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# A shrimp pacifastin light chain-like inhibitor: Molecular identification and role in the control of the prophenoloxidase system

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ABSTRACT

Pacifastin is a recently classified family of serine proteinase inhibitors that play essential roles in various biological processes, including in the regulation of the melanization cascade. Here, a novel pacifastin-related gene, termed *Pm*Pacifastin-like, was identified from a reverse suppression subtractive hybridization (SSH) cDNA library created from hemocytes of the prophenoloxidase *Pm*proPO1/2 co-silenced black tiger shrimp *Penaeus monodon*. The full-length sequences of *Pm*Pacifastin-like and its homologue *Lv*Pacifastin-like from the Pacific white shrimp *Litopenaeus vannamei* were determined. Sequence analysis revealed that both sequences contained thirteen conserved pacifastin light chain domains (PLDs), followed by two putative kunitz domains. Expression analysis demonstrated that the *Pm*Pacifastin-like transcript was expressed in all tested shrimp tissues and larval developmental stages, and its expression responded to *Vibrio harveyi* challenge. To gain insight into the functional roles of *Pm*Pacifastin-like protein, the *in vivo* RNA interference experiment was employed; the results showed that *Pm*Pacifastin-like depletion strongly increased PO activity. Interestingly, suppression of *Pm*Pacifastin-like also down-regulated the expression of the proPO-activating enzyme *Pm*PPAE2 transcript; the *Pm*Pacifastin-like transcript was down-regulated after the *Pm*proPO1/2 transcripts were silenced. Taken together, these results suggest that *Pm*Pacifastin-like is important in the shrimp proPO system and may play an essential role in shrimp immune defense against bacterial infection. These results also expand the knowledge of how pacifastin-related protein participates in the negative regulation of the proPO system in shrimp.


## 1. Introduction

Melanization caused by the activation of the prophenoloxidase (proPO) system is a potent innate defense mechanism that plays a crucial role in pathogen elimination in diverse arthropod species (Amparyup et al., 2013a; Cerenius et al., 2008; Cerenius and Söderhäll, 2004; Kanost and Gorman, 2008). Activation of the proPO system requires microbial identification, followed by sequential activation of a cascade of clip-domain serine proteinases (clip-SPs). In this cascade, a proPO-activating enzyme (PPAE) functions as a terminal proteinase that specifically cleaves and activates the zymogen prophenoloxidase (proPO) to generate phenoloxidase (PO). Active PO then oxidizes phenols to quinones for the production of melanin and other cytotoxic intermediates to encapsulate and kill invading pathogens (Cerenius and Söderhäll, 2004; Nappi and Christensen, 2005).

Melanization and proPO activation must be tightly and elaborately controlled by certain regulatory molecules because quinones and other reactive intermediates produced from the melanization reactions are cytotoxic and deleterious to host cells. Several molecules that contribute to the negative regulation of melanization reactions have been identified, including PO inhibitors (POIs), melanization inhibition proteins (MIPs), and serine proteinase

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inhibitors (SPIs) (Cerenius et al., 2008; Nappi and Christensen, 2005). It has been demonstrated that PO inhibitors inhibit melanization by interfering with PO activity (Lu and Jiang, 2007; Shi et al., 2006), whereas MIPs interfere with the formation of melanin from quinone compounds (Söderhäll et al., 2009; Zhao et al., 2005). In contrast, SPIs appear to participate in the inhibition of proPO activation by interfering with the proteinase activities of PPAEs or the upstream proteinase of PPAEs (Cerenius et al., 2008; Kanost, 1999). A small protein classified as a Kunitz-type SPI was shown to interfere with proPO activation in the insects *Manduca sexta* and *Sarcophaga bullata*; however, their target proteinase remains unknown (Polanowski and Wilusz, 1996; Sugumaran et al., 1985). Since this discovery, several SPIs belonging to the serpin class have been identified in many insect species and their functions in the regulation of the proPO system have been clearly demonstrated (Ahmad et al., 2009; An and Kanost, 2010; An et al., 2011, 2012; De Gregorio et al., 2002; Gupta et al., 2005; Jiang et al., 2003; Ligoxygakis et al., 2002; Liu et al., 2015a; Scherfer et al., 2008; Suwanchaichinda et al., 2013; Tang et al., 2008; Tong et al., 2005; Wang and Jiang, 2004, 2006; Zhu et al., 2003; Zou and Jiang, 2005; Zou et al., 2010). Although, a MIP homolog (*Pm*MIP) was identified in the shrimp *Penaeus monodon*, only the nucleotide sequence and transcript expression profiles have been reported (Angthong et al., 2010). Two kunitz-type SPIs were found in the shrimp *Fenneropenaeus chinensis* (Kong et al., 2013) and *Marsupenaeus japonicus* (Chen et al., 2008); however, the role that these SPIs play in the regulation of the proPO system remains to be investigated. Of the serpin-type SPIs, various serpins have been identified in shrimp, including Lvserpin and Lvserpin7 from *Litopenaeus vannamei* (Liu et al., 2014, 2015b), *Fc*-serpin from *F. chinensis* (Liu et al., 2009), and *Pm*Serpin3, *Pm*SerpinB3, *Pm*Serpin6, and *Pm*Serpin8 from *P. monodon* (Homvises et al., 2010; Somboonwiwat et al., 2006; Somnuk et al., 2012; Wetsaphan et al., 2013). Although *Pm*Serpin3 and *Pm*Serpin8 were shown to inhibit the proPO system *in vitro* (Somnuk et al., 2012; Wetsaphan et al., 2013), little is known about the function *in vivo* and, in most cases, the target proteinase requires further study.

Pacifastin is a high molecular weight heterodimeric protein composed of two covalently linked subunits in which each subunit is encoded by different mRNAs. The pacifastin light chain domain (PLD) contains an N-terminal signal sequence followed by several proteinase inhibitor domains, whereas the heavy chain contains a sequence similar to transferrin (Liang et al., 1997). The PLDs are characterized by six conserved cysteine residues, with the reactive sites located between the last two cysteine residues (Mer et al., 1996; Roussel et al., 2001; Simonet et al., 2002). As with other proteinase inhibitors, each of the PLD domains contains a P1−P1′, a reactive peptide bond (scissile bond) that is involved in binding and is cleaved by the target proteinase. Each P1 residue was primarily responsible for determining the specificity of the target proteinases (Kellenberger and Roussel, 2005; Malik et al., 1999). In insects, some pacifastin-related inhibitors have been identified (Boigegrain et al., 1992; Brehélin et al., 1991; Breugelmans et al., 2009) and a possible role as an inhibitor for the proPO system has been reported (Boigegrain et al., 1992; Brehélin et al., 1991). In crustaceans, PLD-related peptides have been reported from the crayfish *Pacifastacus leniusculus* (Liang et al., 1997) and the crabs *Eriocheir sinensis* (Gai et al., 2008) and *Portunus trituberculatus* (Liu et al., 2015c; Wang et al., 2012). However, information on the function of pacifastin in the regulation of the proPO cascade is only based on results from *in vitro* and *in vivo* studies of the crayfish *P. leniusculus* (Liang et al., 1997; Liu et al., 2007). Recently, a new class of peptidase inhibitors, named panulirin, has been identified and found to act as a competitive and tight-binding inhibitor that inhibits the melanization response to lipopolysaccharides in the spiny lobster *Panulirus argus* (Perdomo-Morales et al., 2013). In addition to the inhibitors described above, some recent findings also demonstrated that a mannose-binding lectin (*Pl*-MBL) and a Caspase 1-like enzyme could interfere with the activation of the proPO-system in the crustacean *P. leniusculus* (Wu et al., 2013; Jearaphunt et al., 2014).

Previously, we identified and characterized the function of several proPO system components as well as their important roles in pathogen defense in the shrimp *P. monodon* (Amparyup et al., 2009, 2012, 2013b; Charoensapsri et al., 2009, 2011, 2014; Jearaphunt et al., 2015). Two proPOs (*Pm*proPO1 and *Pm*proPO2) and two PPAEs (*Pm*PPAE1 and *Pm*PPAE2) were found to be essential for the proPO system and for immune defense against *Vibrio harveyi* and *Fusarium solani* infections (Amparyup et al., 2009; Charoensapsri et al., 2009, 2011, 2014). An LPS and β-1,3-glucan binding protein (*Pm*LGBP), a clip-SP (*Pm*ClipSP2), and two masquerade-like serine proteinase homologues (*Pm*MasSPH1 and *Pm*MasSPH2) were found to be required for pathogen recognition. *Pm*LGBP, *Pm*ClipSP2, and *Pm*MasSPH1 also participated in proPO system activation (Amparyup et al., 2007, 2012, 2013b; Jearaphunt et al., 2015; Jitvaropas et al., 2009). A homologue of clip-SP, *Pm*ClipSP1, was also identified; however, it does not appear to have a function in the shrimp proPO system (Amparyup et al., 2010). Here, we report the identification and characterization of two novel pacifastin-like sequences (*Pm*Pacifastin-like and *Lv*Pacifastin-like) from the black tiger shrimp *P. monodon* and the Pacific white shrimp *L. vannamei*. The transcript expression levels of *Pm*Pacifastin-like gene in various shrimp tissues, at different larval developmental stages, and in response to *V. harveyi* infection were described. To determine the importance of *Pm*Pacifastin-like protein in shrimp, RNA interference (RNAi)-mediated gene suppression of the *Pm*Pacifastin-like transcript was carried out and the function of *Pm*Pacifastin-like protein in the negative regulation of shrimp proPO activation was then biochemically evaluated *in vivo*.

## 2. Materials and methods

### 2.1. Experimental shrimp

Specific pathogen-free (SPF) *P. monodon* were purchased from the Shrimp Genetic Improvement Center, BIOTEC, Thailand. The SPF *L. vannamei* were kindly provided by Charoen Pokphand Company. Shrimp *P. monodon* and *L. vannamei* were acclimatized in aerated tank containing seawater at 20 and 15‰, respectively, for 7 days before being used in the experiments.

### 2.2. Total RNA preparation and cDNA synthesis

Hemolymph was withdrawn from the ventral sinus cavity of the healthy shrimp using a sterile 1-ml syringe with a 25-gauge needle containing an equal volume of anticoagulant AC1 solution (Söderhäll and Smith, 1983). Hemocytes were collected by centrifugation at 800 × g for 10 min at 4 °C. The hemocyte pellets were homogenized in TRI Reagent® (Molecular Research Center) and total RNA was prepared following the manufacturer's instructions. RNA quantity and quality were assessed using UV spectrophotometer and agarose gel electrophoresis, respectively. First-strand cDNA was synthesized using the ImProm-II™ Reverse Transcriptase System kit (Promega) with 1.5 μg of total RNA and 0.5 μg of oligo$(dT)_{15}$ primer and stored at −80 °C until needed.

### 2.3. In vivo gene knockdown of two prophenoloxidase genes in shrimp

Double-stranded RNAs (dsRNAs) corresponding to the *Pm*proPO1 and *Pm*proPO2 transcripts and the control GFP were