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

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ABSTRACT

Melanization is a rapid defense mechanism in invertebrates. The substrate specificity of phenoloxidases (POs) and the role of melanization reaction products were investigated in the black tiger shrimp, *Penaeus monodon*. Two *Pm*POs (*Pm*proPO1 and *Pm*proPO2) were found to display a substrate specificity towards monophenols and diphenols, and exhibit relatively weak activity against 5,6-dihydroxyindole (DHI). Systemic infection of the *Pm*proPO1/2 co-silenced shrimp with the fungus, *Fusarium solani*, led to a significantly increased mortality, suggesting an important role of *Pm*proPOs in shrimp's defense against fungal infection. Using L-DOPA, dopamine or DHI as a substrate, the melanization reaction products exhibited *in vitro* antimicrobial activities towards Gram-negative bacteria (*Vibrio harveyi* and *Vibrio parahaemolyticus*) and Gram-positive bacteria (*Bacillus subtilis*), whereas the lower effect was detected against the fungus (*F. solani*). SEM analysis revealed the morphological changes and damage of cell membranes of *V. harveyi* and *F. solani* after treatment with shrimp melanization reaction products. Together, these findings demonstrate the crucial functions of the proPO system and the importance of melanization reaction products in the shrimp's immune defense.

1. Introduction

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

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

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

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

In the black tiger shrimp, *Penaeus monodon*, several genes implicated in the proPO system, including two proPOs (*Pm*proPO1 and *Pm*proPO2), two PPAEs (*Pm*PPAE1 and *Pm*PPAE2), a LPS- and β-1,3-glucan binding protein (*Pm*LGBP) and a clip-SP (*Pm*ClipSP2), have been identified and their important functions in the shrimp's defense against *Vibrio harveyi* infection have been characterized (Amparyup et al., 2009, 2012, 2013b; Charoensapsri et al., 2009, 2011). In this study, the substrate specificity of the two *Pm*proPO proteins in *P. monodon* shrimp was investigated using RNAi-mediated gene silencing of the *Pm*proPO1 and *Pm*proPO2 genes (and so likely represents the substrate specificity of *Pm*PO1 and *Pm*PO2). *Fusarium solani* is one of the fungal pathogens caused disease problem in shrimp aquaculture. Here, the crucial role of the two *Pm*proPOs in the host defense against infection with the filamentous fungus *F. solani* was subsequently elucidated. The *in vitro* potential effects of the reactive compounds generated from the available phenolic substrates, L-DOPA, dopamine and DHI, during the melanization process in contributing to the killing of selected model pathogenic Gram-negative bacteria, Gram-positive bacteria and a fungus was demonstrated.

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

V. 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 (OD_{600}) 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 OD_{600} 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 OD_{600} 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×*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 *Pm*proPO1 and *Pm*proPO2 transcripts and the control GFP gene were synthesized *in vitro* by PCR amplification (primers in Table 1) using the T7 RiboMAX™ Express