Shrimp Serine Proteinase Homologues

*PmMasSPH-1 and -2 Play a Role in the Activation of the Prophenoloxidase System*

Miti Jearaphunt1, Piti Amparyup1,2, Pakkakul Sangsuriya1,2, Walaiporn Charoensapsri2,3, Saengchan Senapin2,3, Anchalee Tassanakajon1*

1 Center of Excellence for Molecular Biology and Genomics of Shrimp, Department of Biochemistry, Faculty of Science, Chulalongkorn University, Bangkok, Thailand, 2 National Center for Genetic Engineering and Biotechnology (BIOTEC), National Science and Technology Development Agency (NSTDA), Klong 1, Klong Luang, Pathumthani, Thailand, 3 Center of Excellence for Shrimp Molecular Biology and Biotechnology (Centex Shrimp), Mahidol University, Bangkok, Thailand

*anchalee.k@chula.ac.th*

Abstract

Melanization mediated by the prophenoloxidase (proPO) activating system is a rapid immune response used by invertebrates against intruding pathogens. Several masquerade-like and serine proteinase homologues (SPHs) have been demonstrated to play an essential role in proPO activation in insects and crustaceans. In a previous study, we characterized the masquerade-like SPH, *PmMasSPH1*, in the black tiger shrimp *Penaeus monodon* as a multifunctional immune protein based on its recognition and antimicrobial activity against the Gram-negative bacteria *Vibrio harveyi*. In the present study, we identify a novel SPH, known as *PmMasSPH2*, composed of an N-terminal clip domain and a C-terminal SP-like domain that share high similarity to those of other insect and crustacean SPHs. We demonstrate that gene silencing of *PmMasSPH1* and *PmMasSPH2* significantly reduces PO activity, resulting in a high number of *V. harveyi* in the hemolymph. Interestingly, knockdown of *PmMasSPH1* suppressed not only its gene transcript but also other immune-related genes in the proPO system (e.g., *PmPPAE2*) and antimicrobial peptides (e.g., *PenmonPEN3, PenmonPEN5, crustinPm1* and Crus-like*Pm*). The *PmMasSPH1* and *PmMasSPH2* also show binding activity to peptidoglycan (PGN) of Gram-positive bacteria. Using a yeast two-hybrid analysis and co-immunoprecipitation, we demonstrate that *PmMasSPH1* specifically interacted with the final proteinase of the proPO cascade, *PmPPAE2*. Furthermore, the presence of both *PmMasSPH1* and *PmPPAE2* enhances PGN-induced PO activity *in vitro*. Taken together, these results suggest the importance of *PmMasSPHs* in the activation of the shrimp proPO system.
Introduction

Invertebrates primarily rely on innate immunity mediated by cellular and humoral responses to combat invading pathogens. A cellular immune response involves different types of blood cells that recognize conserved pathogen associated molecular patterns (PAMPs) and leads to activation of nodule formation, encapsulation, and phagocytosis of the microorganisms [1–3]. In contrast, the humoral defense involves production and secretion of several immune proteins such as antimicrobial peptides (AMPs), as well as proteinases and their inhibitors that associated with the clotting and melanization cascade in hemolymph [1–3]. Melanization is one of the most rapid and effective humoral responses that is driven by a key enzyme called phenoloxidase (PO) and is tightly regulated by the proPO activation cascade [4–6]. Upon recognition of microbial PAMPs, such as lipopolysaccharide (LPS) from Gram-negative bacteria, peptidoglycan (PGN) from Gram-positive bacteria or β-1,3-glucans from fungi, by pattern- or pathogen-recognition receptors (PRPs) [7], several serine proteinases generate the active PO required for melanin synthesis [4,8,9].

Several reports in insects have demonstrated the essential function of proPO in surviving microbial infection [10–13]. In crustaceans, it has also been demonstrated that the freshwater crayfish Pacifastacus leniusculus is highly susceptible to Aeromonas hydrophila infection after suppression of proPO [14]. The absence of proPO in the kuruma shrimp, Marsupenaeus japonicus, significantly increases mortality and bacterial load in the hemolymph [15]. In the shrimp Penaeus monodon, co-silencing of PmproPO1 and 2 also had increased mortality after the bacterial V. harveyi and the fungus Fusarium solani infections [16,17]. In addition, the melanization reaction products from shrimp hemocyte also had in vitro antimicrobial activities against Gram-negative bacteria (V. harveyi and V. parahaemolyticus), Gram-positive bacteria (Bacillus subtilis) and fungus (F. solani) [17].

Because the highly toxic intermediates generated from the melanization process are harmful to pathogens as well as the host tissue itself, this process must be tightly regulated and localized [18]. Thus, several serine proteinases (SPs) and serine proteinase homologs (SPHs) are involved in controlling the proteolysis steps. SPHs are SP-like enzymes that contain one or more clip-domain(s) at the N-terminus and an SP-like domain at the C-terminus, but the catalytic triad serine has been substituted with glycine, and therefore, SPHs lack proteolysis activity. Several SPHs have been identified in insects and crustaceans and their roles in the proPO cascade have been characterized. In insects, the Anopheles gambiēs SPH, CLIPA8, is required for melanization because knockdown of CLIPA8 will completely abolish melanin synthesis on the surface of the fungus Beauveria bassiana [12]. The Manduca sexta SPHs (SPH1 and SPH2) are also required for generating the active PO and enhancing PO activity via a collaborative function with the proPO activating proteinase-1 (PAP-1) and PAP-3 [19,20]. Moreover, a non-clip domain-containing SPH-3 of M. sexta is required for PO activation, nodule formation, and gene transcription of antimicrobial effectors [21]. The proPO-activating factor-II (PPAF-II) of Holotrichia diomphalia has been characterized as the co-activator of PPAF-I, a clip-domain SP that cleaves proPO into PO, and the clip domain of PPAF-II also plays a role in proPO activation by interacting with PO through its central cleft [22,23]. In Tenebrio molitor, SPH1 but not SPH2 is a proPO activating cofactor that functions together with active PO to regulate melanin production by forming an active melanization complex that efficiently produces melanin on the surface of bacteria and has strong antibacterial activity [24,25]. In addition to proPO activation, SPHs also possess other functions related to embryonic development such as in Drosophila [26] and are also involved in PRP recognition. The crayfish P. leniusculus masquerade-like protein Pl-Masl functions as a PRP that binds LPS, β-1,3-glucan, Gram-negative bacteria and yeast and is involved in the clearance of microorganisms [27], whilst PlSPH1 and PlSPH2 may...
act as the PRP to the Gram-positive bacteria cell wall component PGN [28]. In the mud crab *Scylla paramamosain*, Sp-SPH can also bind various microbial cell wall components such as PGN, LPS and β-1,3-glucans [29].

Our research group has identified and characterized several important proteins in the shrimp proPO system [16,30–33] including the *P. monodon* masquerade-like SPH called *PmMasSPH1* that acts as a multifunctional immune protein [34,35]. Transcription of *PmMasSPH1* has been shown to be induced in response to *V. harveyi* infection [34]. Furthermore, the C-terminal SP-like domain of *PmMasSPH1* can act as a cell adhesive molecule and binds to *V. harveyi*, LPS and virus [35,36]. The N-terminal region of *PmMasSPH1*, containing clip and glycine-rich domains, also has antimicrobial activity against Gram-positive bacteria [35]. In the present study, a newly identified *PmMasSPH2* is described and both *PmMasSPH1* and *PmMasSPH2* are further investigated for their roles in activation of the proPO system using RNA interference (RNAi) and protein-protein interaction approach. Moreover, the PRP properties for *PmMasSPHs* in recognizing and binding to the Gram-positive bacterial cell wall component PGN are also examined.

**Materials and Methods**

**Sample preparation**

Specific pathogen-free (SPF) *Penaeus monodon* (~4 g, fresh weight) were purchased from the Shrimp Genetic Improvement Center, BIOTEC, Thailand. Shrimp were reared in an aerated tank with seawater at 20 ppt salinity for 7 days before the experiments began. The hemolymph was collected and total RNA was extracted using the TRI Reagent (Molecular Research Center) following the manufacturer’s protocol. First-strand cDNA was synthesized using the ImProm-II Reverse Transciptase System kit (Promega) with 1.5 μg of total RNA and 0.5 μg of oligo (dT)15 primer. For the gene expression analysis at different developmental stages, RNA extraction and cDNA synthesis as described above was done on three individual shrimp from four stages including nauplius 3 (N3), protozoea 2 (Z2), mysis 2 (M2) and post-larvae 15 (PL15).

**Sequence analysis**

The sequence similarity search was performed using the BLAST algorithm (http://www.ncbi.nlm.nih.gov/BLAST/). The putative signal peptide cleavage site and the structural protein domains were predicted using the SignalP 3.0 server (http://www.cbs.dtu.dk/services/SignalP/) and the simple modular architecture research tool (SMART) (http://smart.embl-heidelberg.de/), respectively. Multiple amino acid sequence alignments were carried out using the ClustalW2 program (http://www.ebi.ac.uk/Tools/clustalw2/). The phylogenetic tree was created in Molecular Evolutionary Genetics Analysis (MEGA) version 5.2.

**Double-stranded RNAs (dsRNAs) preparation and gene silencing**

dsRNA was generated and purified as described previously [16] using gene specific primers (*PmSPH1*-T7F, *PmSPH1*-T7R, *PmSPH1*-F and *PmSPH1*-R for *PmMasSPH1*, *PmSPH2*-T7F, *PmSPH2*-T7R, *PmSPH2*-F and *PmSPH2*-R for *PmMasSPH2*, and GFPT7-F, GFPT7-R, GFP-F and GFP-R for GFP) (Table 1). For the gene silencing experiment, shrimp (approximately 4 g) were intramuscularly injected with *PmMasSPH1* or *PmMasSPH2* dsRNA at a concentration of 2 μg/g shrimp. Shrimp injected with GFP dsRNA and 150 mM NaCl solution served as the non-related dsRNA and negative controls, respectively. 24 h after injection, shrimp were injected again with corresponding dsRNA and NaCl containing 20 μg of lipopolysaccharide...
Table 1. Primers used in the experiments.

| Primer name          | Sequence (5'-3') | Purpose                     |
|----------------------|------------------|-----------------------------|
| PmSPH1-T7F           | GGATCCTAATACGACTCATACTATAGTTATAACGGACGCGCA | RNAi                        |
| PmSPH1-T7R           | GGATCCTAATACGACTCATACTATAGTTATAACGGACGCGCA | RNAi                        |
| PmSPH1-F             | TTATAACGGACGAGCGCGAACC | RNAi                        |
| PmSPH1-R             | CACGCACTAATACGACTCATACTATAGTTATAACGGACGCGCA | RNAi                        |
| PmSPH2-T7F           | GGATCCTAATACGACTCATACTATAGTTATAACGGACGCGCA | RNAi                        |
| PmSPH2-T7R           | GGATCCTAATACGACTCATACTATAGTTATAACGGACGCGCA | RNAi                        |
| PmSPH2-F             | TGTCGATTAGCTGTTGCGGCGT | RNAi                        |
| PmSPH2-R             | AGCCCCAGCGCTGATGGGTAC | RNAi                        |
| GFPT7-F              | TAATACGACCTATAGGATGTCAGACGAAGCAAGCGGACG | RNAi                        |
| GFPT7-R              | TAATACGACCTATAGGATGTCAGACGAAGCAAGCGGACG | RNAi                        |
| GFP-F                | ATGGTGAGCAAGGCGGAGGA | RNAi                        |
| GFP-R                | TTATAACGGACGACGCGACG | RNAi                        |
| PmSPH1rtF            | TACCCTCACCAGAGCAAGCGA | RT-PCR                     |
| PmSPH1rtR            | CTGGAAGAAAGATCCGAGCGA | RT-PCR                     |
| PmSPH2-322-F         | CAGGGAGTAGCAGTCGATGC | RT-PCR                     |
| PmSPH2-322-R         | TTGTGCTGCCAGACGAGACG | RT-PCR                     |
| PmproPO1-F           | GTCTCTCCCTCGCTGCTCG | RT-PCR                     |
| PmproPO1-R           | GCCGCAGTTCGTTGCGAGTC | RT-PCR                     |
| PmproPO2-F           | GCCAACAGGGAGCGGCTGATG | RT-PCR                     |
| PmproPO2-R           | TCCCTCATGCGGCTGAGACT | RT-PCR                     |
| PPAE1-F              | CTGTCGCTCATTGAGAGGAGG | RT-PCR                     |
| PPAE1-R              | GTAGTAGATGTGTCGCCAGCCT | RT-PCR                     |
| PPAE2-F              | GCCGCCGACGCTCCTGCTGTC | RT-PCR                     |
| PPAE2-R              | ACTCTCGGGAGCGCTGCTG | RT-PCR                     |
| PEN3-F               | GTTCTCTCGGCCTCGTCTCG | RT-PCR                     |
| PEN3-R               | TTTGCATACAACAAAGCTCTA | RT-PCR                     |
| PEN5-F               | TTTGCATATGCTTGCGAGTG | RT-PCR                     |
| PEN5-R               | ACAGATAGTTAAAGTGAAAGAC | RT-PCR                     |
| crustinPm1-F         | CTGTCGAGATGCAAGGATG | RT-PCR                     |
| crustinPm1-R         | AGTCTCTGAGCTGCTGCTG | RT-PCR                     |
| Crus-likePm-F        | CGGCAGGGTTCGAGATTTGACG | RT-PCR                     |
| Crus-likePm-R1       | AATTGAGATGTCGAGAATCGAGCTAT | RT-PCR                     |
| EF1α-F               | GTTCTCTGAGCAAGCTGAAAG | RT-PCR                     |
| EF1α-R               | GTTCTCTGAGCAAGCTGAAAG | RT-PCR                     |
| PmMasSPH2NcoI-F      | CATGCCATGGGGCCAGAATCGAGCTG | Recombinant protein expression |
| PmMasSPH2Xhol-R      | CGCTCTGAGATGCAAAGGATG | Recombinant protein expression |
| PmPPAE2NcoI-F        | CATGCCATGGGGCCAGAATCGAGCTG | Recombinant protein expression |
| PmPPAE2NotI-R        | AATAAATGCGGCGCTCAAGG | Recombinant protein expression |
| PmSPH1-Y2H-F         | GGAATTCCATATGAGGTGGGTGGTGGGAGC | Yeast two-hybrid |
| PmSPH1-Y2H-R         | CGCTCGAGAAAGTACCTGCTGCTG | Yeast two-hybrid |
| PPAE2-Y2H-F          | CATGCCATGGGGCCAGAATCGAGCTG | Yeast two-hybrid |
| PPAE2-Y2H-R          | CGCTCGAGAAAGTACCTGCTGCTG | Yeast two-hybrid |

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(LPS) (Sigma-Aldrich) and 20 μg of laminarin (β-1,3-glucan; Sigma-Aldrich). Shrimp were reared for 48 h after second injection prior to further analysis.

Hemolymph phenoloxidase (PO) activity assay
The total PO activity in shrimp hemolymph was measured as described previously [16]. 48 h after the second dsRNA injection, hemolymph was collected without using any anticoagulant. Total protein concentration was quantified using the Bradford protein assay kit (Bio-Rad). Subsequently, 2 mg total protein was analyzed for PO activity using L-3,4-dihydroxyphenylalanine (L-DOPA) as the substrate. PO activity was defined as ΔA490 per mg total protein/min. The results were analyzed from independent three experiments using a one-way analysis of variance (ANOVA).

Determining bacterial load in PmMasSPHs silenced shrimp
To investigate the involvement of PmMasSPHs in bacterial clearance, shrimp were injected with dsRNA specific to PmMasSPH1, PmMasSPH2 and control GFP as described above. For the second injection, shrimp were given V. harveyi 639 at a concentration of 2×10^5 colony forming units (CFUs). 6 h after bacteria challenge, the hemolymph was collected from each shrimp and serially diluted on LB agar plates and incubated at 30°C overnight. The number of colonies formed on the plate was calculated as CFU/ml.

Gene expression analysis in PmMasSPHs silenced shrimp
To test whether gene silencing of PmMasSPHs could affect the expression of other immune-related gene transcripts, gene expression analysis was carried out in PmMasSPHs silenced shrimp. The hemolymph was collected 48 h after dsRNA injection and then RNA was extracted and cDNA was made as described previously. Gene expression levels of several proPO-related proteins (PmproPO1, PmproPO2, PmPPAE1 and PmPPAE2) and antimicrobial proteins (PenmonPEN3, PenmonPEN5, crustinPm1 and Crus-likePm) were determined by semi-quantitative RT-PCR using the gene specific primers found in Table 1. The PCR conditions were as follows: 94°C 1 min, followed by 25 cycle of 94°C for 30 s, 55°C for 30 s and 72°C for 1 min, and finally 72°C for 5 min for the final step. PCR products were visualized by agarose gel electrophoresis and band intensity was analyzed using a Gel-Pro Analyzer. The expression levels of each gene are shown as relative expression normalized to EF1-α. The statistical significance of the triplicate experiments was determined by one-way ANOVA.

Expression and purification of recombinant proteins
The C-terminal SP-like domain of PmMasSPH2 and the SP domain of PmPPAE2 were amplified using Pfu DNA polymerase (Promega) with the primer pairs PmMasSPH2NcoI-F / PmMasSPH2XhoI-R and PmPPAE2NcoI-F / PmPPAE2NotI-R, respectively (Table 1). The PCR products were purified and cloned into the expression vector pET28b (Novagen). The His-tagged recombinant plasmids of the C-terminal SP-like domain of PmMasSPH1 [35], PmMasSPH2 (this study), the C-terminal SP domain of PmPPAE1 [30] and PmPPAE2 (this study) were used to express recombinant proteins in Escherichia coli Rosetta (DE3) pLysS. After induction with 1 mM IPTG at 37°C for 6 h, bacterial cells were harvested, re-suspended in 20 mM Tris-HCl (pH 8.0), then disrupted by sonication. The supernatant containing soluble protein was purified by nickel affinity chromatography (Ni-NTA Agarose; QIAGEN). In case inclusion bodies were obtained, protein was purified under denaturing conditions with 8 M urea in a Ni-NTA affinity column. The purified protein was subsequently refolded by dialysis.
with 20 mM Tris-HCl (pH 8.0). The protein concentration was then determined using a Bradford protein assay kit (Bio-Rad).

Quantitative determination of PmMasSPHs binding activity to PGN
An ELISA assay was performed to investigate the binding activities of both PmMasSPH1 and PmMasSPH2 to the bacterial cell wall component PGN. A 96-well plate was coated with 2 μg PGN from Bacillus subtilis (InvivoGen) per well in 50 μl water and then incubated at 37°C overnight. After fixing at 60°C for 2 h and blocking with 5% BSA in TBS for 1 h, the PGN-coated plate was washed with TBS three times. 100 μl of recombinant protein at various concentrations was added in each well and incubated at 4°C overnight. After washing, 100 μl of a mouse anti-His antibody (1:5000) was added in each well and incubated at room temperature for 3 h. After washing, the alkaline phosphatase-conjugated goat anti-mouse antibody (1:10000) was added and incubated for 1 h. The plate was then washed twice with TBST and twice with water before adding the substrate (AP Substrate Kit; Bio-Rad). The results were analyzed by Scatchard plot analysis. The dissociation constant (Kd) and the maximum binding (Amax) was calculated from the Y-intercept and slope of linear equation as 1/A = Kd/Amax[L]+1/Amax (A is defined as the absorbance at 405 nm and [L] is the protein concentration).

Protein-protein interaction assay
To determine if there is an interaction between PmMasSPH1 and PmPPAE2, we performed a yeast two-hybrid analysis from Matchmaker GAL4 Two-Hybrid System (Clontech). Full-length cDNA encoding PmMasSPH1 was cloned and fused in-frame with the activation domain (AD) of the pGADT7 vector by PCR amplification using PmSPH1-Y2H-F and PmSPH1-Y2H-R primers (Table 1). Full-length cDNA encoding PmPPAE2 was fused in-frame with the GAL4 DNA binding domain (BD) of the pGBK7 vector using PPAE2-Y2H-F and PPAE2-Y2H-R primers (Table 1). The recombinant plasmids of PmMasSPH1/AD and PmPPAE2/BD were co-transformed into the Saccharomyces cerevisiae strain AH109 using the lithium acetate/dimethyl sulfoxide method [37]. Transformed yeast cells were selected on minimal media plates lacking leucine and tryptophan (-L/-W). Positive interactions were indicated by growth on high stringency medium lacking adenine, histidine, leucine and tryptophan (-A/-H/-L/-W) and by a blue color change from X-α-gal (Apollo Scientific) present in the media. The empty vector pGADT7 was also co-transformation as negative control and an interaction between murine p53 bait fusion (pVA3) and SV40 prey fusion (pTD1) served as a positive control (Clontech).

In addition to the yeast two-hybrid assay, co-immunoprecipitation (co-IP) was also performed between the rPmMasSPHs and rPmPPAEs that were produced in E. coli as described above. The procedure was conducted according to Isono and Schwechheimer [38] with a slight modification. Briefly, to test if rPmMasSPH1 and rPmPPAE2 interact, 40 μg of purified anti-SP-like domain of PmMasSPH1 antibody was mixed with 40 μl of protein A agarose (Sigma) in 20 mM Tris-HCl (pH 8.0) and incubated at 4°C for 3 h. 50 μM of rPmMasSPH1 and 50 μM of the PmPPAE2 were incubated together at 4°C for 3 h and then loaded onto the antibody-tagged resin and further incubated at 4°C for 3 h. After washing 3 times with freshly prepared wash buffer (50 mM Tris-HCl (pH 7.5), 100 mM NaCl, 10% (v/v) glycerol and 0.05% (v/v) Triton X-100), the protein complex was eluted with 2X SDS-PAGE sample loading buffer. The protein complex was then separated by SDS-PAGE, transferred to a nitrocellulose membrane and detected with anti-His antibody. The co-IP assay between PmMasSPH2 and PmPPAEs was also performed using the purified anti-SP-like domain of PmMasSPH2 antibody as described for PmMasSPH1.
Effect of \textit{Pm}MasSPH1 and \textit{Pm}PPAE2 on the PGN-triggered hemolymph PO activity \textit{in vitro}

To identify whether \textit{Pm}MasSPH1 and \textit{Pm}PPAE2 are necessary for activation of proPO after infection by Gram-positive bacteria, PO activity was measured in the presence of PGN and the recombinant proteins. Fresh shrimp hemolymph (HL) was collected and the total protein concentration measured by the Bradford assay (Bio-Rad). Total reaction included 0.8 mg HL with the addition of 0.25 μg PGN and 1 μM of each recombinant protein (\textit{Pm}PPAE2 and \textit{Pm}MasSPH1 or BSA as the protein control). The CAC buffer (10 mM CAC containing 10 mM CaCl₂) was added to a final volume of 174 μl. 26 μl of 3 mg/ml L-DOPA was added and the ΔA₄₉₀ measured at 5 min. Each experimental treatment was performed in triplicate. Data were analyzed using a one-way analysis of variance (ANOVA) followed by Duncan’s test and a p < 0.05 was considered statistically significance.

Results

Sequence analysis of \textit{Pm}MasSPH1 and \textit{Pm}MasSPH2

Based on the shrimp \textit{P. monodon} EST database (http://pmonodon.biotec.or.th/) [39], three full-length cDNAs of clip SPHs (\textit{Pm}MasSPH1, \textit{Pm}MasSPH2 and \textit{Pm}MasSPH3) have been isolated [6]. Among these, a masquerade-like SPH1 (\textit{Pm}MasSPH1) of shrimp \textit{P. monodon} has been cloned and its role in shrimp immunity has been characterized [34,35]. In this study, \textit{Pm}MasSPH1 and the novel SPH \textit{Pm}MasSPH2, were investigated to further elucidate their function in proPO activation. A full-length sequence of \textit{Pm}MasSPH2 was obtained with an open reading frame of 1164 bp encoding 387 amino acid residues. The \textit{Pm}MasSPH2 cDNA sequence was deposited in the GenBank database under accession number FJ620686. Apart from a signal peptide of 20 amino acids at the N-terminus, the mature protein (367 amino acids) has the calculated molecular mass of 39.37 kDa and the predicted pl of 7.52. The conserved clip-domain and serine proteinase (SP)-like domain with the catalytic triad (His₁₈₄, Asp₂₃₄ and Gly₃₃₈) were also found in \textit{Pm}MasSPH2, \textit{Pm}MasSPH1 and other SPHs (Fig. 1). BLAST analysis of \textit{Pm}MasSPH1 and \textit{Pm}MasSPH2 showed high homology to the SPs of crustaceans and insects. High similarity and conserved function involved in proPO cascade have also been reported for the crayfish \textit{Pacifastacus leniusculus} \textit{Pl}SPH1 and \textit{Pl}SPH2, crab \textit{Scylla paramamosain} \textit{Sp}-SPH and beetle \textit{Holotrichia diomphalia} \textit{Hd}PPAF-II [28,29,40], and thus, we aligned these amino acid sequences with both \textit{Pm}MasSPH1 and \textit{Pm}MasSPH2 (Fig. 1). Interestingly, \textit{Pm}MasSPH1 and \textit{Pm}MasSPH2 have only 34.6% sequence similarity but high homology was observed across other species. For instance, \textit{Pm}MasSPH1 has 55.5% similarity to \textit{Pl}SPH2, and \textit{Pm}MasSPH2 and \textit{Pl}SPH1 share 68.2% similarity. Sequence comparison of only the SP-like domain showed consistency in similarity among selected species. The SP-like domains of \textit{Pm}MasSPH1 and \textit{Pm}MasSPH2, \textit{Pm}MasSPH1 and \textit{Pl}SPH2, and \textit{Pm}MasSPH2 and \textit{Pl}SPH1 share 51.6%, 73.6% and 78.9% similarity, respectively. A phylogenetic analysis of the SP-like domain of insect and crustacean PPAFs was also carried out to examine the evolutionary relationships. A phylogenetic tree constructed using the Neighbor-Joining method revealed that \textit{Pm}MasSPH1 and \textit{Pm}MasSPH2 were grouped in a clade of crustacean PPAFs (Fig. 2). \textit{Pm}MasSPH1 is closely related to \textit{P. monodon} serine proteinase-like protein (\textit{Pm}SPL) and other shrimp PPAFs and is grouped in the same clade as \textit{Pl}SPH2. In contrast, \textit{Pm}MasSPH2 is more closely linked to \textit{Pm}SPL3 and \textit{Pl}SPH1 (Fig. 2).
Gene expression in various developmental stages of shrimp larva

We next examined the expression profile of \textit{Pm}MasSPH1 and \textit{Pm}MasSPH2 transcripts to better understand regulation of immune genes during shrimp development. Gene expression patterns of \textit{Pm}MasSPH1 and \textit{Pm}MasSPH2 were obtained and compared to the internal EF1-\textalpha control by RT-PCR analysis at four shrimp larval developmental stages including nauplius 3 (N3), protozoea 2 (Z2), mysis 2 (M2) and post-larvae 15 (PL15). We found that both \textit{Pm}MasSPH1 and \textit{Pm}MasSPH2 were expressed at all the developmental stages we tested but with different expression patterns. Expression levels of the \textit{Pm}MasSPH1 transcripts were stable at all stages, while the \textit{Pm}MasSPH2 transcripts were lower at the early stages and then gradually increased as development progressed (Fig. 3).

Knockdown of \textit{Pm}MasSPHs decreases hemolymph PO activity

Because several proteins together participate in the proPO cascade to defend against invading pathogens, we evaluated the involvement of \textit{Pm}MasSPH1 and \textit{Pm}MasSPH2 in proPO activation by RNAi-mediated gene silencing. Shrimp were injected twice with dsRNA against \textit{Pm}MasSPH1 or \textit{Pm}MasSPH2 with GFP dsRNA or NaCl solution used as control groups. 48 h after the second injection, shrimp hemolymph was collected and gene transcription analyzed. Our results show that shrimp receiving \textit{Pm}MasSPH1 or \textit{Pm}MasSPH2 dsRNA efficiently suppressed \textit{Pm}MasSPH1 or \textit{Pm}MasSPH2 transcript expression (Fig. 4A and B). Injection of GFP dsRNA or NaCl solution had no effect on \textit{Pm}MasSPH1 or \textit{Pm}MasSPH2 mRNA transcript levels.

To examine whether \textit{Pm}MasSPH1 and \textit{Pm}MasSPH2 are involved in the activation of proPO, the total hemolymph PO activity of \textit{Pm}MasSPH1 or \textit{Pm}MasSPH2 silenced shrimp was measured using L-DOPA as a substrate. The hemolymph PO activity of \textit{Pm}MasSPH1 and \textit{Pm}MasSPH2 knockdown shrimp decreased significantly by 66.5\% and 63.7\%, respectively. No significant change in PO activity was observed in the control groups ($p < 0.05$) (Fig. 4C and D). These results indicate that \textit{Pm}MasSPH1 and \textit{Pm}MasSPH2 are involved in proPO activation in shrimp.

\textit{Pm}MasSPH silencing increase bacterial load in shrimp hemolymph

The above results clearly demonstrate that both \textit{Pm}MasSPH1 and \textit{Pm}MasSPH2 have potential roles in proPO activation. Therefore, we investigated whether \textit{Pm}MasSPH1 and \textit{Pm}MasSPH2 have immune activity against shrimp pathogenic bacteria. The \textit{Pm}MasSPH1 or \textit{Pm}MasSPH2 silenced shrimp were challenged with pathogenic \textit{V. harveyi} at $2\times 10^5$ CFU/shrimp. As a control, shrimp injected with GFP dsRNA were also challenged with \textit{V. harveyi}. 6 h after bacterial challenge, hemolymph from individual shrimp was collected and the CFU of \textit{V. harveyi} was determined. Our results show that the number of viable bacterial CFUs in the \textit{Pm}MasSPH1 and \textit{Pm}MasSPH2 silenced shrimp increased 11.5 and 7.9-fold, respectively, compared to the control shrimp injected with GFP dsRNA (Fig. 5A and B). It is worth noting that the \textit{Pm}MasSPH1 silenced shrimp have a higher bacterial hemolymph load than the \textit{Pm}MasSPH2 silenced.
The phylogenetic relationship between serine proteinase domains from PmMasSPH1 and PmMasSPH2 and other serine proteinases. The deduced amino acid sequences of SP-domains from various clip-SPH species were used to generate a phylogenetic tree by the neighbor-joining method. Percent bootstrap values (1000 replicates) are shown at each branch point. PmMasSPH1 (ABE03741); PmMasSPH2 (ACP19560); PmSP, P. monodon serine proteinase (ABW87872); PmSPL, P. monodon serine proteinase-like protein (ABD62888); PmSPL3 (ABO33174); PmMas, P. monodon mas-like protein (AAT42131); FcPPAF, Fenneropenaeus chinensis prophenoloxidase activating factor (AFW98986); FcMas (AFW98983); LvPPAF, Litopenaeus vannamei PPAF (AFW98993); LvSP (AY368151); LvMas (AFW98990); PISPH1, P. leniusculus SPH1 (AAX55746); PISPH2 (ACB41379); MsSPH3, Manduca sexta SPH3 (AF413067); MjSPH, Marsupenaeus japonicus SPH (AB161692); NvSPH21, Nasonia vitripennis SPH21 (NP_001155060); CqSP, Culexquin quefasciatus SP (XP_001868413); AaSP, Aedes aegypti SP (XP_001655705); PtPPAF, Portunus trituberculatus PPAF (ACN87221); CsPAF, Callinectes sapidus PAF (AAS60227); EsPPAF, Eriocheir sinensis PPAF (ACU65942); EdTrypsinlikeSP (ACT78700); LsST, Lucilia sericata saliva trypsin (AEX33291); CpPPAF, Cancer pagurus PPAF (CCE46009); PpSP, Papilio polytes SP (BAM19108); HaSPL1, Helicoverpa armigera SPL1 (ACI32835); TrmPPAF, Tenebrio molitor PPAF (CAC12696); StSPL, Scylla tranquebarica SPL (ADN44616); LoPPAF1, Lonomia obliqua PPAF1 (AAV14958); PxSP, Papilio xuthus SP (BAM17901); PmSPL, Pieris rapae MSPH (ACZ68116); TcSPL4, Tribolium castaneum SP4 (EEZ99183); DmMas, Drosophila melanogaster Mas (AAC46512); Sp-SPH, S. paramamosain SPH (ADG83846).
Fig 3. Expression of PmMasSPH1 and PmMasSPH2 transcripts at various stages of shrimp larval development. Expression profiles of PmMasSPH1 and PmMasSPH2 were examined at four larval stages including nauplius 3 (N3), protozoea 2 (Z2), mysis 2 (M2) and post-larvae 15 (PL15) by semi-quantitative RT-PCR. The elongation factor 1-α (EF1-α) served as an internal control. Each lane represents the result of individual shrimp (n = 3).

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Fig 4. Hemolymph PO activity of PmMasSPH1 and PmMasSPH2 silenced shrimp. The efficiency of gene silencing of PmMasSPH1 and PmMasSPH2 was determined by semi-quantitative RT-PCR. Transcription levels of PmMasSPH1 (A) and PmMasSPH2 (B) were examined in shrimp injected with the corresponding dsRNA. Shrimp injected with GFP dsRNA or NaCl served as control groups. Expression of EF1-α was used as an internal control. Each band represents the pooled cDNA of triplicate samples of each treatment group. Hemolymph PO activity of the PmMasSPH1 (C) and PmMasSPH2 (D) silenced shrimp was also examined. Shrimp injected with GFP dsRNA or NaCl served as control groups. The total hemolymph PO activity was measured as ΔA⁴⁹⁰/mg total protein/min. The data are shown as the mean ± standard deviation (error bars) from three independent experiments. Significantly different means (p < 0.05) are indicated by the lower case letters (a, b) above each bar.

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These results suggest that both \textit{Pm} \textit{MasSPH1} and \textit{Pm} \textit{MasSPH2} are involved in the process of hemolymph bacterial clearance in the shrimp \textit{P. monodon}.

\textbf{\textit{PmMasSPH1} silencing alters expression of other immune genes}

Several studies in insect immunity have shown evidence that a single gene does not strictly regulate one pathway but can play a role in several pathways. Previous studies in the lepidopteran \textit{Manduca sexta} showed that SPH-3 is not only required for proPO activation but is also important for transcription of other immune effector genes [21]. We therefore, examined the effect of \textit{PmMasSPHs} silencing on the expression of other immune genes by semi-quantitative RT-PCR. The hemocyte cDNAs from \textit{Pm} \textit{MasSPH1} or \textit{Pm} \textit{MasSPH2} silenced shrimp was examined for transcript expression of other proPO-related genes (\textit{Pm} \textit{proPO1}, \textit{Pm} \textit{proPO2}, \textit{PmPPAE1} and \textit{PmPPAE2}) and antimicrobial peptide genes (\textit{Penmon} \textit{PEN3}, \textit{Penmon} \textit{PEN5}, \textit{CrustinPm} \textit{1} and Crus-like \textit{Pm}), with \textit{EF1-\alpha} as an internal control. Additionally, expression of these other immune-related genes was examined in the control groups injected with GFP dsRNA and NaCl. As shown in Fig. 6, shrimp injected with \textit{Pm} \textit{MasSPH1} dsRNA have decreased expression of \textit{PmMasSPH1} (89%), \textit{PmPPAE2} (71%) (Fig. 6B), \textit{PenmonPEN3} (54%) (Fig. 6C), \textit{CrustinPm} \textit{1} (69%) (Fig. 6D) and Crus-like \textit{Pm} (65%) (Fig. 6E) when compared to GFP dsRNA control shrimp. This suppression of immune genes by \textit{PmMasSPH1} silencing was not caused by an off-target effect of RNAi because sequence comparison between the region corresponding to \textit{Pm} \textit{MasSPH1} dsRNA and the other immune-related genes have no significant similarity (data not shown). \textit{PmMasSPH2} gene silencing was also performed in the same manner as \textit{PmMasSPH1}. We found that only the \textit{PmMasSPH2} transcripts (98%) were suppressed in this model, while expression of other immune-related genes did not change (data not shown). Taken together, \textit{PmMasSPH1} appeared to be required for proPO activation and is involved in the synthesis of shrimp antimicrobial peptides.

\textbf{In vitro binding of \textit{PmMasSPH1} and \textit{PmMasSPH2} to PGN}

It has been shown that \textit{PmMasSPH1} might act as a PRP because it can bind to the Gram-negative bacteria \textit{V. harveyi}, bacterial cell wall component LPS, and virus [35,36]. However, it is unknown if \textit{PmMasSPHs} binds to Gram-positive bacteria. In this study, \textit{PmMasSPH1} and
PmMasSPH2 were examined for a role in recognition of peptidoglycan (PGN), a Gram-positive bacterial cell wall component. An ELISA assay was used to quantitatively measure the binding activity between the recombinant proteins of mature PmMasSPH1 or mature PmMasSPH2 (with varying concentrations of 0–12.5 μg/ml) with 2 μg of ligand PGN from Bacillus subtilis. GFP served as a recombinant control protein. As shown in Fig. 7, the rPmMasSPH1 and rPmMasSPH2 directly bound PGN in a concentration-dependent manner. The dissociation constant (K_d) of rPmMasSPH1-PGN and rPmMasSPH2-PGN were calculated as 6.51x10^{-9} M and 5.79x10^{-9} M, respectively. These results clearly indicate that PmMasSPH1 and PmMasSPH2 have a functional role as PRPs for PGN.

**PmMasSPH1 interacts with PmPPAE2**

Because it has been demonstrated that some insect SPHs function as co-factors of the terminal PPAEs of proPO activation [11], it is important to investigate whether PmMasSPHs interact with PmPPAEs. Because PmPPAE2 changed expression when PmMasSPH1 was silenced, we examined if there is a protein-protein interaction between PmMasSPH1 and PmPPAE2 using yeast two-hybrid and co-immunoprecipitation (co-IP) assays. In the yeast two-hybrid analysis, both PmMasSPH1/AD and PmPPAE2/BD plasmids were co-transformed into the yeast AH109 strain and plated on selective media. Our results show that PmMasSPH1 specifically binds to PmPPAE2 as indicated by blue colony formation on the selective media-A/-H/-L/-W/X-α-gal (Fig. 8A, lane PmMasSPH1/AD and PmPPAE2/BD). In addition, no self-activation

![Fig 6. The effect of PmMasSPH1 gene silencing on expression of other immune genes.](image-url)
occurred when PmPPAE2/BD was co-transformed with an empty AD plasmid (Fig. 8A, lane empty AD and PmPPAE2/BD). Co-transformation of PmMasSPH1/AD with the control pVA3 plasmid was also performed to confirm that PmMasSPH1 did not interact with the BD plasmid. The yeast two-hybrid assay suggests a genuine interaction between PmMasSPH1 and PmPPAE2.

To further confirm the protein-protein interaction, a co-IP assay with rPmMasSPH1 and rPmPPAE2 was carried out using anti-SPH1-conjugated protein A agarose beads to precipitate the protein complex. As shown in Fig. 8B lane 2, both rPmMasSPH1 and rPmPPAE2 were observed in the elute protein fraction indicating an interaction between the proteins. In addition, other pairwise protein interactions (rPmMasSPH1 and rPmPPAE1, rPmMasSPH2 and

Fig 7. Ability of rPmMasSPHs to bind a Gram-positive bacterial cell wall component. The binding ability of rPmMasSPH1 or rPmMasSPH2 (at varying concentrations 0–12.5 μg/ml) to B. subtilis PGN was quantified by ELISA assay. rGFP was included as a recombinant protein control. The data at each point was fitted to the trend line and is shown as the mean ± standard deviation from three individual experiments.

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Fig 8. Interaction of PmMasSPH1 with PmPPAE2. The protein-protein interaction between PmMasSPH1 and PmPPAE2 were analyzed by yeast two-hybrid and co-IP assays. (A) The yeast two-hybrid results indicate the presence of BD and AD plasmids in transformed cells growing on an-L/-W plate. A putative interaction between PmMasSPH1/AD and PmPPAE2/BD as indicated by growth and blue color on the selective media-A/-H/-L/-W/X-α-gal. The positive control consisted of an interaction between murine p53 bait fusion (pVA3) and SV40 prey fusion (pTD1). The negative controls consisted of yeast cells containing PmPPAE2/BD with an empty AD vector or pVA3 vector and PmMasSPH1/AD. (B) The interaction between PmMasSPH1 and PmPPAE2 was confirmed by co-IP assay. The immunoprecipitated complex between rPmMasSPH1 and rPmPPAE2 was detected using the monoclonal anti-His antibody (lane 2). The rPmMasSPH1 or rPmPPAE2 alone (lanes 1 and 3, respectively) was used as a protein indicator.

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rPmMasSPH1, and rPmMasSPH2 and rPmPPAE2) were also examined by co-IP assay. However, no other positive interactions were found (data not shown). Altogether, the yeast two-hybrid and co-IP analyses indicate a direct interaction between PmMasSPH1 and PmPPAE2.

Enhancement of PGN-triggered hemolymph PO activity by PmMasSPH1 and PmPPAE2 in vitro

Because PmMasSPH1 can recognize PGN and bind to PmPPAE2, we examined the role of these proteins in proPO activation. rPmMasSPH1, rPmPPAE2 and PGN were mixed with shrimp hemolymph and then PO activity was measured using L-DOPA as a substrate. In the presence of PGN, shrimp hemolymph PO activity was induced by approximately 23% compared with the non-activated hemolymph (HL) (Fig. 9). Interestingly, high PO activity from PGN was significantly enhanced by 102% when rPmMasSPH1 and rPmPPAE2 was gradually added to the hemolymph (Fig. 9). In addition, incubation of HL with PGN together with PmPPAE2 and BSA (instead of rPmMasSPH1) resulted in no significant change in PO activity (14% compared to the control HL) (Fig. 9). These results suggest that hemolymph PO activity is slightly activated by PGN and PmMasSPH1 may be a co-factor to PmPPAE2 in P. monodon proPO activation.

Discussion

In invertebrates, including insects and crustaceans, the proPO-activating system is essential for the melanization process that rapidly responds to the intruding pathogens [4–6,11]. The activation of zymogen proPO to active PO, a critical step required for melanin synthesis, depends on the cleavage of terminal clip-serine proteinase and sometimes occurs with the aid of non-catalytic serine proteinase homologues (SPHs) cofactors [4–6,9]. Several studies have shown the requirement of SPHs for proPO activation by enhancing the cleavage of proPO to active PO by PPAEs [20,23,24]. For example, in insects, proPO activation of Holotrichia diomphalia needs PPAF-I to cleave proPO (79 kDa) into PO (76 kDa), and non-catalytic PPAF-II to bind the...
cleaved proPO and generate the catalytically active PO [23]. The process in Manduca sexta requires both PPAE1 (PAP-1) and SPH to simultaneously activate the inactive proPO [20].

In the shrimp P. monodon, PmMasSPH1 has been previously identified and characterized as a multifunctional immune protein because it possesses opsonic abilities, antimicrobial activity against gram-positive bacteria, and binds to LPS, V. harveyi and virus [34–36]. Nevertheless, the function of PmMasSPH1 as it relates to proPO activation and other PmMasSPHs remains unknown. Therefore, in the present study, we examined PmMasSPH1 and the newly identified PmMasSPH2 for their potential roles in proPO activation. The pattern recognition properties of PmMasSPHs were also investigated because knowledge on the recognition of PGN by PmMasSPHs has not yet been reported.

The novel PmMasSPH2 consists of an N-terminal clip-domain and a C-terminal SP-like domain with a catalytic triad containing a Gly residue (instead of a Ser residue) similar to what has been previously identified in PmMasSPH1 and other arthropod SPHs. A multiple sequence alignment showed that PmMasSPH2 has a similar primary structure as PisSPH1, PisSPH2, HedPPAFII and Sp-SPH, containing a one clip-domain at the N-terminus and an SP-like domain at the C-terminus, unlike PmMasSPH1, which contains a glycine-rich domain at the C-terminus. The highest similarity was found between PmMasSPH1 and PisSPH2 and between PmMasSPH2 and PisSPH1, suggesting that a similar role for PisSPHs in proPO activation.

In the developmental expression profile of PmMasSPHs, we found that both transcripts are expressed in all larval stages tested, similar to other proPO-related genes such as PmPPAE1 and PmproPO2 [31], although PmMasSPH2 had a slightly low level of gene expression at the N3 stage. This is in contrast to PmPPAE2 and PmproPO1 transcripts that are expressed at the middle phase of larval development (e.g., mysis and post-larvae stages) [31,41]. It has previously been suggested that PmPPAE1 and PmproPO2 together might function at an early stage of larval development while PmPPAE2 and PmproPO1 may play a role at later developmental stages [31]. However, it was shown in P. monodon that there is no PO activity at the N4 stage, suggesting incomplete proPO activation [41]. In addition, the absence of proPO expression in the middle phase of embryo development has also been reported in the crayfish Pacifastacus leniusculus [42]. Thus, it is possible that the presence of PmMasSPHs at early larval developmental stages of P. monodon might function in immune defense reactions other than in proPO activation. This hypothesis is also supported by previous work that shows that PmMasSPH1 has multiple immune functions including opsonic capabilities, antimicrobial activity and recognition of bacterial pathogens [35].

To evaluate the relevance of PmMasSPHs in the proPO-activating system, in vivo silencing of PmMasSPH1 and PmMasSPH2 using dsRNA was carried out and total shrimp PO activity was then examined. In PmMasSPH1 and PmMasSPH2 silenced shrimp, the hemolymph PO activity was significantly decreased compared to the control GFP dsRNA by approximately 67% and 64%, respectively. In comparison to the other shrimp proPO-related genes, this decrease in PmMasSPHs is quite high and resembles PmproPO1 and PmproPO2 silencing in which the PO activity was reduced by 75% and 73%, respectively [16]. In addition, knockdown of PmPPAE1 and PmPPAE2 in P. monodon reduced the hemolymph PO activity only by 37% and 41%, respectively [31]. All evidence suggests that PmMasSPHs, PmPPAEs and PmproPOs together participate in proPO activation, especially PmMasSPHs that are required factors in the activation cascade because their gene silencing resulted in a significant reduction of PO activity. Similarly, in P. leniusculus, knockdown of PλproPO, PISP1 and PISP2 also disrupt PO activity, indicating their essential roles in proPO activation [14,28].

Because the proPO system is known to rapidly respond to intruding pathogens and silencing of the PmMasSPHs significantly decreases PO activity, the correlation between the reduction in PO activity by PmMasSPH1 and PmMasSPH2 knockdown and bacterial clearance
activity was examined in vivo. The number of viable *V. harveyi* was significantly increased in the hemolymph of *Pm*MasSPHs silenced shrimp thereby suggested the importance of *Pm*MasSPH1 and *Pm*MasSPH2 in bacterial clearance. It was noted that there are differences in the rate of *V. harveyi* clearance between *Pm*MasSPH1 and *Pm*MasSPH2. In comparison to the control GFP dsRNA, the bacterial number in *Pm*MasSPH1 silenced shrimp was higher than in *Pm*MasSPH2 silenced shrimp (11.5-fold versus 7.9-fold, respectively) and may suggest a different way to activate proPO and eliminate the bacteria. In addition to *Pm*MasSPHs, knockdown of *Pm*PPAEs and *Pm*proPOs may also increase bacterial loads and induce high mortality in shrimp [16,30,31]. Likewise, in *P. leniusculus*, silencing of *Pl*proPO decreases PO activity and increases hemolymph bacterial number [14]. These relationships may explain the significant role of *Pm*MasSPHs in promoting the PO activity and subsequently limiting the number of bacteria in shrimp hemolymph.

A previous report in shrimp demonstrated that suppression of proPO genes (*Pm*proPO1 and *Pm*proPO2) alters the expression levels of other proPO-related genes and antimicrobial peptide (AMP) genes [15,32]. In the present study, the effect of *Pm*MasSPHs gene silencing on immune gene expression was therefore investigated. We found that transcription levels of the proPO-related gene (*Pm*PPAE2) and AMP genes (*Penmon*PEN3, *Penmon*PEN5, Crustin*Pm*1 and Crus-like*Pm*) were significantly decreased by suppression of *Pm*MasSPH1 but not *Pm*MasSPH2 transcripts. This suggests that proPO and *Pm*MasSPH1 could contribute to the crosstalk between the proPO-activating system and the AMP synthesis pathway in shrimp. This phenomenon is often found in invertebrate immunity because several studies have reported that a single regulator plays a role in more than one pathway. For example, in the mealworm *Tenebrio molitor*, Tm-SPE is a terminal serine proteinase that cleaves pro-spazle in the Toll pathway to produce AMPs and also activates proPO and the cofactor SPH1 in the melanization process [25]. This is consistent with a report in *Drosophila* showing that the melanization reaction depends on activation of the Toll pathway and removal of the serine protease inhibitor Serpin27A [43]. In *M. sexta*, the hemolymph proteinase HP6 was also shown to activate PPAE1 (proPAPI) in the proPO system and HP8 in the Toll-like pathway [44].

Because it has not yet been reported for the peptidoglycan recognition proteins (PGRPs) in crustaceans, there have been attempts to elucidate the mechanism of proPO activation by peptidoglycan in these animals. In the crayfish *P. leniusculus*, the involvement of *Pl*SPHs in PGN-induced proPO activation has been demonstrated [28]. In the mud crab *Scylla paramamosain*, Sp-SPH is also able to activate the PO activity triggered by PGN [29]. Nevertheless, these crayfish and crab SPHs have no PGN binding motif typically found in PGRPs [45]. Therefore, in the present study, we investigated whether *Pm*MasSPHs can recognize PGN similar to our previous study showing that *Pm*MasSPH1 could bind to the bacterial cell wall component, LPS [35]. Our ELISA assay clearly shows the ability of both *Pm*MasSPH1 and *Pm*MasSPH2 to bind PGN. The slightly different $K_d$ value of *Pm*MasSPH1 (6.51x10^{-9} M) and *Pm*MasSPH2 (5.79x10^{-9} M) to PGN suggests that the binding ability of *Pm*MasSPH1 is stronger than that of *Pm*MasSPH2. Thus, we conclude that *Pm*MasSPH1 recognizes PGN but a different shrimp PRP, *Pm*LGBP, binds to LPS and β-1,3-glucan [32] and subsequently leads to proPO activation. Our results also suggest that aside from the PGRPs, there is another mechanism for PGN-induced immunity in crustaceans probably via other immune related proteins that do not belong to the PGRP family. This was also observed in *P. monodon* QM and C-type lectin proteins that did not contain a PGN binding motif but could interact with PGN and induce melanization activity in vitro [46]. However, the mechanism on how non-PGRP proteins recognize PGN and activate the proPO system requires further investigation.

According to the AMP synthesis pathway in *D. melanogaster*, both Toll and IMD pathways are initially activated by different types of PGN that eventually result in nuclear factor-xB
(NF-κB)-dependent AMP synthesis [47]. In addition, the requirement of PGRP has been shown for the activation of Relish, an NF-κB transcription factor family [48]. Because PmMasSPH1 have the ability to bind PGN and suppression of its transcription affects AMP gene expression, this might suggest that in addition to proPO activation, PmMasSPH1 may play a role in the Toll-induced AMP synthesis pathway. In M. sexta, SPH-3 is not only responsible for the proPO system but is also required for the expression of antimicrobial effector genes [21]. In addition, the silencing of PmMasSPH2 did not affect transcription of other genes and therefore, PmMasSPH2 may play a role only in proPO activation.

To clarify the role of PmMasSPH1 in proPO activation, protein-protein interaction assays were carried out because suppression of PmMasSPH1 altered PmPPAE2 transcript levels. We demonstrated a specific interaction between PmMasSPH1 and PmPPAE2, suggesting that PmMasSPH1 anchors and acts as a cofactor to PmPPAE2 to activate PmproPO1 to active PO. This is supported by an in vitro assay showing that the presence of both PmMasSPH1 and PmPPAE2 can promote hemolymph PO activity triggered by PGN. The ability to bind microorganisms and activate proPO might suggest an effective immune response method. When bound to a pathogen, PmMasSPH1 may recruit other components such as PmPPAE2 and PmproPO1 to generate active PO at the site of pathogen infection. It has been shown in M. sexta that after hemolymph protease HP21 activates proPAP2 and 3 [49], these active proteinases, along with SPHs, then produce active PO at the site of infection [19]. Although the crucial role of PmMasSPH1 in proPO-related immunity is extensive described in this study, the role of PmMasSPH2 is still unclear and requires further investigation. In summary, this study is the first to demonstrate that shrimp SPHs function as pattern recognition proteins for Gram-positive bacteria, act as co-activators of proPO and are involved in AMP synthesis.

**Author Contributions**

Conceived and designed the experiments: MJ PA PS WC SS AT. Performed the experiments: MJ PA PS WC. Analyzed the data: MJ PA PS WC SS AT. Contributed reagents/materials/analysis tools: PA SS AT. Wrote the paper: MJ PA PS WC AT.

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