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

Pakkakul Sangsuriya^{a,b}, Walaiporn Charoensapsri^{b,c}, Sudarat Chomwong^{a,d}, Saengchan Senapin^{b,c}, Anchalee Tassanakajon^a, Piti Amparyup^{a,b,*}

^aCenter of Excellence for Molecular Biology and Genomics of Shrimp, Department of Biochemistry, Faculty of Science, Chulalongkorn University, 254 Phayathai Road, Bangkok 10330, Thailand

^bNational Center for Genetic Engineering and Biotechnology (BIOTEC), National Science and Technology Development Agency (NSTDA), 113 Paholyothin Road, Klong 1, Klong Luang, Pathumthani 12120, Thailand

^cCenter of Excellence for Shrimp Molecular Biology and Biotechnology (Centex Shrimp), Mahidol University, Rama VI Road, Bangkok 10400, Thailand

^dProgram of Biotechnology, Faculty of Science, Chulalongkorn University, 254 Phayathai Road, Bangkok 10330, Thailand

Keywords: Shrimp immunity; *Penaeus monodon*; Serine proteinase inhibitor; Melanization; Prophenoloxidase; Pacifastin; Kunitz

*Corresponding author. Tel.: +66 2 218 5414; Fax: +66 2 218 5414.

E-mail address: piti.amp@biotec.or.th (P. Amparyup).

22 **Abstract**

23 Pacifastin is a recently classified family of serine proteinase inhibitors that play
24 essential roles in various biological processes, including in the regulation of the melanization
25 cascade. Here, a novel pacifastin-related gene, termed *PmPacifastin-like*, was identified from
26 a reverse suppression subtractive hybridization (SSH) cDNA library created from hemocytes
27 of the prophenoloxidase *PmproPO1/2* co-silenced black tiger shrimp *Penaeus monodon*. The
28 full-length sequences of *PmPacifastin-like* and its homologue *LvPacifastin-like* from the
29 Pacific white shrimp *Litopenaeus vannamei* were determined. Sequence analysis revealed
30 that both sequences contained thirteen conserved pacifastin light chain domains (PLDs),
31 followed by two putative kunitz domains. Expression analysis demonstrated that the
32 *PmPacifastin-like* transcript was expressed in all tested shrimp tissues and larval
33 developmental stages, and its expression responded to *Vibrio harveyi* challenge. To gain
34 insight into the functional roles of *PmPacifastin-like* protein, the *in vivo* RNA interference
35 experiment was employed; the results showed that *PmPacifastin-like* depletion strongly
36 increased PO activity. Interestingly, suppression of *PmPacifastin-like* also down-regulated
37 the expression of the proPO-activating enzyme *PmPPAE2* transcript; the *PmPacifastin-like*
38 transcript was down-regulated after the *PmproPO1/2* transcripts were silenced. Taken
39 together, these results suggest that *PmPacifastin-like* is important in the shrimp proPO system
40 and may play an essential role in shrimp immune defense against bacterial infection. These
41 results also expand the knowledge of how pacifastin-related protein participates in the
42 negative regulation of the proPO system in shrimp.

43

44 **1. Introduction**

45 Melanization caused by the activation of the prophenoloxidase (proPO) system is a
46 potent innate defense mechanism that plays a crucial role in pathogen elimination in diverse
47 arthropod species (Amparyup et al., 2013; Cerenius et al., 2008; Cerenius and Söderhäll,
48 2004; Kanost and Gorman, 2008). Activation of the proPO system requires microbial
49 identification, followed by sequential activation of a cascade of clip-domain serine
50 proteinases (clip-SPs). In this cascade, a proPO-activating enzyme (PPAE) functions as a
51 terminal proteinase that specifically cleaves and activates the zymogen prophenoloxidase
52 (proPO) to generate phenoloxidase (PO). Active PO then oxidizes phenols to quinones for the
53 production of melanin and other cytotoxic intermediates to encapsulate and kill invading
54 pathogens (Cerenius and Söderhäll, 2004; Nappi and Christensen, 2005).

55 Melanization and proPO activation must be tightly and elaborately controlled by
56 certain regulatory molecules because quinones and other reactive intermediates produced
57 from the melanization reactions are cytotoxic and deleterious to host cells. Several molecules
58 that contribute to the negative regulation of melanization reactions have been identified,
59 including PO inhibitors (POIs), melanization inhibition proteins (MIPs), and serine proteinase
60 inhibitors (SPIs) (Cerenius et al., 2008; Nappi and Christensen, 2005). It has been
61 demonstrated that PO inhibitors inhibit melanization by interfering with PO activity (Lu and
62 Jiang, 2007; Shi et al., 2006), whereas MIPs interfere with the formation of melanin from
63 quinone compounds (Söderhäll et al., 2009; Zhao et al., 2005). In contrast, SPIs appear to
64 participate in the inhibition of proPO activation by interfering with the proteinase activities of
65 PPAEs or the upstream proteinase of PPAEs (Cerenius et al., 2008; Kanost, 1999). A small
66 protein classified as a Kunitz-type SPI was shown to interfere with proPO activation in the
67 insects *Manduca sexta* and *Sarcophaga bullata*; however, their target proteinase remains
68 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 (*PmMIP*) 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 *L. vannamei* (Liu et al., 2014; 2015c), *Fc-serpin* from *F. chinensis* (Liu et al., 2009), and *PmSerp*3, *PmSerp*B3, *PmSerp*6, and *PmSerp*8 from *P. monodon* (Homvises et al., 2010; Somboonwiwat et al., 2006; Somnuk et al., 2012; Wetsaphan et al., 2013). Although *PmSerp*3 and *PmSerp*8 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

94 the PLD domains contains a P1-P1', a reactive peptide bond (scissile bond) that is involved in
 95 binding and is cleaved by the target proteinase. Each P1 residue was primarily responsible for
 96 determining the specificity of the target proteinases (Kellenberger and Roussel, 2005; Malik
 97 et al., 1999). In insects, some pacifastin-related inhibitors have been identified (Boigegrain et
 98 al., 1992; Brehélin et al., 1991; Breugelmans et al., 2009) and a possible role as an inhibitor
 99 for the proPO system has been reported (Boigegrain et al., 1992; Brehélin et al., 1991). In
 100 crustaceans, PLD-related peptides have been reported from the crayfish *Pacifastacus*
 101 *leniusculus* (Liang et al., 1997) and the crabs *Eriocheir sinensis* (Gai et al., 2008) and
 102 *Portunus trituberculatus* (Liu et al., 2015b; Wang et al., 2012). However, information on the
 103 function of pacifastin in the regulation of the proPO cascade is only based on results from *in*
 104 *vitro* and *in vivo* studies of the crayfish *P. leniusculus* (Liang et al., 1997; Liu et al., 2007).
 105 Recently, a new class of peptidase inhibitors, named panulirin, has been identified and found
 106 to act as a competitive and tight-binding inhibitor that inhibits the melanization response to
 107 lipopolysaccharides in the spiny lobster *Panulirus argus* (Perdomo-Morales et al., 2013). In
 108 addition to the inhibitors described above, some recent findings also demonstrated that a
 109 mannose-binding lectin (*Pl*-MBL) and a Caspase 1-like enzyme could interfere with the
 110 activation of the proPO-system in the crustacean *P. leniusculus* (Wu et al., 2013; Jearaphunt
 111 et al., 2014).

112 Previously, we identified and characterized the function of several proPO system
 113 components as well as their important roles in pathogen defense in the shrimp *P. monodon*
 114 (Amparyup et al. 2009, 2012, 2013b; Charoensapsri et al., 2009, 2011, 2014; Jearaphunt et
 115 al., 2015). Two proPOs (*Pm*proPO1 and *Pm*proPO2) and two PPAEs (*Pm*PPAE1 and
 116 *Pm*PPAE2) were found to be essential for the proPO system and for immune defense against
 117 *Vibrio harveyi* and *Fusarium solani* infections (Amparyup et al., 2009; Charoensapsri et al.,
 118 2009, 2011, 2014). An LPS and β -1,3-glucan binding protein (*Pm*LGBP), a clip-SP

(*PmClipSP2*), and two masquerade-like serine proteinase homologues (*PmMasSPH1* and *PmMasSPH2*) were found to be required for pathogen recognition. *PmLGBP*, *PmClipSP2*, and *PmMasSPH1* 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, *PmClipSP1*, 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 (*PmPacifastin-like* and *LvPacifastin-like*) from the black tiger shrimp *P. monodon* and the Pacific white shrimp *L. vannamei*. The transcript expression levels of *PmPacifastin-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 *PmPacifastin-like* protein in shrimp, RNA interference (RNAi)-mediated gene suppression of the *PmPacifastin-like* transcript was carried out and the function of *PmPacifastin-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 $800 \times g$ 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 *PmproPO1* and *PmproPO2* transcripts and the control GFP were produced *in vitro* using a T7 RiboMAX™ Express Large Scale RNA Production System (Promega) according to the manufacturer's instructions and were further purified as described by Amparyup et al. (2009). For *in vivo* gene silencing of *PmproPO* (*PmproPO1* and *PmproPO2*) transcripts, juvenile *P. monodon* (5 ± 0.2 g, fresh weight) were injected twice intramuscularly with the dsRNAs of the two *PmproPOs* or with GFP (5 µg/g), together with 20 µg of lipopolysaccharide (LPS) from *Escherichia coli*

0111:B5 (Sigma) and laminarin (β -1,3-glucan chain with some β -1,6-linked glucose units) (Sigma) as described by Amparyup et al. (2009). Hemolymph was collected 48 h after the second dsRNA injection and subjected to total RNA isolation using an RNA isolation kit (GE Healthcare) according to the manufacturer's instructions. Analysis of the silencing efficiency of the *PmproPO1* and *PmproPO2* transcripts was carried out using semi-quantitative RT-PCR as described previously (Amparyup et al., 2009). An average of a 92 to 94% reduction of *PmproPO1* and *PmproPO2* expression was observed (data not shown).

2.4. Suppression subtractive hybridization (SSH)

In an attempt to identify genes that were differentially expressed after the *PmproPO1* and *PmproPO2* transcripts were silenced, a PCR-based suppression subtractive hybridization (SSH) cDNA library was constructed following the instructions provided by the PCR Select™ cDNA Subtraction Kit (Clontech), with some minor modifications. Briefly, 1 μ g of the total RNA isolated from hemocytes of the *PmproPO1/2* and GFP dsRNAs injected shrimp (see section 2.3) were reverse transcribed into cDNA using the SMARTer™ PCR cDNA Synthesis Kit (Clontech) following the manufacturer's instructions. The resulting cDNAs were digested with *RsaI* and further used as a driver and tester for the construction of forward and reverse SSH cDNA libraries in accordance with the Clontech PCR-Select cDNA subtraction protocol. The subtracted PCR products were subsequently cloned into the pGEM®-T easy vector (Promega) and transformed into *E. coli* JM109 competent cells (Promega). Recombinant plasmids were randomly selected for plasmid DNA extraction using the Presto™ Mini Plasmid Kit (Geneaid). Recombinant clones were commercially sequenced (Macrogen Inc., Korea), and the resulting cDNA sequences were analyzed using BLAST search algorithms.

2.5. Identification of pacifastin light chain-like genes from shrimp by rapid amplification of the cDNA ends (RACE)

A partial cDNA sequence of *P. monodon* pacifastin light chain-like (*PmPacifastin-like*) protein was obtained from a reverse SSH cDNA library of the *PmproPO1/2* knockdown shrimp using *PmproPO1/2*-silenced cDNA as the driver and GFP dsRNA-injected cDNA as the tester. To obtain the full-length cDNA of the *PmPacifastin-like* gene, two sets of gene specific primers were designed (Table 1) and used for the 5' and 3' rapid amplification of cDNA ends (RACE) using a GeneRacer™ Kit (Invitrogen) according to the manufacturer's instructions. The gene-specific primers *PmPacifastin-like*-R2 and *PmPacifastin-like*-R3 were used for 5' RACE PCRs, whereas *PmPacifastin-like*-F2 and *PmPacifastin-like*-F3 were used for 3' RACE PCRs. The adaptor-ligated cDNA was used as template in a 50 µl PCR reaction volume containing 0.5 µM of each primers, 200 µM of dNTPs, 1 × Advantage® 2 PCR buffer and 1 × Advantage® 2 Polymerase Mix (Clontech). The amplification cycles consisted of 95 °C for 1 min followed by 30 cycles of 94 °C for 15 s, 55 °C for 30 s and 72 °C for 3 min. The final extension was then carried out at 72 °C for 10 min. The amplified RACE products were cloned into pGEM®-T easy vector (Promega) and commercially sequence (Macrogen Inc., Korea). The full-length cDNA of *PmPacifastin-like* was then amplified by PCR using Q5® High-Fidelity DNA polymerase (NEB) with the primer pair *PmPacifastin-like*-ORF-F and *PmPacifastin-like*-ORF-R (Table 1). The PCR reaction was carried out in 25 µl reaction volume contained *P. monodon* hemocytes cDNA as template, 0.5 µM of each primers, 200 µM of dNTPs, 0.25 µl DNA polymerase and 1X reaction buffer. The PCR protocol consisted of denaturation at 98 °C for 30 s, followed by 30 cycles of 98 °C for 10 s, 55 °C for 30 s, and 72 °C for 3 min. Amplified products were cloned and sequenced on both strands to obtain the complete *PmPacifastin-like* sequence.

A partial nucleotide sequence of *LvPacifastin-like* was obtained from a blast search of a full-length sequence of *PmPacifastin-like* against the *L. vannamei* EST database. Based on the retrieved EST sequence, a gene specific primer *LvPacifastin-like*-5UTR (Table 1) was

designed and use for the amplification of the full-length cDNA of *LvPacifastin*-like gene. The semi-nested PCR were conducted using a pair of primers *LvPacifastin*-like-5UTR and *PmPacifastin*-like-ORF-F for the first round of amplification and *PmPacifastin*-like-ORF-F and *PmPacifastin*-like-ORF-R for the second round of amplification. The PCR reactions and conditions were performed as above using cDNA from hemocytes of *L. vannamei* as template.

2.6. DNA sequence analysis

DNA and protein analysis were carried out using the EXPASY web server (<http://www.expasy.org/>). The homology search was carried out with BLAST program in the NCBI database (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>). Multiple sequence alignments were conducted by ClustalW (Thompson et al., 1994) and a Neighbor-Joining (NJ) tree was constructed using Molecular Evolutionary Genetics Analysis (MEGA 6) software (Tamura et al., 2013). An analysis for protein domains was accomplished by SMART database (Letunic et al., 2012).

2.7. Analysis of *PmPacifastin*-like gene expression in shrimp tissues and larval developmental stages

The semi-quantitative RT-PCR was employed to analyze the expression profiles of the *PmPacifastin*-like mRNA in different tissues and developmental stages of shrimp *P. monodon*. Total RNA was isolated from the tissues of hemocyte, hepatopancreas, gills, lymphoid organ, intestine and heart and from four developmental stages including nauplius 3, protozoa 2, mysis 2 and post-larvae 15 using the TRI Reagent® (Molecular Research Center) according to the manufacturer's instructions as described (Charoensapsri et al., 2011). Total RNA was then used as template for first-strand cDNA synthesis using the ImProm-II™ Reverse Transcriptase System kit (Promega) according to the manufacturer's protocols. The primers used for RT-PCR are listed in Table 1. The elongation factor 1 α (EF1 α) gene was

used as an internal control. The PCR conditions consisted of an initial denaturation step at 94 °C for 1 min followed by 30 cycles at 94 °C for 30 s, 55 °C for 30 s and 72 °C for 30 s. The final extension step was performed at 72 °C for 10 min. The amplified products were analyzed by agarose gel electrophoresis.

2.8. Temporal expression of *PmPacifastin*-like transcripts in shrimp hemocytes following infection with the pathogenic bacteria *Vibrio harveyi*

Quantitative real-time PCR (qRT-PCR) was used to measure the changes in *PmPacifastin*-like transcript levels in *P. monodon* hemocytes after injection with the bacteria *V. harveyi*. The experimental shrimp were divided into 3 groups (10 shrimp per group) and injected with 50 µl of *V. harveyi* (1×10^6 CFUs/ml) suspended in sterile saline buffer (150 mM NaCl). For the control group, each shrimp from 3 groups (10 shrimp per group) was injected with 50 µl of sterile saline buffer. At 0, 6, 24, and 48 h, hemolymph was withdrawn and centrifuged at $800 \times g$ for 10 min at 4 °C. The hemocyte pellets were collected and stored at -80 °C for subsequent total RNA isolation. The extraction of total RNA and first-strand cDNA synthesis were performed following the methods described in section 2.2. The extracted total RNAs from three shrimp per treatment at each time point were pooled. Quantitative real-time PCR analysis was performed as described by Amparyup et al. (2007) using the *PmPacifastin*-like- F / -R primer pair (Table 1). EF1 α was amplified as the internal control and reference standard to verify the quantitative real time PCR reaction. Three replicates were performed for each template, and three independent replicates were utilized for each data point. The Ct values of the infected hemocyte samples at each time point were normalized with the saline-injected samples. The mathematical model of Pfaffl (2001) was used to determine the relative expression ratio.

2.9. PO activity assay of *PmPacifastin*-like gene knockdown shrimp

To elucidate the importance of the *PmPacifastin*-like gene in the negative regulation of the shrimp proPO system, the *PmPacifastin*-like gene was suppressed by its corresponding dsRNA, and the PO activity in shrimp hemolymph was then determined. The dsRNAs of *PmPacifastin*-like and GFP genes were synthesized and purified as described in section 2.3 using gene specific primers (*PmPacifastin*-like -T7F / -T7R, *PmPacifastin*-like -F / -R) (Table 1). For the *in vivo* gene silencing experiment, three groups of SPF shrimp (~2 g, fresh weight) were injected intramuscularly with 25 μ l of 150 mM NaCl alone or in addition to 2.5 μ g/ g shrimp of *PmPacifastin*-like dsRNA or GFP dsRNA, as described above. Forty-eight hours after the second injection, shrimp hemolymph was withdrawn and PO activity was determined as described by Amparyup et al. (2009). In brief, 2 mg of total protein was analyzed for PO activity using 3 mg/ml L-3,4-dihydroxyphenylalanine (L-DOPA) as the substrate. After incubation for 30 min, the PO activity was recorded as the ΔA_{490} /mg total protein/min. The results were analyzed from three independent experiments using a one-way analysis of variance (ANOVA) followed by Duncan's test.

2.10. Gene expression analysis in proPO-system gene silenced shrimp

To determine the effect of gene silencing on the expression of shrimp proPO-system genes (*PmproPOs*, *PmPPAE1*, *PmPPAE2*, and *PmPacifastin*-like), expression analysis was carried out in silenced shrimp using semi-quantitative RT-PCR with gene specific primers. The dsRNAs of *PmproPOs*, *PmPPAE1*, *PmPPAE2*, and *PmPacifastin*-like were prepared, and gene silencing experiments were carried out for each gene as described by Amparyup et al. (2009), Charoensapsri et al. (2009), Charoensapsri et al. (2011) and in section 2.9, respectively. The expression levels of each gene are shown as relative expression normalized to EF1 α . The experiment was performed in triplicate and statistical analysis was performed using one-way ANOVA followed by Duncan's test.

3. Results and Discussion

3.1. Identification and sequence analysis of shrimp *PmPacifastin*-like and *LvPacifastin*-like genes

The prophenoloxidase (proPO) system is a tightly regulated defense mechanism that is involved in the biosynthesis of melanin and in the generation of toxic reactive intermediates for pathogenic sequestration. Activation of the proPO system requires recognition of the microbial pathogen by host pattern-recognition proteins (PRPs) and the proteolytic cleavage of the proPO zymogen to the active phenoloxidase (PO) enzyme via a cascade of clip-domain serine proteinases (clip-SP).

Accumulating evidence indicates that PO plays a pivotal role in the proPO activating system. Additionally, the cross-talk between arthropod proPO and Toll pathways via the clip-SP cascade has been demonstrated in both insects and crustaceans (Amparyup et al., 2013; An et al., 2009, 2010; Kan et al., 2008; Kim et al., 2008; Roh et al., 2009). In this study, we initially identified the genes that differentially regulated by *PmproPOs* using suppression subtractive hybridization (SSH) of the *PmproPO* gene-silenced hemocyte cells from *P. monodon*. Surprisingly, a SSH clone from a reverse subtracted library of the proPO-knockdown shrimp was found to exhibit sequence homology to the pacifastin light chain gene of the crayfish *P. leniusculus* (53%). We thus named this gene *P. monodon* pacifastin light chain-like (*PmPacifastin*-like). Pacifastin is a novel member of the serine proteinase inhibitor (SPI) family that has recently been identified in many arthropods (Breugelmans et al., 2009) and in some non-arthropods (Roeding et al., 2007). In crustaceans, only three pacifastin-related genes have been reported to date, including in the freshwater crayfish *P. leniusculus* (Liang et al., 1997), the swimming crab *Portunus trituberculatus* (Wang et al., 2012), and the Chinese mitten crab *Eriocheir sinensis* (Gai et al., 2008). Thus, we selected the *PmPacifastin*-like gene for further characterization.

307 The full-length cDNA of *PmPacifastin*-like was successfully amplified from *P.*
308 *monodon* hemocytes using RACE-PCR assays. The cDNA was composed of a 5'
309 untranslated region (UTR) of 66 bp, an open reading frame (ORF) of 2223 bp that encoded
310 740 deduced amino acid residues, and a 3' UTR of 507 bp. The *PmPacifastin*-like sequence
311 was deposited in the GenBank database under accession number KR905467. A putative
312 signal peptide of *PmPacifastin*-like protein was located at the first 19 amino acid residues.
313 Therefore, the mature *PmPacifastin*-like protein (721 amino acids) had an estimated
314 molecular weight of approximately 78.57 kDa and an isoelectric point of 5.56. Primary
315 structural analysis of the *PmPacifastin*-like protein revealed thirteen domains within the
316 protein sequence with typical characteristics of the pacifastin light chain domain (PLD) (Fig.
317 1). The PLDs shared high homology, which could be observed in a highly conserved cysteine
318 array (Cys1 – Xaa₉₋₁₂ – Cys2 – Asn – Xaa – Cys3 – Xaa – Cys4 – Xaa₂₋₃ – Gly – Xaa₃₋₆ –
319 Cys5 – Thr – Xaa₃ – Cys6), as was found in other member of the pacifastin family. The P1
320 residues for enzyme specificity were observed between the last two cysteine residues of the
321 PLDs (Fig. 1).

322 Interestingly, except for the PLD, *PmPacifastin*-like contained two additional kunitz
323 motifs, that is, one of the SPIs, at the C-terminus (Fig. 1). This newly identified sequence
324 highlighted the uniqueness of SPIs because it was a combination of the motifs of different
325 SPI families, that is, pacifastin and kunitz, and the protein sequence was not found in any
326 known pacifastin. To verify this ambiguous sequence, pacifastin cDNA was also isolated
327 from the hemocytes of Pacific white shrimp *L. vannamei* for comparison. The specific primer
328 designed for *L. vannamei* could successfully amplify the entire *LvPacifastin*-like ORF, which
329 consisted of 2214 bp encoding 737 deduced amino acid residues. With the exclusion of the
330 signal peptide (amino acid residues 1-19), the molecular mass of mature *LvPacifastin*-like
331 protein (718 amino acids) was approximately 78.33 kDa, with an isoelectric point of 5.47.

This *Lv*Pacifastin-like cDNA sequence was deposited in the GenBank database under accession number KR905468. Similarly to the *Pm*Pacifastin-like gene, an analysis for protein domains in the *Lv*Pacifastin-like gene also revealed thirteen PLDs and two kunitz domains. Sequence comparison between *Pm*Pacifastin-like and *Lv*Pacifastin-like genes shared high similarity (80%) (Fig. 1). It was concluded that a combination of pacifastin and kunitz motifs existed in the protein sequence of both *P. monodon* and *L. vannamei*. Therefore, this is the first putative serine proteinase inhibitor identified in penaeid shrimp that contains both pacifastin and kunitz domains.

BLASTP analysis of the entire *Pm*Pacifastin-like protein sequence showed the greatest homology to the pacifastin light chain of many crustaceans, including crayfish *P. leniusculus* (48% similarity), crab *P. trituberculatus* (49% similarity), and crab *E. sinensis* (52% similarity). Most of the sequence similarity was contributed by the PLDs composed of the conserved array of six cysteine residues. Thirteen PLDs from *P. monodon* and *L. vannamei*, nine PLDs from *P. leniusculus*, eight PLDs from *P. trituberculatus*, and six PLDs from *E. sinensis* were aligned (Fig. 2) and exhibited the highly conserved six cysteine residues that form three predicted disulfide bridges (Cys1 – Cys4, Cys2 – Cys6 and Cys3 – Cys5) that would stabilize a typical folding of pacifastin protein (Gáspári et al., 2002; Kellenberger et al., 2003; Mer et al., 1994, 1996; Roussel et al., 2001). The variation of amino acid residues at the reactive bond P1-P1' (Kellenberger and Roussel, 2005) were revealed among PLDs from different species (Fig. 2), indicating differences in inhibitory specificity.

The thirteen PLDs from *Pm*Pacifastin-like and *Lv*Pacifastin-like proteins seem to represent the greatest repeated number. A similar finding was observed in the insect *Bombyx mori*, in which it was suggested that the variation in the number of inhibitor domains might be due to a gene duplication event and variation in mRNA splicing (Breugelmans et al.,

2009). Unlike insects, no dibasic cleavage site that flanked the PLD domains (Simonet et al., 2003) was revealed in the shrimp *PmPacifastin*-like proteins; therefore, the proteins were not separated into several inhibitory units. Pacifastins identified in other crustaceans also do not contain dibasic cleavage sites. In addition, in *P. leniusculus*, the nine-PLD domain of pacifastin was purified as a single molecule from the hemolymph (Liang et al., 1997). Taken together, these findings imply that the thirteen-PLD domain in shrimp is a single inhibitory unit that is responsible for proteinase inhibition. Because the reactive site P1-P1' interacts directly with the catalytic residues of targeted enzymes, P1 residues are critical for enzyme specificity (Kellenberger and Roussel, 2005). Bulky, aromatic residues, such as phenylalanine, leucine, and methionine, at the P1 position specifically inhibit chymotrypsin, while the basic residues, such as lysine and arginine, are specific to trypsin (Kellenberger and Roussel, 2005; Simonet et al., 2002). In swimming crab *P. trituberculatus*, the recombinant protein of mature *PtPLC* and separated PLD domains were found to inhibit both trypsin and chymotrypsin activity *in vitro* (Liu et al., 2015b). Therefore, the *PmPacifastin*-like protein would be expected to specifically inhibit trypsin (with *PmPLD*7, 9, 11 and 13) and chymotrypsin (with *PmPLD*1, 3 and 5). Likewise, *LvPacifastin*-like protein would inhibit both trypsin (with *LvPLD*4, 7, 9, 11 and 13) and chymotrypsin (with *LvPLD*1 and 3). In addition, it has been suggested that shrimp pacifastin-like proteins would preferably inhibit trypsin over chymotrypsin due to the greater contribution of trypsin-specific PLD domains.

To evaluate the evolutionary relationships of *PmPacifastin*-like and *LvPacifastin*-like proteins, phylogenetic trees were constructed to compare these shrimp protein sequences with homologues from other pacifastin-related proteins in crustaceans and insects. To analyze phylogenetic relationships in specificity of the inhibitory domain, PLDs from different species were aligned separately. It was clear that the PLDs from *PmPacifastin*-like and *LvPacifastin*-like proteins grouped in a clade of proteins from crustaceans. The different

PLDs were closely linked based on enzyme specificity to trypsin or chymotrypsin (Fig. 3).
Notably, some PLDs from mosquito (*Cq*PLD1 and *Cq*PLD8) were grouped in the clade
containing shrimp PLDs rather than with other insects (Fig. 3).

In contrast to pacifastin, kunitz is the SPI that is widely found in a variety of many
organisms, such as plants, insects, and microbes (Laskowski and Kato, 1980; Ranasinghe and
McManus, 2013). Nevertheless, the presence of inhibitory domains in both PLD and kunitz
has never been reported for any SPI. In the insect *B. mori*, kunitz domains were organized
with other proteinase inhibitor domains, such as WAP and Antistasin (Zhao et al., 2012).
Notably, the kunitz domains in shrimp *Pm*Pacifastin-like proteins appeared to be different
from kunitz-type SPI found in the shrimps *F. chinensis* (Kong et al., 2013) and *M. japonicus*
(Chen et al., 2008) because they were placed in different clades of the phylogenetic tree. This
difference might be due to the insertion of three amino acid residues between Cys1 and Cys2,
which differs from a typical kunitz domain that has a cysteine pattern of Cys1 – Xaa8 – Cys2
– Xaa15 – Cys3 – Xaa7 – Cys4 – Xaa12 – Cys5 – Xaa3 – Cys6 (Chen et al., 2001; Paesen et al.,
2009; Schmidt et al., 2005). The variation of the cysteine patterns in kunitz domains has also
been reported in the tick *Ixodes scapularis* (Dai et al., 2012).

Based on the different patterns of cysteines, tick kunitz proteins can be classified into
three groups with diverse function. Group I is responsible for the suppression of serine
proteinase, while group II seems to have evolved a new function involved in ion channel
modulation (Dai et al., 2012). Interestingly, the kunitz domains of *Pm*Pacifastin-like and
*Lv*Pacifastin-like proteins were placed in a different clade from Japanese and Chinese shrimp
kunitz-type SPI but instead were grouped closely to the hypothetical protein of the water flea
Daphnia pulex (Fig. 4). This result clearly indicated that the kunitz domains in *Pm*Pacifastin-
like and *Lv*Pacifastin-like proteins were not related to shrimp kunitz-type SPI. It would be
interesting to investigate whether the additional kunitz domains in shrimp *Pm*Pacifastin-like

proteins would help increase the efficiency of proteinase inhibition or would play some other function as described for the *I. scapularis* kunitz proteins.

3.2. Expression profiles of *PmPacifastin*-like transcripts in various tissues and at different larval developmental stages in shrimp

Gene expression analyses in different shrimp tissues and at different developmental stages were initially performed to investigate the biological and physiological functions of *PmPacifastin*-like in shrimp. Semi-quantitative RT-PCR was employed to determine the *PmPacifastin*-like gene expression in various tissues, including hemocytes, hepatopancreas, gill, lymphoid organ, intestine, and heart. It was found that the *PmPacifastin*-like transcripts were expressed in all of the tested tissues, but the highest expression level was detected in gills (Fig. 5A). This finding is consistent with the tissue distribution of other pacifastin-related genes previously reported in crustaceans. *EsPLC* mRNA transcripts were detected at high levels in the hepatopancreas, gills, and hemolymph of *E. sinensis*, and *PtPLC* of *P. trituberculatus* was highly expressed in the hemocytes, gills, hepatopancreas, and stomach (Gai et al., 2008; Wang et al., 2012). Because gills are considered to be the major site of immune reactions in shrimp (Tassanakajon et al., 2013) and because gill tissue is heavily infiltrated by circulating hemocytes, high mRNA expression of *PmPacifastin*-like in gills might imply an essential role of *PmPacifastin*-like in shrimp immunity.

The expression profile of *PmPacifastin*-like transcripts in various developmental stages of shrimp larva was examined using semi-quantitative RT-PCR analysis. As shown in Fig. 5B, the *PmPacifastin*-like transcripts were expressed in all shrimp larval stages, including nauplius, protozoa, mysis, and post-larvae. This result is in accord with previous observations in the crayfish *P. leniusculus*, which revealed that pacifastin transcripts were present during the middle phase of crayfish embryo development (Zhang et al., 2010). In the shrimp *P. monodon*, the relationship of the expression profiles of proPO, PPAAE, and SPH

genes during larval development stages has been investigated (Charoensapsri et al., 2011; Jearaphunt et al., 2015). *PmPPAE1*, *PmproPO2*, *PmMasSPH1*, and *PmMasSPH2* transcripts were detected during all of the larval stages, whereas *PmPPAE2* and *PmproPO1* transcripts were primarily expressed during the late stages of larval development (mysis and post-larvae). The presence of pacifastin-like transcripts in this study suggest that the proPO cascade has an effect on shrimp larvae. However, further investigation is needed to reveal these effects.

Serine proteinase inhibitors not only play roles in controlling the proteolysis cascade but also contribute to host immunity (Kanost, 1999; Ranasinghe and McManus, 2013; Rimphanitchayakit and Tassanakajon, 2010). Because the immune system in shrimp larva is not completely established, the continuous expression of *PmPacifastin*-like mRNA might help protect shrimp larva against pathogens. In addition to immune function, there is evidence that pacifastin is involved in the reproduction process in locusts (Breugelmans et al., 2008). It would be interesting to determine whether the *PmPacifastin*-like gene is involved in the growth and development of shrimp; however, further investigation is required in this area.

3.3. Transcriptional analysis of *PmPacifastin*-like gene after bacterial challenge

Several members of the shrimp proPO system are immune genes that are responsive to infection by pathogenic bacteria (Amparyup et al., 2007, 2012, 2013b; Charoensapsri et al., 2009; Jang et al., 2011; Pang et al., 2014). Here, the functional role of the *PmPacifastin*-like gene in the shrimp proPO system was demonstrated. Therefore, it is important to examine whether *PmPacifastin*-like transcripts were affected by bacterial infection. A time course analysis of *PmPacifastin*-like mRNA expression in hemocytes of shrimp injected with the Gram-negative bacteria *V. harveyi* and controls injected with saline solution was carried out using quantitative real time-PCR. The expression of *EF1 α* transcripts was used as a reference at each time point. As shown in Fig. 6, the expression of *PmPacifastin*-like mRNA

in hemocytes exhibited temporal fluctuations during the progress of the *V. harveyi* infection. Compared with 0 h post infection, *PmPacifastin*-like transcripts decreased by 0.43-fold at 6 h and then reached a maximum level of 1.35-fold at 24 h before dropping slightly to 1.13-fold at 48 h. It is thus expected that the *PmPacifastin*-like gene is an immune-related gene that responds to *V. harveyi* infection in shrimp, which might explain why high expression of *PmPacifastin*-like transcripts is needed to control excess serine proteinases. Up-regulation of pacifastins, *EsPLC*, and *PtPLC* genes were also described in the crabs *E. sinensis* and *P. trituberculatus* challenged with various pathogenic bacteria, although maximum levels of expression were reached at different time periods post infection (Gai et al., 2008; Wang et al., 2012). The high expression levels at different times post infection could be due to differences in the virulence of pathogens among species. Evidence for the role of pacifastin in pathogen defense was clearly revealed in *P. leniusculus*. The pacifastin mRNA transcripts in crayfish were up-regulated at 3 h after *A. hydrophila* challenge (Liu et al., 2007). Knockdown of pacifastin in *P. leniusculus* not only increased PO activity but also delayed mortality caused by infection with the highly pathogenic bacteria *A. hydrophila* (Liu et al., 2007). The improved mortality rate in pacifastin-silenced *P. leniusculus* could be due to enhanced immune activities, including bacterial clearance activity, phagocytosis, and nodule formation; correlation between these mechanisms needs to be clarified (Liu et al., 2007). Collectively, it would imply that *PmPacifastin*-like might play an essential role in shrimp defense against bacterial infection, probably through control of the proPO system as described in the species above.

3.4. Effect of *PmPacifastin*-like silencing on hemolymph phenoloxidase (PO) activity

The functional role of *PmPacifastin*-like gene in shrimp *P. monodon* defense was investigated using dsRNA-mediated gene silencing. Shrimp were injected intramuscularly with *PmPacifastin*-like dsRNA; controls were injected with GFP dsRNA or NaCl buffer.

After the dsRNA injection, shrimp hemocytes were collected for RT-PCR analyses of the *PmPacifastin*-like transcripts. Silencing of the specific *PmPacifastin*-like gene was achieved in shrimp that received the *PmPacifastin*-like dsRNA, while no gene suppression effects were observed in the control GFP dsRNA and NaCl groups (Fig. 7). The relative expression levels of *PmPacifastin*-like mRNA were significantly lower in *PmPacifastin*-like silenced shrimp, with approximately 90% reductions compared with the NaCl-injected group (Fig. 7). *PmPacifastin*-like dsRNA was specific and effective in suppressing *PmPacifastin*-like mRNA expression. Compared with other proPO-related genes in *P. monodon*, gene silencing of serine proteinases, such as *PmPPAEs* (Charoensapsri et al., 2009; 2011), and serine proteinase inhibitors, such as *PmPacifastin*-like gene, seem to show greater inhibitory effects than *PmproPO* genes because *PmproPO* dsRNAs showed no complete *PmproPO* transcript inhibition (Amparyup et al., 2009). This result suggests that mRNA levels of both serine proteinase and serine proteinase inhibitor are appropriately maintained to control the proteolytic cascade in the shrimp proPO system.

Some reports in arthropods demonstrated the relevance of pacifastin as an inhibitor for proPO activation (Boigegrain et al., 1992; Brehélin et al., 1991; Liang et al., 1997; Liu et al., 2007). Therefore, to examine whether the *PmPacifastin*-like gene was involved in the shrimp proPO system, PO activity was quantitatively determined in *PmPacifastin*-like silenced shrimp. After the silencing of *PmPacifastin*-like transcripts, shrimp hemolymph was sampled for a PO activity assay, using L-DOPA as the substrate, and compared with shrimp injected with the control GFP dsRNA or NaCl solution. PO activity of the *PmPacifastin*-like silenced shrimp increased significantly to approximately 2-fold higher than that of the control groups (Fig. 7). Thus, *PmPacifastin*-like protein is likely to participate in the shrimp proPO pathway and to play a role as an inhibitor in proPO activation. This result is in agreement with findings from the crayfish *P. leniusculus* in which the silencing of pacifastin resulted in

higher PO activity (Liu et al., 2007). In *P. leniusculus*, a 44% reduction of pacifastin transcripts enhanced PO activity by 1.4-fold compared with the control group, whereas in shrimp *P. monodon*, a 90% reduction of *PmPacifastin*-like transcripts increased PO activity by up to 2-fold. This finding strongly suggests that there is a direct correlation between *PmPacifastin*-like and PO activity. Therefore, *PmPacifastin*-like protein should be considered to be an important member of the shrimp proPO system, although serine proteinase targets for *PmPacifastin*-like protein remain to be elucidated.

3.5. Effect of RNAi-mediated suppression of *PmPacifastin*-like gene on proPO-associated gene expression

To gain a greater understanding of the regulatory role of the *PmPacifastin*-like gene in the proPO cascade, the expression levels of other proPO-related gene transcripts were examined in the *PmPacifastin*-like silenced shrimp. Interestingly, as shown in Fig. 8, suppression of the *PmPacifastin*-like gene not only affected its corresponding transcripts but also significantly suppressed the levels of *PmPPAE2* mRNA transcripts. Nevertheless, there was no effect of *PmPacifastin*-like silencing on the expression levels of *PmproPO1*, *PmproPO2*, and *PmPPAE1* (Fig. 8). Thus, we inferred that regulation of the inhibitor *PmPacifastin*-like and the proteinase *PmPPAE2* at the transcriptional level is presumably an essential mechanism that regulates the proteinase cascade in shrimp. However, it was found that knockdown of two *PmproPO* genes significantly decreased the transcriptional levels of *PmPacifastin*-like, which was consistent with the SSH library (Fig. 8). This result strongly supported an essential role of *PmproPO*s in controlling the proPO pathway in shrimp via gene regulatory mechanisms. Previously, we showed that disruption of *PmproPO* gene expression could affected the expression of a terminal serine proteinase (*PmPPAE2*) and a pattern recognition protein (*PmLGBP*) (Amparyup et al., 2012). Likewise, suppression of *PmMasSPH1* also reduced the expression of *PmPPAE2* transcripts (Jearaphunt et al., 2015).

Together, these findings suggest that systematic regulations are required for controlling the proPO system in the shrimp *P. monodon*.

4. Conclusion

The goal of this study was to identify target genes that were differentially regulated by the *PmproPO1* and *PmproPO2* transcripts in hemocytes of the shrimp *P. monodon*. A pacifastin light chain-related gene, designated as *PmPacifastin-like*, was discovered among the down-regulated genes from an SSH library. There are a limited number of pacifastin-related genes in crustaceans; here, we cloned and described novel pacifastin-like genes in two penaeid shrimp *P. monodon* and *L. vannamei*. Both *PmPacifastin-like* and *LvPacifastin-like* sequences contain thirteen conserved pacifastin light chain domains (PLDs) followed by two putative kunitz domains. The expression profile of *PmPacifastin-like* transcripts was shown to be induced in response to infection by the pathogenic bacteria *V. harveyi*. The molecular mechanisms of *PmPacifastin-like* in the regulation of the proPO system were described; suppression of the *PmPacifastin-like* gene caused a significant increase in PO activity in knockdown shrimp. Additionally, silencing the *PmPacifastin-like* gene also contributed to a reduction in transcript levels of a serine proteinase *PmPPAE2*. Data from this study could enhance our understanding of the function of the *PmPacifastin-like* gene in controlling the proPO system and thus provide insights into the systemic regulation of proPO-related genes in shrimp.

Acknowledgment

This work was supported by research grants from the Thailand Research Fund to AT (TRF Senior Scholar No RTA5580008) and the Thailand Research Fund and Thailand National Center for Genetic Engineering and Biotechnology (BIOTEC) to PA (Grant No. RSA5580046), Higher Education Research Promotion and National Research University

557 Project of Thailand, Office of the Higher Education Commission Grant WCU-58-010-FW,
558 the Japan Science and Technology Agency (JST)/ the Japan International Cooperation
559 Agency (JICA), Science and Technology Research Partnership for Sustainable Development
560 (SATREPS), and the Visiting Fellowship under the Japan Society for the Promotion of
561 Science (JSPS) core-University Program. A student fellowship granted to S.C. by the
562 Thailand Graduate Institute of Science and Technology (TGIST) program of National
563 Science and Technology Development Agency (NSTDA) is gratefully acknowledged.
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847

Figure Legends

Figure 1. The deduced amino acid sequence alignment of the black tiger shrimp *P. monodon* *PmPacifastin*-like and the Pacific white shrimp *L. vannamei* *LvPacifastin*-like.

The deduced amino acids are numbered on the right margin. The putative signal peptides are underlined and italicized. The thirteen conserved PLDs are boxed, whereas the conserved cysteine are marked by gray highlight. The P1 residues in PLDs are shaded by dark gray. The enzyme specificity regarding to the P1 position are indicated by the black arrowhead for trypsin and the white arrowhead for chymotrypsin. The two kunitz domains at C-terminus are shaded in gray.

Figure 2. Multiple sequence alignment of the predicted amino acid sequence of PLDs from *PmPacifastin*-like and *LvPacifastin*-like with other crustacean PLDs. Thirteen PLDs of *PmPacifastin*-like (*PmPLD*1 to 13) and *LvPacifastin*-like (*LvPLD*1 to 13) were aligned with the PLDs from crayfish *P. leniusculus* (*PlPLD*1-9), swimming crab *P. trituberculatus* (*PtPLD*1-8) and Chinese mitten crab *E. sinensis* (*EsPLD*1-6). The numbers indicate the position of amino acids in the protein sequence. All PLDs shared the conserved array of six cysteine residues (Cys1 – Xaa₉₋₁₂ – Cys2 – Asn – Xaa – Cys3 – Xaa – Cys4 – Xaa₂₋₃ – Gly – Xaa₃₋₆ – Cys5 – Thr – Xaa₃ – Cys6). The conserved cysteine residues are shaded by dark grey, while other conserved residues including asparagine, glycine and threonine are shaded by light gray. The specific pattern of three disulfide bridges is indicated by bracket. The reactive residues P1-P1' are represented by two black arrowheads.

Figure 3. The phylogenetic relationship between PLDs from shrimp *PmPacifastin*-like and *LvPacifastin*-like and other pacifastin-related proteins. A phylogenetic tree generated by neighbor-joining method was carried out to compare the protein sequences of separated PLDs from *PmPacifastin*-like (*PmPLD*1-13) and *LvPacifastin*-like (*LvPLD*1-13) with other pacifastin-related proteins. Those were included crayfish *P. leniusculus* (*PlPLD*1-9,

873 AAC64661), crab *P. trituberculatus* (PtPLD1-8, AEX20235), crab *E. sinensis* (EsPLD1-6,
874 ACF35640), mosquito *Culex quinquefasciatus* (CqPLD1-8, EDS34849), butterfly *Danaus*
875 *plexippus* (DpPLD1-5, EHJ66762) and jewel wasp *Nasonia vitripennis* (NvPLD1-6,
876 XP_008204060). The inhibitory specificity for trypsin and chymotrypsin in each PLD is
877 indicated. Percentage bootstrap values (1000 replicates) are shown at each branch point.

878 **Figure 4. The phylogenetic relationship between kunitz domain from *PmPacifastin*-like**
879 **and *LvPacifastin*-like to other kunitz-related proteins.** The separated kunitz domains from
880 *PmPacifastin*-like and *LvPacifastin*-like and other kunitz-related proteins were determined the
881 evolutionary relationship by neighbor-joining method. Those amino acid sequences used for
882 comparison were from shrimp *F. chinensis* (AGU16239), shrimp *M. japonicus* (AAR92097),
883 cattle *Bos Taurus* (NP_001001554), tick *Haemaphysalis longicornis* (BAI99730), tick
884 *Araneus ventricosus* (AFX95921), horsefly *Tabanus yao* (ACS72289), ant *Harpegnathos*
885 *saltator* (EFN87117), frog *Rana chensinensis* (ACV66786), water flea *Daphnia pulex*
886 (EFX79782) and nematode *Oesophagostomum dentatum* (KHJ84558). Percentage bootstrap
887 values (1000 replicates) are shown at each branch point.

888 **Figure 5. Expression analysis of *PmPacifastin*-like mRNA in various tissues and larval**
889 **developmental stages of *P. monodon*.** (A) Tissue distribution of *PmPacifastin*-like was
890 analyzed by semi-quantitative RT-PCR in the tissue of hemocytes, hepatopancreas, gills,
891 lymphoid organs, intestines and heart. (B) Developmental expression profile of *PmPacifastin*-
892 like was examined at four larval stages including nauplius 3, protozoa 2, mysis 2 and post-
893 larvae 15 by semi-quantitative RT-PCR. The elongation factor 1 α (EF1 α) served as an
894 internal control in all analyzes.

895 **Figure 6. Temporal expression of *PmPacifastin*-like transcripts in shrimp hemocytes**
896 **after *Vibrio harveyi* challenge.** Hemocytes were collected from *V. harveyi*-injected shrimp at
897 indicative time post challenge. Samples from three shrimp at each time point were pooled and

898 measured tree time using SYBR Green quantitative real-time PCR. The expression levels of
899 *PmPacifastin*-like mRNA relative to the internal reference gene *EF1 α* , and normalized with
900 the saline-injected sample are indicated. Data are shown as the mean $\pm$ 1 SD, derived from
901 triplicate samples per assay and three independent assays

902 **Figure 7. *In vivo* gene silencing of *PmPacifastin*-like in *P. monodon* shrimp.** Shrimp were
903 injected *PmPacifastin*-like dsRNA (2.5 μ g/ g shrimp). Control were shrimp injected with GFP
904 dsRNA or NaCl. At 48 h after the second dsRNA injection, hemolymph were collected for
905 analysis of the silencing efficiency and determination of the PO activity. (A) The efficiency
906 of *PmPacifastin*-like gene silencing determined by semi-quantitative RT-PCR. *EF1 α* was
907 used as an internal control. Each lane represents cDNA from an individual shrimp. (B) The
908 hemolymph PO activity of the *PmPacifastin*-like silenced shrimp. PO activity was determined
909 using L-DOPA as substrate and defined as ΔA_{490} /mg total protein/min. The data are derived
910 from three independently replicated experiments and shown as the mean $\pm$ SD (error bars).
911 The different lower case letters above each bar indicate means with significantly different (*p*
912 < 0.05).

913 **Figure 8. Effect of gene silencing of *PmPacifastin*-like, *PmPPAEs* and *PmproPOs* on the**
914 **expression of the proPO-associated genes.** (A) The effect of *PmPacifastin*-like gene
915 silencing on the expression levels of *PmPacifastin*-like, *PmproPO1*, *PmproPO2*, *PmPPAE1*,
916 and *PmPPAE2* transcripts. (B) The effect of *PmPPAE1* and *PmPPAE2* gene silencing on the
917 expression levels of *PmPacifastin*-like transcripts. (C) The effect of *PmproPO1* and
918 *PmproPO2* co-silencing on the transcription levels of *PmPacifastin*-like transcripts. The gene
919 knockdown efficiency was evaluated by semi-quantitative RT-PCR using the gene-specific
920 primers for each gene (Table 1). *EF1 α* served as the internal reference gene. The average
921 relative expressions are representative of three independent repeats and displayed as the mean

922 $\pm$ SD (error bar). Significant difference compared with control is indicated by an asterisk ($p <$
923 0.05).

Table 1. Primers used in this study.

Primer name	Sequence (5' to 3')	Usage
PO1i-F	CACCCTGAGAGCTCCCACTT	RNAi
PO1i-R	GAGATCCTGCAGTGTAACGT	RNAi
PO1iT7-F	GGATCCTAATACGACTCACTATAGGCACCCT GAGAGCTCCCACTT	RNAi
PO1iT7-R	GGATCCTAATACGACTCACTATAGGGAGATC CTGCAGTGTAACGT	RNAi
PO2i-F	CGTCTTCCTCGGCCGCTCCT	RNAi
PO2i-R	CCACGGGGATACCGCTGCC	RNAi
PO2iT7-F	GGATCCTAATACGACTCACTATAGGCGTCTT CCTCGGCCGCTCCT	RNAi
PO2iT7-R	GGATCCTAATACGACTCACTATAGGCCACGG GGATACCGCTGCCA	RNAi
GFP-F	ATGGTGAGCAAGGGCGAGGA	RNAi
GFP-R	TTACTTGTACAGCTCGTCCA	RNAi
GFPT7-F	TAATACGACTCACTATAGGATGGTGAGCAAG GGCGAGGA	RNAi
GFPT7-R	TAATACGACTCACTATAGGTTACTTGTACAG CTCGTCCA	RNAi
<i>PmPacifastin</i> -like-R2	AGACCAAAGCATGGCCCAGAATCGA	5'RACE, Transcription study
<i>PmPacifastin</i> -like-R3	TGGGATCCACAGGTGGGTGACGGCA	5'RACE
<i>PmPacifastin</i> -like-F2	CCGACCGCCACCAGTGGTAGTAACA	3'RACE, Transcription study
<i>PmPacifastin</i> -like-F3	ACGCTTCAGTCCCGATGACCCTAT	3'RACE
<i>PmPacifastin</i> -like-ORF-F	ATGAAGACTTTGTGTGTGTTGCTG	Full-length amplification
<i>PmPacifastin</i> -like-ORF-R	TCAAAGCAATACATCCATAGAGGT	Full-length amplification
<i>LvPacifastin</i> -like-5UTR	AGAACGCTGGTGAGAGTGGTAGGC	Full-length amplification
<i>PmPacifastin</i> -like-F	GGCCAAGGCGCATGCACTAAGA	RNAi
<i>PmPacifastin</i> -like-R	CTGCCCCATGTACCCGTAGCGA	RNAi
<i>PmPacifastin</i> -like-T7F	GGATCCTAATACGACTCACTATAGGGGCCAA GGCGCATGCACTAAGA	RNAi
<i>PmPacifastin</i> -like-T7R	GGATCCTAATACGACTCACTATAGGCTGCCC CATGTACCCGTAGCGA	RNAi
PO1-F	GGTCTTCCCCTCCCGCTTCG	Transcription study
PO1-R	GCCGCAGGTCCTTTGGCAGC	Transcription study
PO2-F	GCCAAGGGGAACGGGTGATG	Transcription study
PO2-R	TCCCTCATGGCGGTCGAGGT	Transcription study
PPAE1-F	CGTCTGCTTCATTGAGGGAGTG	Transcription study
PPAE1-R	GTAGTAGATGGTGCCCCAGCCT	Transcription study
PPAE2-F	GCGGCGGTCACGCTCCTTGTTT	Transcription study
PPAE2-R	ACTCTCGGGGGCACGCTTGTTG	Transcription study
EF1 α -F	GGTGCTGGACAAGCTGAAGGC	Transcription study
EF1 α -R	CGTTCCGGTGATCATGTTCTTGATG	Transcription study

Figure 1

<i>Pm</i> Pacifastin-like	MKTLCLVLLLLGVVAVVESSALLGAQRGVTLPPHFTCAPGSRWKVECNCTCSETGLAAC	60
<i>Lv</i> Pacifastin-like	MKTLCLVLLLLFGVLVAVVESSALLGVQRTVSLPPHFTCAPGSRWKDDCNCTCSDTGISAC	60
<i>Pi</i> Pacifastin	MK--ALLILVMTVAAHGASLEQPDPTASDLDPKSLCAPGSRWKNECNWCSADHGLALC	58
<i>Pm</i> Pacifastin-like	TLMACFGDAQSSDEPVCEDEGRWKIDDNCWCKCIRGSPACSSRPCCDNHQPLARVDTEED	120
<i>Lv</i> Pacifastin-like	TLMACFGDAQSSDESVCDEDEGRWKIDECNWCKCIRGSPACSSRPCCDDHQPLTRVDGEDD	120
<i>Pi</i> Pacifastin	TLMGCFPGYKAAQGESVCESEGRWKADDNCWCRCLDGSPSCTKRLCRTKLAKGMFASQTE	118
<i>Pm</i> Pacifastin-like	IPPGGVCTRGSAAA--WKPDNCWCTCTRD-GNAVCNFKACLDGYVPDPTQK--QCENGSKW	174
<i>Lv</i> Pacifastin-like	IPPGGICKPGSAAA--WKPDNCWCTCTQDGSASCNIRACLPDYVQDPREK--VCENGSKW	175
<i>Pi</i> Pacifastin	ETE--CYGDPDTNRWRIECNWCRCVNGKG--SCTRKGCPQVINGIGLANTNECEGTPTW	173
<i>Pm</i> Pacifastin-like	LVDNNTCVCVDERARCTQKACATSRRESSELPDPAVCKPGSMWKEDCNICRCSSTGKAAAC	234
<i>Lv</i> Pacifastin-like	KLDNNTCVCVDERARCTRKACDVSR-TTELPPDAVCKPGSFWKEECNICRCSSTGRAVAC	234
<i>Pi</i> Pacifastin	TKGCNNTCSVNGSAQCTTEECKLQVQSPSVBAVAFRSGGRTGKCRPDADHDDSLPDYG---	230
<i>Pm</i> Pacifastin-like	IMRGCAAGTTEDASEPKCSHGSKWKRDECNWCDVHGEFNCTERVCGHPTPHISQDEKEE	294
<i>Lv</i> Pacifastin-like	TVKACIGTTENADEPKCTDGSWKRRDDCNWCDVEGKIRCTQRCVGHPTFQIRQFEKEE	294
<i>Pi</i> Pacifastin	-----	-----
<i>Pm</i> Pacifastin-like	MCTEGSRWRVSCNWCCTCRNGT-GGCTKRGCPDPDYDQRTDDPFCCEANSMWKDCNWCNCHN	353
<i>Lv</i> Pacifastin-like	MCTEGSRWRVSCNWCSCMNGT-GGCTKKGCPDPFEERTDAPECEGTSRWKKDCNWCNCHN	353
<i>Pi</i> Pacifastin	QCVFGRWKKDCNWCCTCTETAIGMCTLIGC-----	260
<i>Pm</i> Pacifastin-like	GRGICITTLACNTPFSAFTVHVSQPTVEVTVTRDEAFCTEGSRWLRSNCSCRCIEGKAACK	413
<i>Lv</i> Pacifastin-like	GRGICITTRACIS---TEAVHVSQPTVEVTVIQQEPECTEGSRWLQSCNCSCRCIEGKAACK	410
<i>Pi</i> Pacifastin	-----	-----
<i>Pm</i> Pacifastin-like	RRCLPDFKPSPEDSVCEGNSSWRVDCNWCNCVNGKAICTIKACGVQRPRFPVVTVTRE	473
<i>Lv</i> Pacifastin-like	RRCLPDFEPSPEPVCCHGNSSWRVDCNWCNCANGKAICTYKACGVQRQAPFPVAVVTRE	470
<i>Pi</i> Pacifastin	----LNYEPKPGEAICTDGSWKDDCNWCTCNNGSASCCTEKLCOYKPDGSLP-----	308
<i>Pm</i> Pacifastin-like	EGATCTEGSRWRLGCNWCRCIK-GQGACTKRGCTAGDAPAPDDFICEENSSWKVDCNWCN	532
<i>Lv</i> Pacifastin-like	EGATCTEGSHWRLDCNWCRCIK-GQGACTKRGCSADEAPGPDDFVCEGTSSWKVDCNWCN	529
<i>Pi</i> Pacifastin	DNDMCVPGSRWKDECNWCCEANGAAPTCTRMGCSSEYKPPQGEAVCIDGSRWKVDCNWCN	368
<i>Pm</i> Pacifastin-like	CVDGKAVCTEMGCGPEGRSLGSGDIQENEPKPCINGTTWSKDCNRCSCINGVGRCTRNRF	592
<i>Lv</i> Pacifastin-like	CFDGKAVCTEMACDLEGRSFGSVGVQENPTFKPCINGTTWSADCNKCSVNGVGRCTRRF	589
<i>Pi</i> Pacifastin	CNNGSSACTEKLCLKPGG-----QCTEGESWRQDCNMCSCTGLRICSVKG	414
<i>Pm</i> Pacifastin-like	CRHPPVDHICNLPAINEEVEFCRGFESLWTFDSYDRRCMEIIYGGCGGTENLFETKAACE	652
<i>Lv</i> Pacifastin-like	CRHPPVNEICNLPSVYEDVDRCRGFESLWTFDSYEGRCAAIYGGCGGTENLFETKAACE	649
<i>Pi</i> Pacifastin	CPPTPT-----	420
<i>Pm</i> Pacifastin-like	AKCTRTTRQGSRLRSEGSQEDKCKLDLDSGPCFGLFHRYGYNSANGRCEQEVFGGCGGNANN	712
<i>Lv</i> Pacifastin-like	AKCTRAAQGSRLRSAGSQEDKCKLKLDSGPCFGLFYRYGYNSANGRCEQEIFGGCGGNANN	709
<i>Pi</i> Pacifastin	-----	-----
<i>Pm</i> Pacifastin-like	FETVQECDDQCGGGAPVSGKDTSMVDLL	740
<i>Lv</i> Pacifastin-like	FETAQECDDQCGGGVFPVSGKDTSMVDLL	737
<i>Pi</i> Pacifastin	-----	-----

Figure 2

			Cys2-Cys6	
			Cys1-Cys4	Cys3-Cys5
<i>PlPLD9</i>	388	CTEG---ESWRQDCNMCSCTGL-RI	CSVKGC	415
<i>ESPLD6</i>	248	CEGD---SSWRQDCNNCRV-NGT-SA	CSKRLC	275
<i>PmPLD7</i>	296	CTEG---SRWRVSCNWCTCR-NGT-GG	CTKRCG	323
<i>LvPLD7</i>	296	CTEG---SRWRVSCNWCSCM-NGT-GG	CTKKGC	323
<i>PmPLD9</i>	389	CTEG---SRWLRSNCSCRCI-EGK-AA	CTKRRG	416
<i>LvPLD9</i>	386	CTEG---SRWLQSCNSCRCI-EGK-AA	CSKRRG	413
<i>PmPLD11</i>	478	CTEG---SRWRLGCNWCRCI-KGQ-GA	CTKRCG	505
<i>LvPLD11</i>	475	CTEG---SHWRLCDNWCRCI-KGQ-GA	CTKRCG	502
<i>PtPLD3</i>	121	CKEG---SRWRVSCNWCTCR-GGK-GA	CTEMAC	148
<i>EsPLD3</i>	122	CEEG---SRWRVSCNWCTCE-HGR-GV	CTEAAAC	149
<i>PtPLD1</i>	34	CEGRPLVDRWRKDCNWCSCN-EGR-VR	CSRQLC	64
<i>EsPLD1</i>	37	CTGNSAADRWKDCNWCSCR-NGR-GT	CTRRLC	67
<i>PtPLD7</i>	292	CQGELGIDRWKDCNWCNCR-NGA-GA	CTKRQC	322
<i>PlPLD3</i>	122	CYGDPTDNRWRIECNWCRCV-NGK-GS	CTRKGC	152
<i>PmPLD4</i>	168	CENG---SKWLVDNCTVCV-DER-ARCT	QKAC	195
<i>LvPLD4</i>	169	CENG---SKWLVDNCTVCV-DER-ARCT	CRKAC	196
<i>PtPLD2</i>	75	CEGS---PTWKDDNCTRCA-GGR-AV	CTAKHC	102
<i>EsPLD2</i>	78	CEGN---PTWKDDNCSCSCV-DGR-AI	CTSEYC	105
<i>PtPLD8</i>	333	CEGN---PSWMKDCNRCRCV-DGR-AV	CTTKFC	360
<i>PlPLD4</i>	167	CEGT---PTWTKGNTCSCV-NGS-AQ	CTTEEC	194
<i>PmPLD13</i>	566	CING---TTWSKDCNRCSCI-NGV-GR	CTRNF	593
<i>LvPLD13</i>	563	CING---TTWSADCNKCSCV-NGV-GR	CTRRFC	590
<i>PmPLD8</i>	336	CEAN---SMWKKDCNWCNCH-NGR-GI	CTTLAC	363
<i>LvPLD8</i>	336	CEGT---SRWKKDCNWCNCH-NGR-GI	CTTRAC	363
<i>EsPLD4</i>	165	CHGD---ARWKKDCNWCRCV-NGR-GI	CTTLAC	192
<i>PtPLD4</i>	164	CHGS---SRWKKDCNWCRCV-NGR-GI	CTTKAC	191
<i>PmPLD10</i>	429	CEGN---SSWRVDCNWCNVC-NGK-AI	CTIKAC	456
<i>LvPLD10</i>	426	CHGN---SSWRVDCNWCNCA-NGK-AI	CTYKAC	453
<i>PmPLD12</i>	518	CEEN---SSWKVDCNWCNVC-DGK-AV	CTEMGC	545
<i>LvPLD12</i>	515	CEGT---SSWKVDCNWCNCE-DGK-AV	CTEMAC	542
<i>PmPLD2</i>	78	CDEG---SRWKIDDCNWKCCI-RGS-PAC	SSRPC	106
<i>LvPLD2</i>	78	CDEG---SRWKIDDCNWKCCI-RGS-PAC	SSRPC	106
<i>PlPLD2</i>	76	CSEG---SRWKADDCNWCRCI-DGS-PS	CTKRLC	104
<i>PtPLD6</i>	246	CEDG---SVWKTDDCNICRCI-DGM-SA	CTKRLC	274
<i>PmPLD6</i>	251	CSHG---SKWKRDENWCDCV-HGE-FN	CTERV	279
<i>LvPLD6</i>	251	CTDG---SKWKRDDCNWCDCV-EGK-IR	CTQRV	279
<i>PlPLD6</i>	272	CTDG---SKWK-DDCNWCTCN-NGS-AS	CTEKL	299
<i>PlPLD8</i>	354	CIDG---SRWK-VDCNWCTCN-NGS-SA	CTEKL	381
<i>PmPLD1</i>	37	CAPG---SRWKVEDNTCTCSETGL-AA	CTLMAC	65
<i>LvPLD1</i>	37	CAPG---SRWKDDNTCTCSDTGI-SA	CTLMAC	65
<i>PlPLD1</i>	35	CAPG---SRWKNECNWCSCADHGL-AL	CTLMGC	63
<i>PlPLD7</i>	313	CVPG---SRWKDECNWCWCEANGA-AP	CTRMGC	341
<i>EsPLD5</i>	209	CVPG---SRWLQECNWCVCNDNGN-AI	CSLRAC	237
<i>PlPLD5</i>	232	CVPG---SRWKKDCNWCSTETAI-GM	CTLIG	260
<i>PmPLD3</i>	127	CTRG---SAWKPDNWCCTCTRDGN-AV	CNLRAC	155
<i>LvPLD3</i>	127	CKPG---SAWKPDNWCCTCTQDGMAS	CNLRAC	156
<i>PmPLD5</i>	210	CKPG---SMWKEDCNICRCSSTGKAAA	CTMRGC	239
<i>LvPLD5</i>	210	CKPG---SFWKEECNTRCSSTGRAVA	CTVKAC	239
<i>PtPLD5</i>	206	CVPG---SRWLVDCNWGCSDDGRRSSF	CTLMAC	235

Figure 3

Figure 4

Figure 5

Figure 6

Figure 7

Figure 8

(A) *PmPacifastin*-like knockdown shrimp

(B) *PmPPAE1* and *PmPPAE2* knockdown shrimp

(C) *PmproPO1/2* knockdown shrimp