosine), but less against the catechol compound (DHI). This finding is in accord with the reported substrate specificity of other arthropod POs in insects and crustaceans (Asano and Ashida, 2001; Aspán et al., 1995; Gollas-Galván et al., 1999; Hall et al., 1995; Sugumaran, 1998, 2002). For example, the active POs from the insect *M. sexta* preferred to oxidize dopamine and its *N*-acylated derivatives than DOPA and tyrosine (Hall et al., 1995; Sugumaran, 1998, 2002). In the silkworm, *Bombyx mori*, the purified proPOs displayed substrate specificities and specific activities towards the *o*-diphenols as: dopamine > *N*-acetyldopamine > L-DOPA (Asano and Ashida, 2001). In crustaceans, the purified proPO from the crayfish *P. leniusculus* readily hydroxylated monophenols and oxidized *o*-diphenols, but did not oxidize *p*-diphenols (Aspán et al., 1995). A similar substrate specificity was also reported in the brown shrimp, *P. californiensis*, in which the purified proPO was capable of oxidizing monophenols and *o*-diphenols, but not *p*-diphenols (Gollas-Galván et al., 1999).

Based on their substrate specificity, arthropod POs have been classified into the two types of tyrosinase-POs and catecholoxidase-POs (Barrett and Trevella, 1989; Decker et al., 2007). The tyrosinase-POs exhibit both monophenolase and diphenolase activities and are involved in the hydroxylation of monophenols to *o*-diphenols and the further oxidation of *o*-diphenols to *o*-quinones. In contrast, catecholoxidase-POs contain only a diphenolase activity and are implicated only in the oxidation of diphenolic substrates. Since *PmproPO1* and *PmproPO2* (and so presumably *PmPO1* and *PmPO2*) displayed the same monophenolase and diphenolase activities, we consequently classified them both as tyrosinase-POs.

Melanin biosynthesis has been demonstrated to be a potent, rapid and efficient immune effector that plays crucial roles in the defense against invading pathogens in invertebrates. The importance of PO in eliminating microbial pathogens has been reported in many insects and crustaceans (Amparyup et al., 2009, 2013a 2013b; Binggeli et al., 2014; Cerenius et al., 2008, 2010; Charoensapsri et al., 2009, 2011; Eleftherianos et al., 2007; Fagutao et al., 2009; Kan et al., 2008; Liu et al., 2007; Nappi and Christensen, 2005; Yassine et al., 2012; Zhao et al., 2007, 2011). In the insect *M. sexta*, silencing of PO resulted in a much higher host susceptibility to the virulent bacterium *Photorhabdus* infection (Eleftherianos et al., 2007). Moreover, melanization was also shown to be important for insect defense against fungal infection. The melanization response of the mosquito *Anopheles gambiae* was reported to be implicated in its defense against infection with the entomopathogenic fungus *Beauveria bassiana* (Yassine et al., 2012). Recently, melanization was shown to significantly contribute to *Drosophila* survival to fungi and Gram-positive bacterial infections (Binggeli et al., 2014). In the crustacean crayfish *P. leniusculus*, PO activity is required for the crayfish to defend against infection with the bacterium *Aeromonas hydrophila* (Liu et al., 2007). In penaeid shrimp, the importance of proPO system has also been demonstrated. Gene knockdown of *Marsupenaeus japonicus* proPO precursor resulted in a significant increase in the bacterial load and a sharp increase in shrimp mortality even in the absence of microbial challenge (Fagutao et al., 2009). In shrimp *P. monodon*, we previously showed that the two *PmproPOs* are active components that play a crucial role in protecting shrimp from infection with the highly pathogenic bacterium *V. harveyi* (Amparyup et al., 2009). However, no *in vivo* experiment demonstrates the roles of the proPO system in the fungal defense. Here, we employed the *in vivo* gene silencing experiment to investigate the roles of *PmproPO1* and *PmproPO2* against the fungal infection. In accordance with the previous reports (Binggeli et al., 2014; Yassine et al., 2012), a significant increase in the

cumulative mortality was observed after *PmproPO1* and *PmproPO2* silenced shrimp were challenged with the pathogenic fungus *F. solani*. This result suggests an antifungal role of two proPO genes (*PmproPO1* and *PmproPO2*) in the defense against *F. solani* infection in shrimp.

The melanization reaction frequently takes place at the site of injury or infection in which the melanin products are deposited on or near a pathogen to serve as a physical shield in the invertebrates from the invading pathogens. During melanization, highly reactive quinone intermediates are generated (Gillespie et al., 1997; Nappi and Christensen, 2005; Nappi et al., 1995). Quinones are capable of entering cytotoxic reactions, both enzymatic and non-enzymatic redox cycling, with their corresponding semi-quinone radicals and cause the production of cytotoxic free radicals, including the reactive oxygen species of superoxide anions, hydrogen peroxide and hydroxyl radicals, or to polymerize to form melanin (Arakawa, 1994; Leem et al., 1996; Nappi and Christensen, 2005; Nappi and Vass, 1998; Nappi et al., 1995; Zhao et al., 2007). To date, many aspects of melanin formation have been studied in arthropods (Reviewed by Cerenius et al., 2008; Kanost and Gorman, 2008). PO-catalyzed formation of quinones, semiquinones and DHI has been shown to be a potent defense mechanism to kill microbial pathogens (Cerenius et al., 2010; Kan et al., 2008; Zhao et al., 2007, 2011). The melanin precursors L-DOPA and DHI were detected in the hemolymph of immune reactive *Drosophila melanogaster* larvae parasitized by *Leptopilina boulardi* (Nappi et al., 1992), whereas dopamine was detected as a major melanin precursor in *M. sexta* plasma (Hopkins et al., 1984). However, little is known about the potential roles and reactivity of melanization reaction products generated by active POs in shrimp immunity. In this study, the *in vitro* cell viability test of the antibacterial and antifungal activity of the melanization reaction products generated from shrimp POs using L-DOPA, dopamine or DHI as the substrate were evaluated. The Gram-negative bacteria *V. harveyi* and *V.*

parahaemolyticus and the Gram-positive bacterium *Bacillus subtilis*, as well as the filamentous fungus *F. solani*, were used as model pathogens. The results demonstrated that the active compound(s) produced during the *P. monodon* melanization process effectively suppressed the growth of these bacterial and fungal species *in vitro*.

V. harveyi and *F. solani* are the major pathogenic bacteria and fungal species in penaeid shrimp. We tested the cytotoxic effect of the phenolic substrates on the morphology of microbial cells. L-DOPA, dopamine and DHI were used individually as the substrate at 2 mM as compared with the over dose (20 mM). SEM analysis revealed that DHI (2 mM), but neither L-DOPA nor dopamine, is cytotoxic and exhibited strong bactericidal and fungicidal activities against *V. harveyi* and *F. solani*, respectively. However, at 20 mM whilst DHI still displayed the highest bactericidal and fungicidal activities this was followed by dopamine and L-DOPA, respectively. The substrate concentration at 2mM were then selected for examining the toxicity of shrimp melanization reaction product against *V. harveyi* and *F. solani*. Interestingly, SEM analysis revealed that the melanization reaction products of *P. monodon* showed an effective antibacterial and antifungal activity against the selected microbes *in vitro*, consistent with the previous reports in *M. sexta*, *T. molitor* and *P. leniusculus* (Cerenius et al., 2010; Kan et al., 2008; Zhao et al., 2007, 2011).

PTU is a known inhibitor of PO enzymes and blocks the conversion of DOPA to dopaquinone and the conversion of DHI to melanochrome (Körner and Pawelek, 1982). Our SEM results clearly showed that 0.1 mM PTU significantly inhibited the antimicrobial activity of the PO-mediated melanization reaction of *P. monodon*. Albeit indirectly, these results support the involvement of intermediates of the proPO cascade in the generation of O_2^- in *P. monodon*. These results suggest that melanization reaction products of shrimp *P. monodon* exhibit both antibacterial and antifungal activities against the gram-negative

bacteria and fungus pathogens, emphasizing the important role of melanization in shrimp immunity.

Melanization is known to be crucial for killing invading microbes in a wide range of arthropods. The proteolytic activation of the proPO precursor as well as the PO-mediated reactive intermediates is an integral part of the shrimp's defense against microbial infection. In this study, both *PmproPO1* and *PmproPO2* exhibited substrate specificity toward mono- and di-phenols and were classified as tyrosinase-type POs. The results also demonstrated that two *PmproPOs* are important for the fungal defense against the pathogenic fungus *F. solani* infection in *P. monodon* shrimp. In addition, the antimicrobial effects of the reactive compounds generated during the PO activation were demonstrated and found to play a critical role in the killing of a variety of microbes, namely the representative Gram-negative bacteria (*V. harveyi* and *V. parahaemolyticus*), Gram-positive bacteria (*B. subtilis*) and filamentous fungus (*F. solani*). These findings provide the evidence supporting the important role of melanization in shrimp immune defense against microbial infections.

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Figure legends

Figure 1. Substrate specificity analysis of *P. monodon* POs. (A) Specificity and efficacy of gene silencing experiment of *PmproPO1* and *PmproPO2* transcripts by sq-RT-PCR using the gene-specific primers for each gene (Table 1). EF1- α was served as the internal reference gene. Each lane represents the cDNA from each of two individual shrimp. PO enzymatic activities were measured in the hemolymph of the *PmproPO1*- and *PmproPO2*-silenced shrimp using (B) the monophenol L-tyrosine, (C, D) the *o*-diphenols L-DOPA and dopamine, respectively, and (E) the catechol compound DHI as the substrate. The graphs show the mean ($\pm$ 1 SE) of PO activity at 30 min after the indicated substrates were added, and are derived from three independent repeats. Means with a different lowercase letter are significantly different ($P < 0.05$).

Figure 2. Cumulative mortality of *PmproPO1/2* co-silenced *P. monodon* after *F. solani* infection. *PmproPO1/2* co-silenced shrimp were injected with *F. solani* and then the shrimp mortality was recorded daily over a 5 dpi period and expressed as the percent cumulative mortality. Data are shown as the mean ($\pm$ 1 SE), and are derived from three independent experiments.

Figure 3. Melanization reaction products of *P. monodon* exhibit antibacterial and antifungal activities *in vitro*. Melanization reaction products were prepared from SHLS alone (no substrate) or SHLS with 2 mM L-DOPA, dopamine or DHI as the substrate. The (A, B) Gram-negative bacteria *Vibrio harveyi* and *V. parahaemolyticus*, respectively, the (C) Gram-positive bacteria *B. subtilis*, and (D) the filamentous fungus *F. solani* were treated with the above shrimp melanization reaction products with or without PTU, prior to further culture on LB (bacteria) or PDA (fungus) overnight and then the total viable colony number (CFUs)

was evaluated. Data are shown as the mean ($\pm$ 1 SE), and are derived from three independent repeats. Asterisk indicates a statistical significant difference between means ($P < 0.05$).

Figure 4. *In vitro* effect of phenolic substrates and *P. monodon* melanization reaction products on the morphology of *V. harveyi* cells. SEM images (10,000-20,000 x magnification) of *V. harveyi* treated alone with the indicated phenolic substrates (L-DOPA, dopamine and DHI) at 2 mM and 20 mM, or treated with shrimp melanization reaction products prepared from SHLS alone (no substrate) or SHLS with the indicated substrate (2 mM) in the presence and/or absence of 0.1 mM PTU. Images shown are representative of at least three such fields of view per sample and three independent experiments. Scale bars represent 1 μ m.

Figure 5. *In vitro* effect of phenolic substrates and melanization reaction products on the morphology of *F. solani* cells. SEM images (7,500-10,000 x magnification) of *F. solani* treated with the indicated phenolic substrate (L-DOPA, dopamine and DHI) at 2 mM or 20 mM, or treated with shrimp melanization reaction products prepared from SHLS alone (no substrate) or SHLS with the indicated substrate (2 mM). Images shown are representative of at least three such fields of view per sample and three independent experiments. Scale bars represent 1 μ m.

Table 1

Nucleotide sequences of the primers used.

Primer	Sequence (5'–3')	GenBank accession number	Purpose
PO1i-F	5'-CACCTGAGAGCTCCCACTT-3'	AF099741	RNAi
PO1i-R	5'-GAGATCCTGCAGTGTAACGT-3'	AF099741	RNAi
PO1iT7-F	5'- <u>GGATCCTAATACGACTCACTATAGG</u> CACCTGAGAGCTCCCACTT-3'	AF099741	RNAi
PO1iT7-R	5'- <u>GGATCCTAATACGACTCACTATAGG</u> GAGATCCTGCAGTGTAACGT-3'	AF099741	RNAi
PO2i-F	5'-CGTCTTCCTCGGCCGCTCCT-3'	FJ025814	RNAi
PO2i-R	5'-CCACGGGGATACCGCTGCCA-3'	FJ025814	RNAi
PO2iT7-F	5'- <u>GGATCCTAATACGACTCACTATAGG</u> CGTCTTCCTCGGCCGCTCCT-3'	FJ025814	RNAi
PO2iT7-R	5'- <u>GGATCCTAATACGACTCACTATAGG</u> CCACGGGGATACCGCTGCCA-3'	FJ025814	RNAi
GFP-F	5'-ATGGTGAGCAAGGGCGAGGA-3'	U55761	RNAi
GFP-R	5'-TTACTTGTACAGCTCGTCCA-3'	U55761	RNAi
GFPT7-F	5'- <u>TAATACGACTCACTATAGG</u> ATGGTGAGCAAGGGCGAGGA-3'	U55761	RNAi
GFPT7-R	5'- <u>TAATACGACTCACTATAGG</u> TTACTTGTACAGCTCGTCCA-3'	U55761	RNAi
PO1RT-F	5'-GGTCTTCCCCTCCCGCTTCG-3'	AF099741	sq-RT-PCR
PO1RT-R	5'-GCCGCAGGTCCTTTGGCAGC-3'	AF099741	sq-RT-PCR
PO2RT-F	5'-GCCAAGGGGAACGGGTGATG-3'	FJ025814	sq-RT-PCR
PO2RT-R	5'-TCCCTCATGGCGGTGCGAGGT-3'	FJ025814	sq-RT-PCR
EF1 α -F	5'-GGTGCTGGACAAGCTGAAGGC-3'		sq-RT-PCR
EF1 α -R	5'-CGTTCCGGTGATCATGTTCTTGATG-3'		sq-RT-PCR

The T7 polymerase binding site as a 5' flanking sequence is underlined.

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5