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The immune function of prophenoloxidase from red swamp crayfish (*Procambarus clarkii*) in response to bacterial infection

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ABSTRACT

Prophenoloxidase (proPO) is the zymogen form of phenoloxidase (PO), a key enzyme in melanization cascade that has been co-opted in invertebrate immune reactions. There have been reported that proPO plays many essential roles in the crustacean immune system. However, little is known about the function of proPO from red swamp crayfish (*Procambarus clarkii*) which is an important cultured species worldwide. Here, we cloned and expressed proPO gene from red swamp crayfish (*Pc*proPO). Subsequently, specific antibody against *Pc*proPO was generated. The immune function of *Pc*proPO was further characterized *in vitro* and *in vivo*. The results showed that the expression of *Pc*proPO mRNA could be significantly up-regulated during the challenge of Gram-positive-negative (*Vibrio parahaemolyticus*) and Gram-positive-positive bacterial (*Staphylococcus aureus*). Furthermore, the purified recombinant *Pc*proPO protein had a strong affinity binding to both bacteria and polysaccharides. *In vivo* knockdown of *Pc*proPO could significantly reduce the crayfish bacterial clearance ability, resulting in the higher mortality of the crayfish during *V. parahaemolyticus* infection. In addition, *in vitro* knockdown of *Pc*proPO in the hemocytes significantly reduced the phenoloxidase (PO) activity and the bacterial clearance ability, indicating that *Pc*proPO might involve in hemocyte-mediated melanization. Our results will shed a new light on the immune function of *Pc*proPO in the crayfish.

1. Introduction

Red swamp crayfish (*Procambarus clarkii*) as a world invasive species, since it was introduced to China in 1929, though, it turned into one of the most popular varieties in aquaculture in recent years [1]. Based on the data of the Ministry of Agriculture in China, the total annual production of *P. clarkii* reached nearly 1,000,000 metric tons in 2017, exceeding the total economic output of 260 billion. However, the increasing cultivation of red swamp crayfish has been constrained in recent years by various pathogens such as *White spot syndrome virus* (WSSV), *Vibrio parahaemolyticus*, and *Spiroplasma eriocheiris,* causing serious economic losses to crustacean aquaculture industries annually [2–6] Despite the lack of an adaptive immune system in red swamp crayfish, they exert stronger selection pressure on the innate immune system to defend against the pathogens [7]. Studying the innate immunity of *P. clarkii* is helpful in improving our knowledge on invertebrate biology, as well as benefits the development of *anti*-disease strategy in aquaculture [8,9].

Crustaceans primarily rely on a fixed non-specific immune mechanism as a structural barrier to prevent the pathogen entry and spread. The mobile non-specific immune system has two principal components, the humoral and cellular systems both of which are activated upon immune challenge [10]. Prophenoloxidase (proPO) is a critical enzyme in innate immune response involving in multiple physiological processes, such as melanization, cytotoxic reactant production, particle encapsulation, hemocyte attraction and inducing

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Table 1
Primers sequences used in this study.

Primer	Sequence (5′–3′)	Purpose
*Pc*ProPo-F	TGCCTTAGGGGTGTTTTA	RT-PCR
*Pc*ProPo-B	CAGGGTGACTGGTCTTGG	
18S–F	ACCGATTGAATGATTTAGTGAG	RT-PCR
18S-R	TACGGAAACCTTGTTACGAC	
*Pc*ProPo-*Eco*R1-F	GTCGAATTCCTCTCTGATAACGTCACG	Recombinant plasmid
*Pc*ProPo-*Not*1-B	GACGCGGCCGCATTTAATTGTTCGACTTCTGAAG	
ds*Pc*proPO-F	GCGTAATACGACTCACTATAGgTGGGGCCGGATTCACGA	dsRNA
ds*Pc*proPO-B	GCGTAATACGACTCACTATAGgTATCTTTGTAACGAATG	
dsGFP-F	GCGTAATACGACTCACTATAGGTGGTCCCAATTCTCGTGGAAC	dsRNA
dsGFP-R	GCGTAATACGACTCACTATAGGCTTGAAGTTGACCTTGATGCC	

phagocytosis, and the formation of nodules and capsules [11–13]. During pathogen infection or physical injury, a serine protease cascade leads to the activation of proPO by proPO-activating proteinase (PAP) at a conserved proteolytic cleavage site near the N-terminus to generate active phenoloxidase (PO) [11]. In general, activation of the proPO system is controlled by a multistep pathway. The active PO possesses o-hydroxylase and o-diphenoloxidase activities that convert a variety of monophenolic and o-diphenolic substrates to o-quinones [14,15]. Quinones and other reactive intermediates directly kill microbial pathogens and parasitoids [16,17]. ProPO can also be activated extracellularly by microbial products without proteolytic cleavages such as lipopolysaccharides from bacteria and β-1,3-glucans from fungi [10,12]. Over the past decade, the proPO system has been relatively well studied, as a result its prominence in the invertebrate immune system has been confirmed [10]. Notably, in recent years, the proPO structures from *Manduca sexta*, *Marsupenaeus japonicas,* and *Anopheles gambiae* have been investigated, which provided us new insights into the mechanism of proPO activation [18,19]. Many studies have examined the factors which can activate proPO system and immune response of the proPO against pathogen invasion, little is yet known about the interaction of proPO of *P. clarkii* and its immune functions.

In the present study, we have obtained recombinant protein *Pc*proPO and uncovered the temporal and spatial expression patterns in *P. clarkii*. We showed that the recombinant protein bound to various bacteria and polysaccharides. Furthermore, we have investigated the immune function of *Pc*proPO via *in vivo* and *in vitro* knocked down of *Pc*proPO gene of *P. clarkii*. Our results could provide insight into immunological function of the *Pc*proPO during bacterial infection.

2. Materials and methods

2.1. Materials

The healthy *P. clarkii* (8–10 g) were obtained from the Baishazhou aquatic product market in Wuhan, China. The animals were maintained in tanks with filtered and aerated freshwater at 28 °C. Lipopolysaccharide (LPS; from *Escherichia coli* serotype 055:B5), lipoteichoic acid (LTA; from *Staphylococcus aureus*), and peptidoglycan (PGN; from *Micrococcus luteus*) were purchased from Sigma (St. Louis, MO, USA). The bacteria *S. aureus*, *Streptococcus agalactiae*, *Bacillus subtilis*, *V. parahaemolyticus*, *Aeromonas hydrophila,* and *Edwardsiella ictaluri* were maintained in our laboratory. The primary polyclonal antibody *anti-Pc*proPO was raised in rabbit and stored in our laboratory. Secondary antibodies dye-linked goat *anti*-rabbit IgG antibody was purchased from Gene Tech (Shanghai), and FITC conjugated *anti*-rabbit IgG was purchased from Abbkine, USA.

2.2. Bacterial challenge and sample collection

The LB broth was used to culture *S. aureus* and *V. parahaemolyticus* at 37 °C overnight and washed two times with sterile PBS at 5000 *g* for 10 min. The crayfish were divided into three groups (24 crayfish in each group). Each crayfish was injected with 25 μl of 10^7 CFU/ml (CFU, Colony Forming Units) *S. aureus* and *V. parahaemolyticus*, respectively. Crayfish in the control group were injected with the same amount of saline water. For tissues distribution analysis, the hemocytes, heart, hepatopancreas, gill, stomach, and intestine were collected from at least five normal healthy shrimp. For expression pattern assay, the hepatopancreas was collected at 0, 3, 6, 12, and 24 h post injection (hpi) for RNA extraction.

2.3. Total RNA extraction and cDNA synthesis

The total RNA was isolated from various tissues using the Trizol reagent (Takara, Dalian, China). Approximately 1 μg of total RNA was used to synthesize the first-strand cDNA using PrimeScript™ RT reagent Kit with gDNA Eraser (Takara, Dalian, China) following the manufacturers' instruction and then stored at −20 °C.

2.4. Recombinant expression of PcproPO

The *Pc*proPO domain was analyzed using the *SMART* software (http://smart.Embl-heidelberg.de/), basing on the reference gene (accession no. EF595973.1). Specific primers were designed basing on the target sequences to clone into the prokaryotic expression vector pGEX-5X-1. All the primers used in this study were given in Table 1. The recombinant plasmid containing *Pc*proPO gene (r*Pc*proPO) was transformed into *E. coli* BL21 (DE3) competent cells (Dingguo, China) and then the recombinant protein was induced under the optimized conditions. The proteins were purified using a Micro Protein PAGE Recovery Kit (Sangon, Shanghai, China), quantified by Standard Bradford assay [20] and determined in 10% SDS-PAGE, then stored at −80 °C.

2.5. Expression analysis of PcproPO by quantitative real-time (qRT-PCR)

The expression pattern and the tissue distribution of *Pc*proPO gene in various tissues were studied using qRT-PCR in a real-time thermal cycler (Roche, Switzerland) following the protocol of the SYBR real-time PCR Premixture (Vazyme, China). 18s rRNA was stably expressed along with the sample throughout the experiments and was used as the internal control. Each sample was measured at least triplicates according to the procedure as follows: preincubation at 95 °C for 30 s followed by 40 cycles of 95 °C for 5 s, 55 °C for 20 s, and 72 °C for 20 s, finally at 4 °C for 5 min. The relative expression ratio of the target genes versus the 18s rRNA gene was calculated using the $2^{-\triangle\triangle CT}$ method. SPSS software was used to analyze the obtained data, and all data were given in terms of relative mRNA expression.

2.6. Analysis of PcproPO by cell staining and immunofluorescence assay

The hemocytes were isolated from the hemolymph of three healthy crayfish by centrifugation at 700*g* for 10 min, at 4 °C, and washed thrice

with PBS. For immunofluorescence assay (IFA), after blocking for 2 h with 5% non-fat milk at room temperature (RT), hemocytes were incubated with *anti-Pc*proPO (1:500 in 2% non-fat milk) for 2 h at RT and washed thrice using PBS. Later, the FITC conjugated *anti*-rabbit IgG antibody was added (1:10,000 in 2% non-fat milk) and incubated in the darkness for 1 h at 37 °C. After washing 3 times, hemocytes were stained with 4-6- diamidino-2-phenylindole dihydrochloride (DAPI, Guge Biology, Wuhan) for 10 min at RT in the darkness. Subsequently, the hemocytes were washed 3 times and observed under a fluorescence microscope (Olympus BX51, Japan). May-Giemsa stain assay was used to observe the morphology of hemocytes, following the methods of the manufacturer's. Briefly, the hemocytes were tiled onto the glass slide, dye A was used to stain cell for 1 min and continued with dye B for 6 min, water was used to wash the excess stains. The stained slides were observed for hemocytes morphological structures under light microscope.

2.7. Bacterial binding assay

The bacterial binding assay was performed according to the previously described method [21] with slight modification. Briefly, overnight cultured six different bacteria's viz., Gram-negative and Gram-positive-positive bacteria: *S. aureus*, *S. agalactiae* and *B. subtilis*; Gram-negative and Gram-positive-negative bacteria: *V. parahaemolyticus*, *A. hydrophila,* and *E. coli* were harvested and washed 3 times by centrifugation at 5000 *g* for 5 min and finally resuspended in PBS. The recombinant r*Pc*proPO protein at a final concentration of 50 μg/ml was incubated at 28 °C for 1 h in the tubes with the above mentioned six bacteria. The combinations were washed six times and resuspended in 50 μl of PBS then transferred onto a nitrocellulose membrane after separating by 10% SDS-PAGE. The membranes were blocked with 5% nonfat milk diluted in TBS (10 mM Tris-HCl, 150 mM NaCl, pH 8.0) for 1 h at RT with gentle shaking. Then the membranes were incubated with primary *anti-Pc*proPO rabbit antibody (1:1000) overnight at 4 °C. After washing with TBST (TBS + 0.02% Tween) for three times, the membranes were incubated with dye-linked goat *anti*-rabbit IgG antibody (1:10,000 dilution in blocking reagent) for 1 h at RT with gentle shaking. Finally, the membranes were thoroughly washed with TBST and band was visualized using Odyssey infrared imaging system (Li-Cor Biosciences, USA).

2.8. Polysaccharide-binding assay

Binding of *Pc*proPO to polysaccharides was analyzed by an enzyme-linked immunosorbent assay (ELISA) as previously described with minor modification [21]. Briefly, microtiter wells were coated with 5 μg of LPS, LTA, or PGN overnight at RT. After washing 3 times with TBS, the wells were incubated with 200 μl of negative goat serum (Boster Biological Technology, China) in TBS for 2 h at RT and continued washing for four times with TBST. The plates were incubated with twofold diluted recombinant *Pc*proPO protein in TBS containing negative goat serum (100 μl/well) for 3 h at RT and washed four times with TBST. 100 μl of the rabbit *anti-Pc*proPO antiserum diluted (1:500) with TBST containing negative goat serum was added to each well, and the wells were then incubated for 1 h at RT and followed by washing four times with TBST. The wells were rewashed after incubating with 100 μl of the HRP-conjugated goat *anti*-rabbit IgG (1000 × dilution with TBST containing negative goat serum) for 1 h at 37 °C. Subsequently, 200 μl of the color solution 3, 3′, 5, 5′ -tetramethylbenzidine (TMB, Beyotime Institute of Biotechnology, China) was added to each well for 10 min and then stopped by adding 50 μl of 2 M H_2SO_4. Control experiments were performed using GST-tag proteins instead of the recombinant proteins. The absorbance was measured at a wavelength of 450 nm using a microplate reader (Molecular Devices, USA). The average OD value of the wells containing only GST-tag was subtracted to obtain the specific activity from all OD values.

2.9. PO enzyme activity assay

The activity assay for the PO enzyme was established according to methods [17]. The whole hemolymph was collected and followed by the addition of 1 mM 4-methylcatechol in 0.1 M potassium phosphate (pH 6.0) as substrate. After 5 min, the PO enzyme activities were measured using microplate reader at 405 nm absorbance.

2.10. In vivo knockdown of PcproPO and bacterial clearance analysis in crayfish

To repress *Pc*proPO activity *in vivo*, the specific primer sequences (Table 1) were linked to the T7 promoter by using the commercial transcription T7 kit (Fermentas, USA) and followed the methods as previously described [22]. Simultaneously, dsRNA for the green fluorescent protein (GFP) gene was also prepared and used as negative control. The ability of the bacterial clearance in knockdown crayfish was analyzed as described previously [23]. In brief, after 24 h post-treatment of dsRNA, the crayfish was injected with *V. parahaemolyticus* and *S. aureus* (10^7 CFU/ml), respectively. At 60 min post-bacterial infection, the whole hemolymph was collected and immediately gradient diluted with PBS. Fifty microliters of each of the diluted hemolymph were plated onto LB agar plates overnight at 37 °C, and bacteria were quantified by CFU. For relative expression analysis, the total RNAs were extracted from the knockdown crayfish hemocytes for 24 h, and the knockdown shrimp challenged with *V. parahaemolyticus* and *S. aureus* (10^7 CFU/ml) for 12 h, so as to evaluate the effect of RNAi by qRT-PCR.

2.11. Survival assay

To test the importance of proPO in the immune defense, the *Pc*proPO or dsGFP knockdown and normal crayfish groups (n = 20) were injected with 50 μl of *V. parahaemolyticus* (1×10^7 CFU/ml) and monitored every 12 hpi for signs of disease and survival rate over the 84 hpi. Three independent trials were performed for all treatments.

2.12. In vitro knockdown analysis in crayfish hemocytes

In vitro gene silencing assay was performed as described previously [24]. Briefly, the freshly collected hemocyte cells were seeded into 12-well culture plates (5×10^5 per well) with L-15 medium supplemented with 5 μM 2-mercaptoethanol, 1 μM 2-phenylthiourea, 60 μg/ml penicillin, 50 μg/ml streptomycin, 50 μg/ml gentamycin and 2 mM L-glutamine [25]. After replacing fresh culture medium for 2 h, 5 μg of proPO or GFP dsRNA was added into each well and continued incubation for another 24 h at 25 °C. Later, the cells were separately incubated for 3 h with *V. parahaemolyticus* and *S. aureus* at a final concentration of 5×10^6 per well, and 50 μl suspensions were plated onto LB agar plates then quantified by CFU count. *In vitro* melanization responses of the knockdown hemocytes incubated for 3 h with *V. parahaemolyticus* and *S. aureus* at 1:10 ratio were observed under light microscope. *Pc*proPO protein expression in the crayfish hemocytes was analyzed by Western blot as described above.

3. Results

3.1. PcproPO involved in antibacterial immunity

The mRNA transcripts were measured by qRT-PCR to investigate the distribution of the *Pc*proPO expression in various tissues. The mRNA expression levels of *Pc*proPO was relatively higher in hemocytes than the other tested tissues (Fig. 1A). Furthermore, the challenge of *S. aureus* and *V. parahaemolyticus* could significantly activate the PO activity of crayfish compared with that of the control group (Fig. 1B). The time course expression profile of *Pc*proPO was analyzed in crayfish hemocytes challenged with *S. aureus* and *V. parahaemolyticus*. We

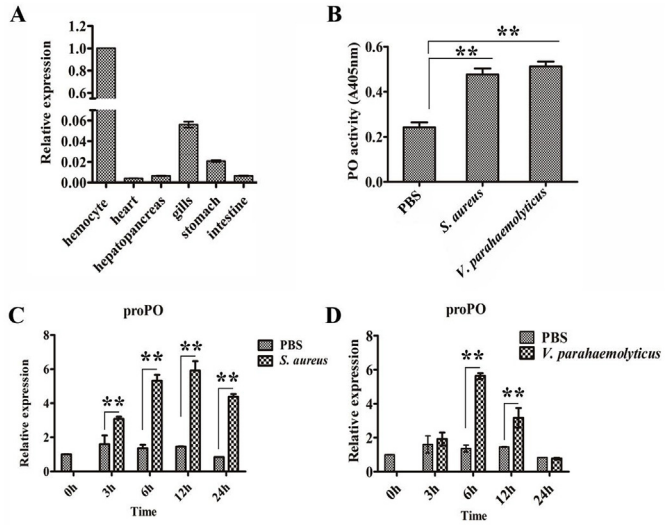

Fig. 1. Tissue distribution and expression model of *Pc*proPO. (A) qRT-PCR was used to detect tissue distribution of *Pc*proPO in *P. clarkii*. (B) The PO enzyme activity assay in the crayfish hemolymph stimulated by *S. aureus* and *V. parahaemolyticus*. (C) The hepatopancreas expression analysis of proPO at different time challenged with (C) *S. aureus* and (D) *V. parahaemolyticus*. The asterisks (**) indicate significant differences ($p < 0.01$) between the challenged and control crayfish. Error bars represent ± SD of the three independent PCR amplification.

observed that the *Pc*proPO expression was gradually increased and significantly up-regulated at 6 hpi in both *S. aureus* and *V. parahaemolyticus* infected crayfish hemocytes (Fig. 1C and D). The presented data indicated that the *Pc*proPO of crayfish was involved in immune response during bacterial challenge.

3.2. Purification and subcellular localization of *Pc*proPO

Recombinant expression of *Pc*proPO with high yield was obtained in *E. coli* BL21 (DE3) harboring the prokaryotic expression plasmid r*Pc*proPO, and a strong band for purified protein about 72 kDa with GST-tag could be seen (Fig. 2A). The polyclonal antisera raised in rabbit (*anti-Pc*proPO) showed a specific band at the correct position, which further clarified the expression of specific recombinant *Pc*proPO protein (Fig. 2A and B). Subcellular localization of the *Pc*proPO proteins in the crayfish hemocytes by indirect immunofluorescence assay and May–-Giemsa staining revealed that a large portion of proteins could be found in the granular hemocytes (GHc) (Fig. 2C and D).

3.3. *Pc*proPO binding assay

To study the bacterial binding effect of *Pc*proPO, six kinds of bacteria that include three Gram-negative and Gram-positive-positive and three Gram-negative and Gram-positive-negative bacteria were used. The Western blot results showed that r*Pc*proPO protein had a strong affinity and bound to both Gram-negative and Gram-positive bacteria, which was not observed in the GST-tagged proteins (Fig. 3A). ELISA analysis with polysaccharides (LPS, LTA, and PGN) obtained from both the Gram-negative and Gram-positive bacteria, confirmed further that the binding effect was due to the polysaccharides present on the bacterial surfaces (Fig. 3B, C, and D). Taken together these data claims that the r*Pc*proPO protein of crayfish has the ability to interact directly to the bacteria.

3.4. *proPO involved in bacterial clearance in vivo*

To test whether *Pc*proPO is involved in crayfish against bacterial invasion directly, the juvenile *P. clarkia* were treated for dsRNA-

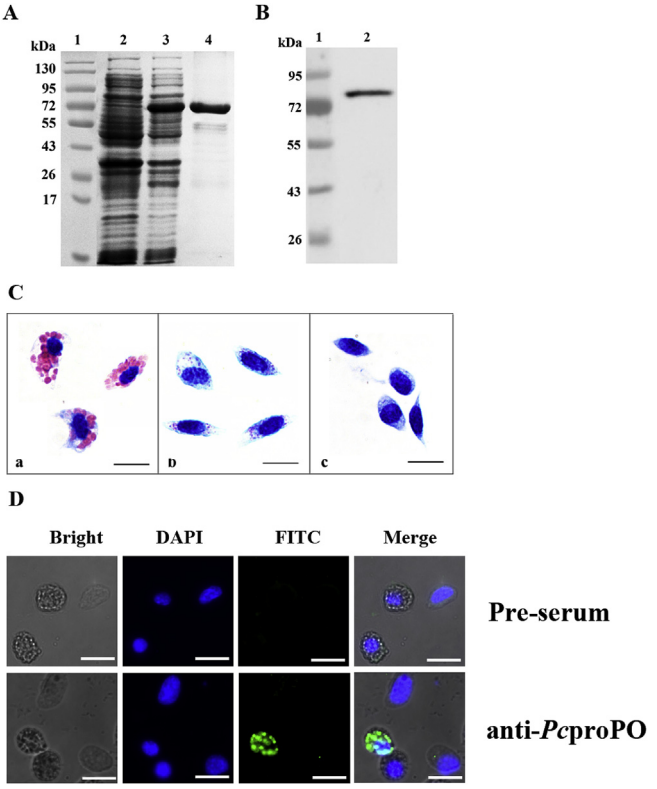

Fig. 2. Purification and subcellular localization of *Pc*proPO. (A) Prokaryotic recombinant expression and purification of *Pc*proPO. Lane 1: protein marker; lane 2: *E. coli* containing a recombinant vector; lane 3: IPTG induced *E. coli* containing a recombinant vector; lane 4: purified *Pc*proPO protein. (B) Specificity analysis of *Pc*proPO rabbit antiserum by Western blot. Lane 1: protein marker; lane 2: hemocyte *Pc*proPO protein detected with rabbit polyclonal antisera. (C) May–Giemsa staining of *P. clarkii* hemocyte sub-population. Ca: the granular hemocytes (GHc); Cb: the semi-granular hemocytes (SGHc); Cc: hyaline cells. (D) Subcellular localization of *Pc*proPO by indirect immunofluorescence, *anti-Pc*proPO rabbit serum or pre-serum (serum from normal rabbit) to locate *Pc*proPO and FITC for detection, the nucleus was stained with DAPI.

mediated gene silencing, and the mRNA expression levels of *Pc*proPO was analyzed. As shown in Fig. 4A, the expression level of *Pc*proPO in crayfish treated with GFP-dsRNA was high, whereas, *Pc*proPO-dsRNA treatment has decreased the *Pc*proPO protein expression level. Subsequently, analysis of the PO enzyme activity proved that the *Pc*proPO-dsRNA knockdown group was significantly depreciated in enzyme production, compared to the control group (Fig. 4B). To further elucidate the function of proPO in *P. clarkii* against bacteria invasion, differently post-treated crayfishes were injected with *S. aureus* and *V. parahaemolyticus*. The mRNA transcript levels of *Pc*proPO in *S. aureus* treated groups were significantly higher than the normal (PBS) group. However, in the *Pc*proPO-knockdown group, the *Pc*proPO expression level was much lower than that of dsGFP group (Fig. 4C). Additionally, knockdown crayfishes were subjected to bacterial clearance analysis, and the result showed that the number of *S. aureus* in the ds*Pc*proPO group was significantly higher than that of the dsGFP treated group (Fig. 4D). Similar effects were observed in the *Pc*proPO expression level and bacterial clearance experiments when the knockdown crayfishes were infected in the parallel combination with *V. parahaemolyticus* (Fig. 4E and F). The above results indicated that *Pc*proPO could indirectly involve in the antibacterial immunity.

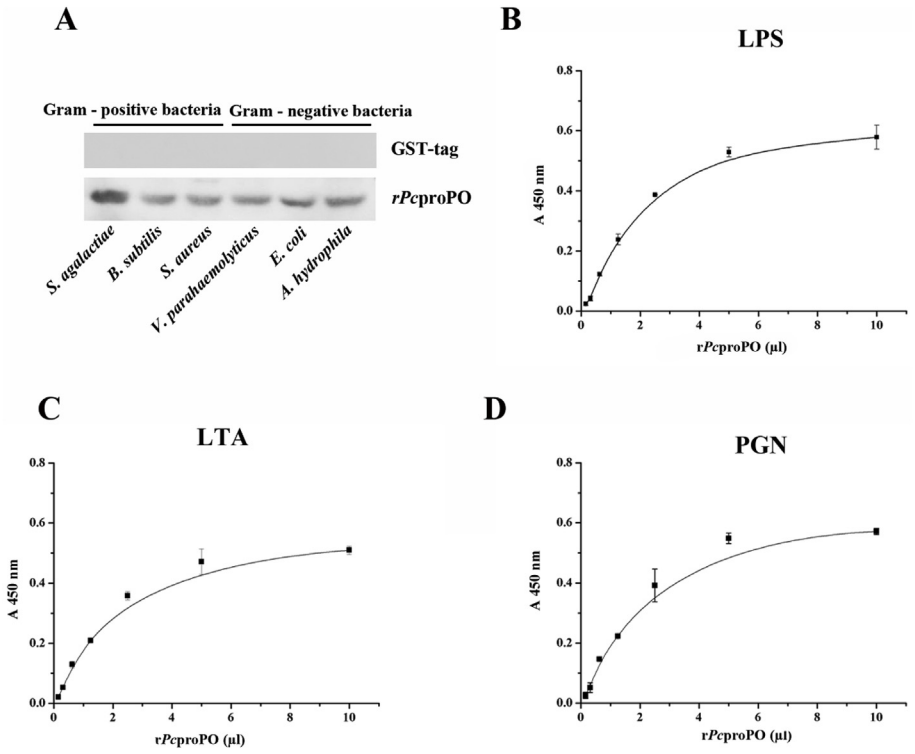

Fig. 3. *In vitro* Bacterial and Polysaccharides-binding assay. (A) Purified r*Pc*proPO binding to both Gram-positive and Gram-negative bacteria. Purified r*Pc*proPO binding to Polysaccharides (B) lipopolysaccharide [LPS] (C) lipoteichoic acid [LTA] and (D) peptidoglycan [PGN]. Data shown are the mean ± SEM derived from the three repeats.

3.5. The effect of PcproPO on the survival rate of crayfish infected with V. parahaemolyticus

The potential role of *Pc*proPO gene in the survival of crayfish was evaluated in the normal and knockdown crayfish by challenging with *V. parahaemolyticus*. In the *Pc*proPO knockdown group, the infection with *V. parahaemolyticus* led to rapid mortality (less than 15% survival within 60 h), which was significantly higher than that of the control groups, either treated with either GFP dsRNA or the normal crayfish (Fig. 5).

3.6. The immune function of PcproPO in the hemocyte

To elucidate the role of phenoloxidase in the host defense against bacterial pathogens, dsRNA was employed to target the proPO gene in the crayfish hemocytes. Quantitative RT-PCR and Western blot assay determined the efficiency of RNAi-mediated transcript depletion. Experiment results showed that the primary hemocytes cultures treated with proPO-dsRNA for 24 h, significantly decreased ($P < 0.01$) the proPO mRNA transcript and protein expression levels, rather than the expression levels of GFP-dsRNA treated hemocytes (Fig. 6A and B). PO enzyme activity in the dsRNA treated hemocytes showed the PO activity was significantly decreased in the ds*Pc*proPO group than that of the dsGFP group (Fig. 6C). We also determined whether *in vitro* silencing of the proPO gene could influence the proliferation of *V. parahaemolyticus* and *S. aureus*. The CFU counts of both pathogens were significantly ($P < 0.01$) abundant in the hemocytes pretreated with ds*Pc*proPO than the control (dsGFP) at 3 h (Fig. 6D and E). Melanization is an essential immune mechanism in invertebrates. As shown in Fig. 6F, the dsGFP treated wells showed more black dots distributed in the medium compared to that of ds*Pc*proPO wells. When the bacteria *S. aureus* and *V. parahaemolyticus* were used to stimulate the knockdown hemocytes, the black dots were increased in dsGFP wells. However, the number of black dots in ds*Pc*proPO wells was not significant change.

4. Discussion

Prophenoloxidase is a primary immune response in many invertebrates, which is one of the more potent humoral constituents that requires several associated proteins for activation. It plays an essential role in the innate immune responses as a non-self-recognition system through association with various cellular responses of hemocytes such as phagocytosis, melanization, cytotoxic reactant production, particle encapsulation, and nodules and capsules formation [11,12,26]. Presently known shrimp proPOs exhibit the typical characteristic of arthropod proPOs, including two functional copper-binding sites (similar to hemocyanins), a proteolytic activation site, and no hydrophobic signal peptide [27,28]. Over the past decade, several *in vivo* or *in vitro* gene silencing assays of proPO had been performed on *Pacifastacus leniusculus*, *Penaeus monodon*, *Marsupenaeus japonicus, Scylla Paramamosain,* and *Litopenaeus vannamei* and fairly well studied due to its importance in the invertebrate immune system. On the contrary, other findings demonstrate that in some insects the proPO system is not essential for defense against microbial infections [24,29–36]. However, the proPO gene expression from the hemocyte during pathogen invasion still remains controversial. In the present study, we found that the *Pc*proPO gene was mainly expressed in hemocytes of crayfish by RT-PCR, as previously reported in *L. vannamei*, *P. monodon*, *P. leniusculus*, and *Scylla serrata* [32,37–42]. Interestingly, the transcription of *Pc*proPO was also detected in gill, heart and hepatopancreas, as reported in *Cherax quadricarinatus*, *P. clarkii*, *S. paramamosain*, *Portunus trituberculatus,* and *L. vannamei* [24,43–45]. It may be due to infiltration of hemocytes in various tissues in decapods which possess an open circulation system. In order to determine if induction of immune system correlated with PO system, we stimulated the shrimp with *S. aureus* and *V. parahaemolyticus* and analyzed for the enzyme levels, the results showed that the PO activity level in hemolymph was significantly increased, corroborating the results of *C. quadricarinatus*, *Marsupenaeus japonicus* and *Carcinoscorpius rotundicauda* [17,33,46]. Expression pattern analysis of shrimp infected with *S. aureus* and *V. parahaemolyticus* revealed both the two kinds of bacteria could activate the *Pc*proPO transcription, some previous study also stated that during bacterial

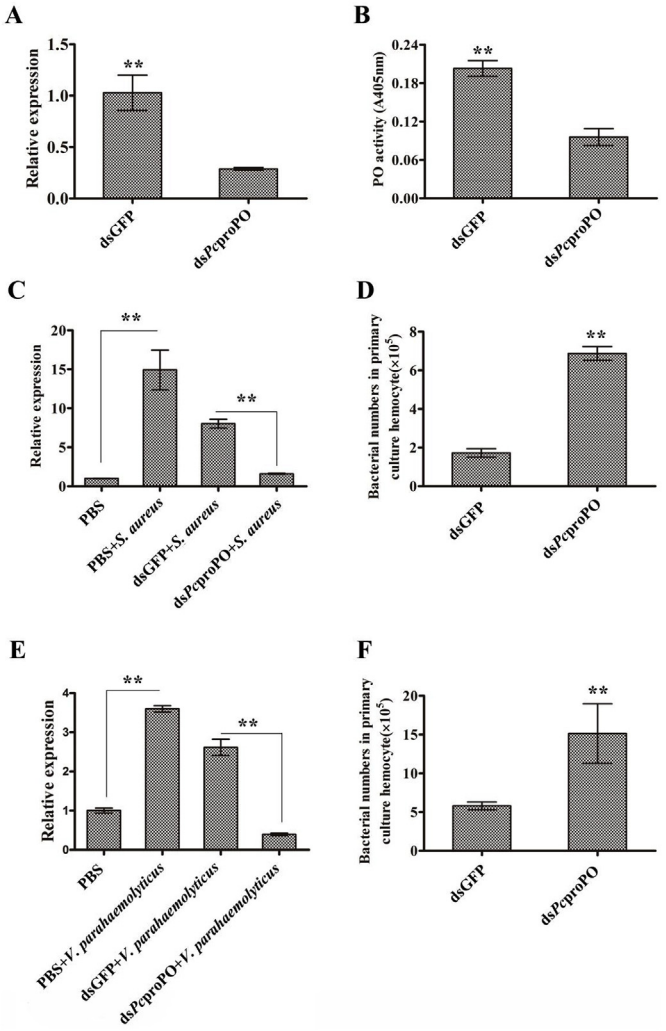

Fig. 4. *In vivo* bacterial clearance of knockdown crayfish. Knockdown of proPO specific gene by RNAi using proPO-dsRNA, GFP-dsRNA was used as the control. (A) mRNA expression analysis from the hemocytes of shrimp treated with dsRNA for 24 h by qRT-PCR. (B) Total hemolymph phenoloxidase (PO) enzyme activity was assayed using 4-methylcatechol and detected with a microplate reader at A405 nm. (C) The transcript level of *Pc*proPO from the hemocytes of knockdown shrimp 12 hpi with *S. aureus* (D) The number of *S. aureus* in hemolymph by CFU method. (E) The transcript level of *Pc*proPO from the hemocytes of knockdown shrimp 12 hpi with *V. parahaemolyticus*. (F) The number of *V. parahaemolyticus* in hemolymph by CFU method. The significant difference across dsRNAs group is indicated with two asterisks at $P < 0.01$.

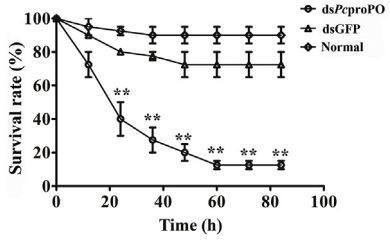

Fig. 5. *In vivo Pc*proPO knockdown in crayfish susceptibility test to *V. parahaemolyticus*. Survival analysis of dsGFP and *Pc*proPO knockdown shrimp groups challenged with *V. parahaemolyticus* (5×10^5 CFUs) infection. The shrimp mortality was recorded for every 12 hpi, and the data are shown as the mean ± 1 SEM survival rate (%), derived from three independent experiments.

infection the mRNA expression of proPO was up-regulated, suggesting that proPO might be involved in bacterial clearance [24,45].

The first Penaeid proPO was successfully purified from the hemocytes of *P. californiensis* in 1999, then a number of proPO genes have been identified and cloned from various shrimp species, including *P. semisulcatus, P. monodon, M. japonicas, L. vannamei* and *F. chinensis* [32,47,48]. The relative molecular mass of proPO is about 70–80 kDa that integrated with two copper atoms [49]. In the present study, we cloned one proPO gene from *P. clarkii,* and the Western blot result showed the molecular mass is around 72 kDa. The indirect immunofluorescence result displayed that the *Pc*proPO was localized in the granular hemocytes, which is consistent with the previous study of *S. paramamosain* [24]. Although the proPO system has been extensively studied in the invertebrates, very little focus was given on direct interaction of pathogens with proPO. Here, bacterial and polysaccharide binding assay was employed with *rPc*proPO protein, the results showed that the recombinant protein could strongly bound to different bacteria and the polysaccharides from the bacterial origin. Similarly, the proPO of *Portunus pelagicus* was able to agglutinate yeast, which implied the proPO could interact with various microbes [50]. It is well known that proPO plays an essential role in melanization, which could help to kill the invading pathogens, revealing that the proPO might interact with pathogens. Furthermore, the homology of arthropod hemocyanins share the similar structures (tryosinase domain and C-terminal domain of hemocyanin) and functions such as PO activity [51]. In previous studies, it was already established that the tryosinase domain and C-terminal domain of hemocyanin both contained strong affinity abilities to bacterial or polysaccharide [52,53]. Our results also revealed similar functions for proPO when treated with either polysaccharides or bacteria. However, the affinity ability of proPO still needs to be further explored.

The primary culture of hemocytes has been a powerful tool for gene functional studies in crustaceans [54], especially for the proPO [55]. In recent years, proPO had been investigated using gene silencing in crustaceans. In this work, we used a dsRNA-based approach to obtain an efficient knockdown of *P. clarkii* proPO gene both *in vivo* and *in vitro*. The data from Western blot, qRT-PCR analysis, PO enzyme activity, and bacterial clearance assay clearly showed a strong and selective reduction of proPO protein and mRNA levels. Moreover, knockdown of the proPO had lost the ability to control bacterial growth compared to that of dsGFP. These results are in line with other data recently collected in a model of *S. paramamosain*, *M. japonicus* and *P. monodon* [24,32,48]. In order to further study the role of proPO in the immune system, we investigated survival rate by challenging with *V. parahaemolyticus,* the survival of the proPO knockdown groups was greatly reduced than that of the other groups. Jang et al. also found that knockdown of the proPO in *L. vannamei* decreased the probability of survival during *V. harveyi* infection [34]. An immediate response in the cellular defense during pathogen invasion is probably initiated by melanization resulting in the stimulation of the proPO system. In our data, we found that knockdown of the proPO also reduced the melanization in hemocytes compared with the dsGFP group. These data suggested that the proPO gene might play an indispensable role in the control of systemic bacterial infections, and could help us to elucidate the defense role of the proPO-activating system in *P. clarkii.*

In conclusion, the proPO from *P. clarkii* is also localized in hemocytes and the proPO mRNA transcription was detected to be up-regulated after *S. aureus* and *V. parahaemolyticus* challenge. Bacterial and polysaccharide assay suggested that proPO could interact with bacteria directly. *In vivo* and *in vitro* experiments indicated that proPO participated in response to bacterial stress. Altogether the presented data support the evidence that the proPO system in *P. clarkii* is critical for a strong defense mechanism against bacterial pathogens. Based on the previous studies and our obtained results, it tempts us to speculate the role of shrimp proPO as follows. The shrimp proPO belongs to the hemocyanin superfamily, sharing a similar domain structure and

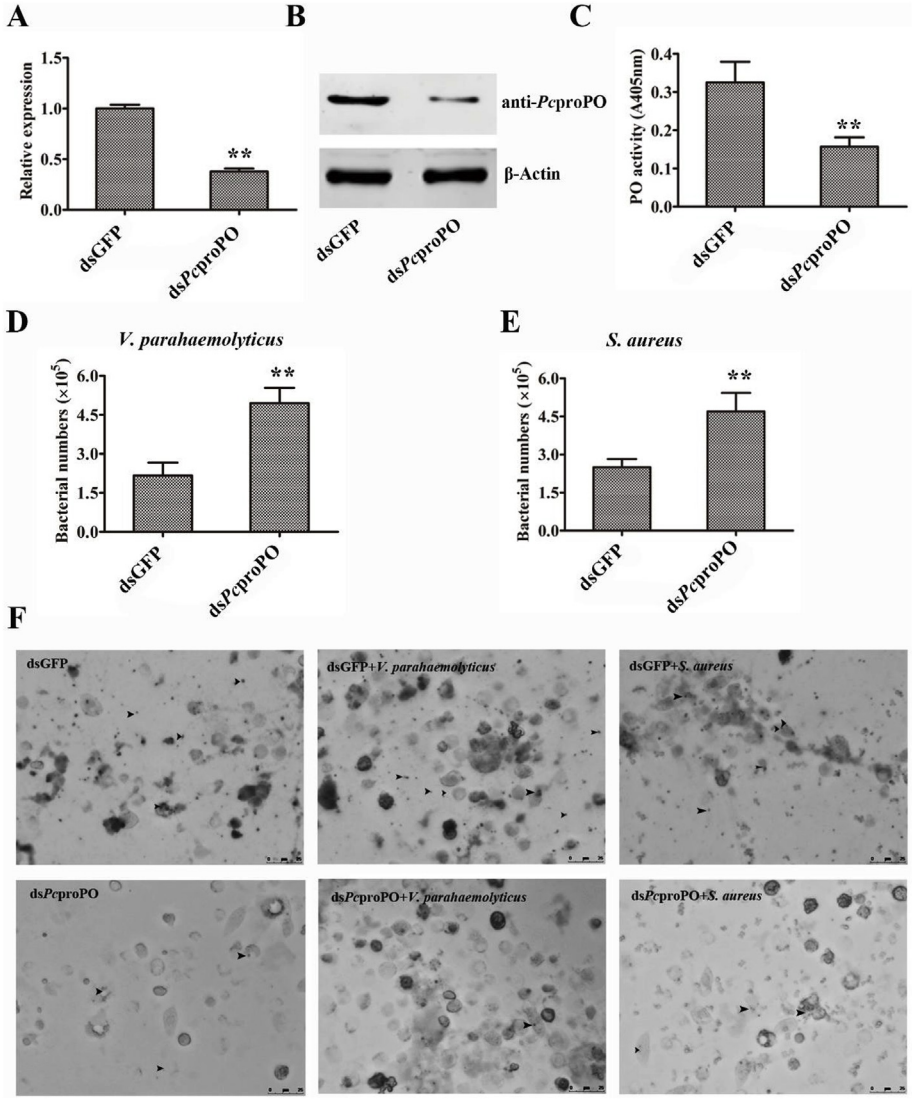

Fig. 6. *In vitro* bacterial clearance of *Pc*proPO knockdown crayfish hemocytes. (A) The mRNA expression level of *Pc*proPO in crayfish hemocytes treated with proPO or GFP dsRNA for 24 h. (B) The protein expression level of dsRNA knockdown hemocytes for 24 h by western blot. (C) The phenoloxidase (PO) enzyme activity of hemocyte treated with proPO or GFP dsRNA for 24 h. The hemocyte pretreated with dsRNA for 24 h were incubated with *V. parahaemolyticus* (D) and *S. aureus* (E) for 3 h, and calculated by CFU. (F) The photomicrographs of dsRNA pretreated hemocytes incubated with or without *V. parahaemolyticus* or *S. aureus* observed under the light microscope for melanization, several black dots could be seen around the cells and in the medium (arrowheads). Scale bar 25 μm.

immunological functions with other hemocyanins, they might evolve from a common ancestor. However, shrimp proPO has been shown to be localized only in the cytoplasm of hemocytes, while hemocyanins are confined in serum of hemolymph. It is reasonable to believe that shrimp proPO plays immunological functions within the hemocytes. By contrast, other hemocyanins carry out pathogen clearance in the serum. The underlying mechanism of their evolution remain enigmatic and needs to be elucidated in the future.

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