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THE CHARACTERIZATION OF PROPHENOLOXIDASE AND PHENOLOXIDASE FROM RED CLAW CRAYFISH, *CHERAX QUADRICARINATUS*

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Abstract: In this study, a prophenoloxidase (*proPO*) gene was cloned from hemocytes of red claw crayfish, *Cherax quadricarinatus*. The open reading frame (ORF) of *proPO* gene is 1995 bp and encodes a 665 amino acids (aa) protein. The predicted molecular mass of the protein was 75.7 kD with an estimated pI of 6.23. It contained two putative tyrosinase copper-binding motifs with six histidine residues and one thiol-ester-like motif, which showed similar structural features of proPOs from other crustaceans. The amino acid sequence of *C. quadricarinatus* proPO showed similarity of 68%, 63%, 63% and 59% with that of the proPO of *Pacifastacus leniusculus*, *Homarus gammarus*, *Homarus americanus* and *Procambarus clarkii*, respectively. The recombinant proPO were expressed in *Escherichia coli* BL21 with pET-28a expression vector. After protein purification and antibody prepared, the titer of rabbit anti-proPO serum was above 1∶12800. The mRNA transcription of *proPO* and PO activity in *C. quadricarinatus* were high in hemocytes, hepatopancreas and gills, lower values were seen in other tissues (nerve, heart, intestine and muscle). The immune characterization of *proPO* gene was studied after *Spiroplasma eriocheiris* and *Aeromonas hydrophila* stimulation. After *S. eriocheiris* or *A. hydrophila* challenging, the mRNA transcription of *proPO* gene and PO activity in hemocytes, hepatopancreas and gill of *C. quadricarinatus* were up-regulated at different times to resist different stimulation. These results indicated that it was potentially involved in the acute response against invading bacteria in *C. quadricarinatus*.

Key words: prophenoloxidase; *Spiroplasma eriocheiris*; *Aeromonas hydrophila*; *Cherax quadricarinatus*
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The red claw crayfish, *Cherax quadricarinatus* belongs to the phylum Arthropoda, class Crustacea, order Decapoda, family Parastacidae and genus *Cherax*. It is native to Australia and Papua New Guinea, and is a tropical species [1]. It was introduced to China and cultured intensively and semi-intensively as an important species in aquaculture in Jiangsu, Zhejiang, Fujian and Guangdong Provinces [2, 3]. Recently, the frequent outbreak of diseases has caused drastic decrease in its production and catastrophic economic losses in the industry. Understanding the immune defense mechanisms of crayfish may be conducive to the development of better disease control strategies in the crustacean farming.

It is known that invertebrates lack adaptive immune system, and rely instead on innate immune system against invading pathogens. Once pathogens like bacteria or viruses enter the host, they activate a complex system of innate defense mechanisms. First, it initiates the prophenoloxidase (proPO)-activating system is initiated in the presence of several microbial wall components like β-1,3-glucan, lipopolysaccharide (LPS) and peptidoglycan, which leads to the production of phenoloxidase (PO) by an prophenoloxidase-activating enzyme (ppA), resulting in melanisation through a complex enzymatic cascade [4]. PO catalyses to formate the quinone by phenolic compounds. And quinine spontaneously becomes melanin. Quinone has antifungal functions and melanin is involved in wound healing and in encapsulation reactions [5].

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The molecular biology study of *C. quadricarinatus* is little, there are few genes were reported including Heat shock protein 70, *DDX*5 gene and so on [2, 3, 6]. The *proPO* gene has not reported and studied in *C. quadricarinatus*. The *proPO* gene was cloned and its characterizations were studied in this paper. The main objectives of the present study were (1) to clone and identify the full-length cDNA of *proPO* from *C. quadricarinatus*, (2) to investigate the *proPO* mRNA transcription and specific enzyme activity of PO in different tissues, (3) to examine the temporal transcription of proPO mRNA and the variation of specific PO activity in *C. quadricarinatus* post *Spiroplasma eriocheiris* and *Aeromonas hydrophila* challenge.

1 Materials and methods

1.1 Animal and RNA isolation

C. quadricarinatus [(100±5) g] were purchased from a market in Nanjing, China, and cultivated in 100 L tanks. The hemolymph from the base of crayfish last leg was obtained using a 2 mL syringe, quickly added into anticoagulant solution (glucose, 2.05 g; citrate, 0.8 g; NaCl, 0.42 g; double distilled water was added to 100 mL). Samples were immediately centrifuged at 2000 g, 4℃ for 10min to collect the hemocytes. The extraction of total RNAs from hemocytes was conducted by the Trizol, following the manufacturer's instructions. The total RNA concentration was determined by measuring the absorbance at A_{260}. Electrophoresis was used to check the RNA integrity.

1.2 Reverse transcription and gene cloning

Five μg of total RNA was reverse-transcribed into cDNA with an M-MLV RTase cDNA Synthesis Kit (Takara, Japan). The sequences of degenerate primer pairs, proPO-F and proPO-R (Tab. 1) were designed based on the highly conserved nucleotide of *proPO* gene by using the CLUSTAL program [7] to clone the sequence of *proPO* gene of *C. quadricarinatus*. PCR was performed using 1.5 mmol/L $MgCl_2$, 0.2 mmol/L dNTP, 0.2 mmol/L of each of primers, 1 U *Taq* DNA polymerase (Takara, Japan) and 5 ng of cDNA. The amplification program consisted of 5min at 94℃ followed by 35 cycles of 94℃ for 30s, 52℃ for 40s, 72℃ for 40s and a final elongation step of 72℃ for 5min. PCR amplicons were size separated and visualized on an ethidium bromide stained 1% agarose gel. Amplicons of expected sizes were purified with an Agarose Gel DNA Purification Kit (Takara, Japan), and then subcloned into the pMD-19T cloning vector (Takara, Japan). Positive clones containing inserts of an expected size were sequenced using M13 primers, and sequenced at Invitrogen, Shanghai.

1.3 Rapid amplification of cDNA ends (RACE)

The *proPO* gene partial cDNA sequence from *C. quadricarinatus* was extended by using 5′ and 3′ RACE (SMART™ cDNA kit). A total of three gene-specific primers (Tab. 1) were designed based on the genes partial cDNA sequences. The 3′ RACE PCR reaction was carried out in a total volume of 50 μL containing 2.5 μL (800 ng/μL) of the first-strand cDNA reaction as a template, 5 μL of 10 × Advantage 2 PCR buffer, 1 μL of 10 mmol/L dNTPs, 5 μL (10 mmol/L) gene-specific primer, 1 mL of Universal Primer A Mix (UPM; Clonetech, USA), 34.5 μL of sterile deionized water, and 1 U 50 × Advantage 2 polymerase mix (Clonetech, USA). For the 5′ RACE, UPM was used as forward primers in PCR reactions in conjunction with the reverse gene-specific primers. PCR amplification conditions for both the 3′ and 5′ RACE were as follows: 5 cycles at 94℃ for 30s, 72℃ for 3min; 5 cycles at 94℃ for 30s, 70℃ for 30s, and 72℃ for 3min; 20 cycles at 94℃ for 30s, 68℃ for 30s, and 72℃ for 3min. After linking into the vector, the samples were sequenced at Invitrogen.

1.4 Sequence analysis of *proPO*

The cDNA sequence and deduced amino acid sequence of *proPO* gene were analyzed using the BLAST algorithm (http://www.ncbi.nlm.nih.gov/blast). Translation and protein analyses were performed using ExPASy tools (http://us.expasy.org/tools/). The ClustalW Multiple Alignment program (http://www.ebi.ac.uk/clustalw/) was used to create the multiple sequence alignment. An unrooted phylogenetic tree was constructed based on the amino sequences alignment by the neighbor-joining (NJ) algorithm embedded in MEGA 4 program. The reliability of the branching was tested by bootstrap resampling (1000 pseudo-replicates).

1.5 Protein expression, purification and antibody preparation

ProPO-ORF-F and proPO-ORF-R were designed to amplify the ORF of *C. quadricarinatus* proPO gene. After digested with restriction enzymes (*Eco*R Ⅰ and *Xho* Ⅰ), the production was ligated into the pET-28a expression vector (Novagen, USA). The resulting recombinant plasmids pET-proPO transformed to *E. coli* BL21 (DE3) (Trans, China) for protein expression. The recombinant proteins were analyzed by SDS-PAGE. High-Affinity Ni-NTA (nickel-nitrilotriacetic acid) Resin (Jinsite, China) was used to purify the recombinant proteins according to the instructions. The New Zealand White rabbits were immunized with 100 μg of purified protein that was homogenized in complete Freund's adjuvant for three times at 2-week intervals. A boost injection in incomplete Freund's adjuvant was given for another week. Rabbit serum was collected seven days after the last immunization. Antibody titer was determined from the immunized rabbits by ELISA. Briefly, the optimum concentration

(0.5 μg per well) of recombinant protein diluted in 0.05 mol/L carbonate buffer, was coated onto a 96-well plate (Corning, USA) and then incubated 12h at 4℃ with blocking solution (5% skimmed milk powder in TBS). The plate was washed three times with TBST. Next, rabbit antiserum (primary antibody) of different dilutions was added to each well and incubated for 1h. After washing with TBST, the plates were incubated with 100 μL of a 1∶5000 dilution of HRP-conjugated goat anti-rabbit IgG for 30min. The plates were washed again and 100 μL of TMB/H_2O_2 substrate was added to each well. Optical density was measured at 450 nm with a Model 680 micro-plate reader (Bio-Rad, USA).

1.6 Tissue distribution of *proPO* mRNA transcription and PO activity

A sample of total RNA at 5 μg from *C. quadricarinatus* tissues including nerve, heart, intestine, hemocytes, gill, and hepatopancreas were reverse-transcribed respectively into cDNA with a PrimeScript RT reagent Kit (Takara, Japan), respectively. Real-time PCR was carried out with a Mastercycler ep realplex (Eppendorf, Germany) to study the transcription of *proPO* mRNA in tissues, respectively. The PCR reaction was performed in a 25 μL volume with a SYBR Premix Ex Taq^{TM} Kit (Takara, Japan), 2 μmol/L of each specific primer and 1 μL of cDNA in Mastercycler ep realplex (Eppendorf, Germany). The primers pair using in real-time PCR was list in Tab. 1. The primers *β-actin*-F and *β-actin*-R were used to amplify β-actin fragment that was used as a housekeeping gene. The relative transcription level of *proPO* was calculated according to the $2^{-\Delta\Delta CT}$ method [8]. Statistical analysis was performed using SPSS software (Ver11.0). Data represent the mean ± standard error (S.E.). Statistical significance was determined by one-way ANOVA and post-hoc Duncan multiple range tests. The significance level was set at $P<0.05$.

Tab. 1　Degenerate and specific primers used for proPO cloning in the experiment

Name	Sequence (5′-3′)
Primers designed to clone *proPO* gene	
proPO-F	CACCACTGGCACTGGCAYYT
proPO-R	CGGTCGAASGGRAAGCCCAT
proPO-3F1	GGAGGAGCCGAAAGGACCCAGGAAATG
proPO-3F2	CCAATGCCGTGTCCTTCTGTGGGATCCT
proPO-5R1	TTTCCACACGGTTGAG
proPO-5R2	TCGTACCTGGCGACCATTTGTT
proPO-5R3	AGAGTTCACCTTTACGGTCCCC
proPO-ORF-F	AGG*GAATTC*ATGGCTGCCACAGACCAGAC
proPO-ORF-R	GAC*CTCGAG*CTACTTCTTGAGCTGGTTGTTG
Real-time quantitative primers	
proPO-qPCR-F	CCTCGTCCACCACCTGCTG
proPO-qPCR-R	GGCTCCTCCACCTTGTCCTTC
β-actin-F	ATCACTGCTCTGGCTCCTGCTACC
β-actin-R	CGGACTCGTCGTACTCCTCCTTGG

Three unchallenged *C. quadricarinatus* were dissected in cold PBS to isolate tissues, including hepatopancreas, gill, muscle, heart, nerve, intestine and hemocytes. The dissected tissues were added to nine volume of cold lysis buffer (415 mmol/L NaCl, 100 mmol/L glucose, 10 mmol/L cacodylic acid, 5 mmol/L $CaCl_2$, pH 7.0) and homogenized. Cell debris was removed by centrifuging at 3000 g for 10min to prepare crude PO samples after sonication at 40 W for 2min. PO activity was quantified by monitoring the rate of dopachrome formation by using a colorimetric assay [9]. 100 μL of HLS or crude PO sample was mixed with 250 μL 100 mmol/L PBS (pH 6.8) in a 1.5 mL Eppendorf tube. Then, 350 μL of the pre-cold 15 mmol/L DOPA dissolved in PBS was added to the Eppendorf tube. The tube was immediately capped, inverted, warmed at 30℃, blanked on its own absorbance and monitored for changes in optical density at 490 nm for over 10min. PO activity was recorded as the maximum change in absorbance over any one minute interval (ΔOD_{490} nm/min) during the first 10min of the assay. The total protein of different samples was determined by the Bradford method using BSA as standards.

1.7 *proPO* mRNA transcription and PO activity under pathogen stimulation

The *C. quadricarinatus* were cultivated for 1 week before treatment. *S. eriocheiris* was maintained in pure culture in R2 liquid medium at 30℃. The *A. hydrophila* was grown at 28℃ in TSB medium. The *C. quadricarinatus* in the experimental group I (30 individuals) received an injection of 100 μL *S. eriocheiris* (10^7

spiroplasmas/mL) individually. The *C. quadricarinatus* in the experimental group II (30 individuals) received an injection of 100 μL *A. hydrophila* (10^5 cells/mL) individually. The *C. quadricarinatus* (30 individuals) receiving an injection of 100 μL PBS (pH=7.0) individually, were used as control groups. Every 3 individuals for one group were randomly collected at 0h, 1h, 3h, 6h, 12h, 24h, 48h and 72h post-injection.

The total RNA from hemolymph, gill and hepatopancreas was extracted from different groups by the Trizol technique. After reverse-transcribed into cDNA, real-time RT-PCR was carried out to measure the transcription levels of *proPO* gene. The total crude PO from different tissues (hemolymph, gill and hepatopancreas) was prepared. The PO activity at different time after pathogen challenge was measured. The methods of experiment and analysis were list in 1.6.

2 Results

2.1 Sequence of *proPO* cDNA

The full-length *proPO* gene cDNA of *C. quadricarinatus* consisted of 2951 bp, including 75 bp in the 5′-untranslated region, an open reading frame (ORF) of 1995, and 881 bp in the 3′-untranslated region including a stop codon (TAG), putative polyadenylation consensus signal (AATAAA) and a poly (A) tail. The ORF of *proPO* cDNA was found to be composed of 665 amino acids (aa). The calculated molecular mass of the *proPO* was 75.7 kD with an estimated pI of 6.23. The *C. quadricarinatus proPO* cDNA sequence and their deduced amino acid sequence were submitted to the NCBI GenBank under accession number JQ954858.

A thiol ester motif (GCGWPEHL) from 558 to 565 that is present in the complement components, C3, C4 and a2-macroglobulin, was also observed in *C. quadricarinatus proPO*. No hydrophobic signal was found in the *proPO* sequence. Three N-linked glycosylation sites and two putative tyrosinase copper binding motifs with six histidine residues (177, 181, 203 in copper A, and 337, 341, and 377 in copper B) were conserved in what.

2.2 Phylogenetic analysis of proPO

The amino acid sequence of *C. quadricarinatus* proPO showed similarity of 68%, 63%, 63% and 59% with that of the proPO of *Pacifastacus leniusculus*, *Homarus gammarus*, *Homarus americanus* and *Procambarus clarkii*, respectively. The phylogenetic analysis indicated that the proPO of *C. quadricarinatus* is homologous to that of *P. leniusculus* and *P. clarkii*. The proPO of *C. quadricarinatus*is is close to lobsters proPO and far away from crabs proPO (Fig. 1).

2.3 Protein expression, purification and antibody preparation

Express this as recombinant protein, proPO, with an apparent molecular weight of around 66 kD, was detected by SDS-PAGE (Fig. 2, lane 2), which could be purified by High-Affinity Ni-NTA Resin (Fig. 2,

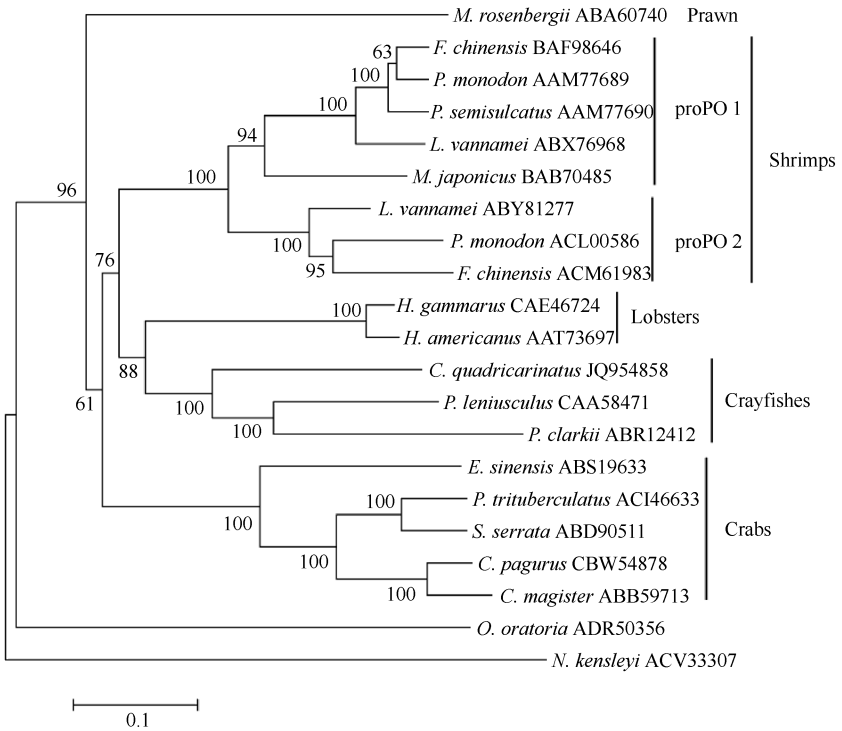

Fig. 1 Neighbor-joining phylogentic tree of proPO amino acid sequences from different species of animals

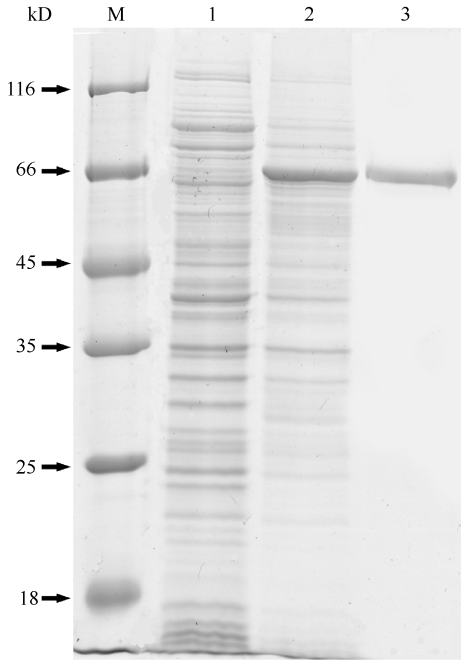

Fig. 2　Analysis of recombinant *C. quadricarinatus* proPO by SDS-PAGE
Lane M, molecular weight markers; lane 1, *E. coli* BL21 (DE3); lanes 2, *E. coli* BL21 (DE3) with pET-proPO after protein expression; lane 3, purified recombinant fusion proteins proPO

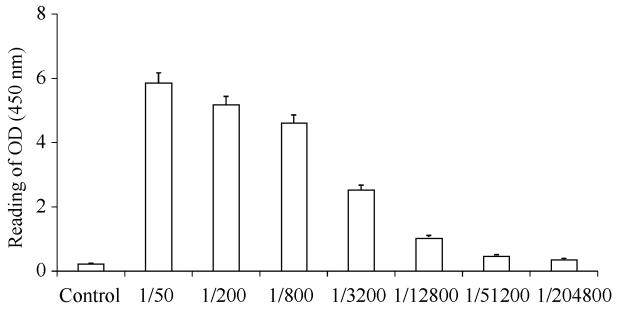

Fig. 3　Antibody titer was determined by ELISA

lanes 3). The titer of anti-proPO antibody against rabbit was determined by ELISA (Fig. 3). The titer of anti-proPO serum was above 1∶12800 and below 1∶51200 as tested by ELISA.

2.4　Tissue distribution of *proPO* mRNA transcription and PO activity

As determined by real-time RT-PCR, *proPO* transcription was widely observed in the nerve, hepatopancreas, hemocytes, gill, heart, intestine and muscle of *C. quadricarinatus* (Fig. 4A). The transcription of *proPO* was highest in hemocytes, followed by hepatopancreas and gills, lower levels were seen in other tissues (heart, intestine, muscle and nerve).

Similar to *proPO* mRNA transcription in tissues of *C. quadricarinatus*, the PO activities also could be detected in all examined tissues by the traditional colorimetric method. The highest activities were observed

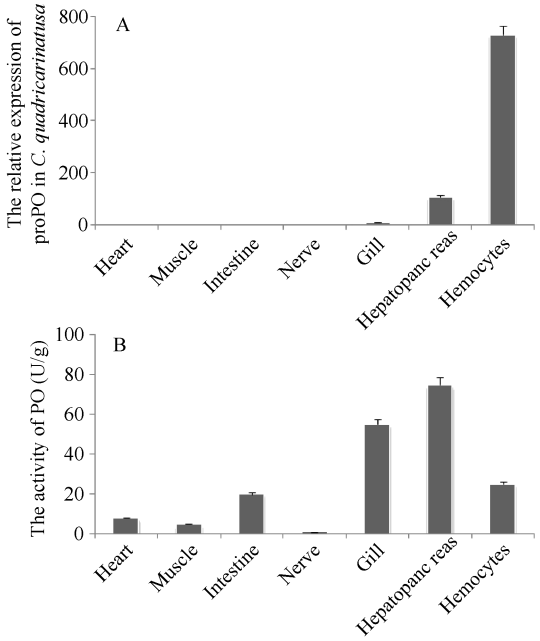

Fig. 4　Tissue-dependent proPO mRNA transcripts (A) and PO activity (B) of *C. quadricarinatus*

in hepatopancreas, followed by hemocytes and gill, and lower PO activities were seen in other tissues (intestine, heart, muscle and nerve) (Fig. 4B).

2.5　*ProPO* mRNA transcription pattern under pathogens stimulations

The proPO mRNA transcription pattern after pathogens challenge are shown in Fig. 5. The transcript of *proPO* mRNA in hemocytes was up-regulated at 48 and 72h after the *S. eriocheiris* or *A. hydrophila* challenge. The *proPO* mRNA transcription in hemocytes was also up-regulated at 6h after the *A. hydrophila* stimulation (Fig. 5A). The *proPO* mRNA in hepatopancreas was up-regulated at 1h after the *S. eriocheiris* or *A. hydrophila* challenge. And after *S. eriocheiris* challenge, *proPO* mRNA in hepatopancreas was also up-regulated at 3, 6 and 12h (Fig. 5B). The *proPO* mRNA transcription pattern in gills after *A. hydrophila* challenge is up-regulated at 24, 48 and 72h. The transcript of *proPO* mRNA in the gills was up-regulated at 1 and 72h after the *S. eriocheiris* challenge (Fig. 5C).

2.6　PO activities variation in *C. quadricarinatus* following pathogen stimulation

Similar to *proPO* mRNA transcription, the PO activities were increased during the time course of hemocytes, hepatopancreas and gills post *S. eriocheiris* or *A. hydrophila* stimulation (Fig. 6). There were two peaks of PO activities in hemocytes post-stimulation. The first peak was observed at 1h post-injection, which was 1.88-fold to that in control group (P>0.05). Afterwards, the PO activities decreased to normal level at 3h. As time progressed, the

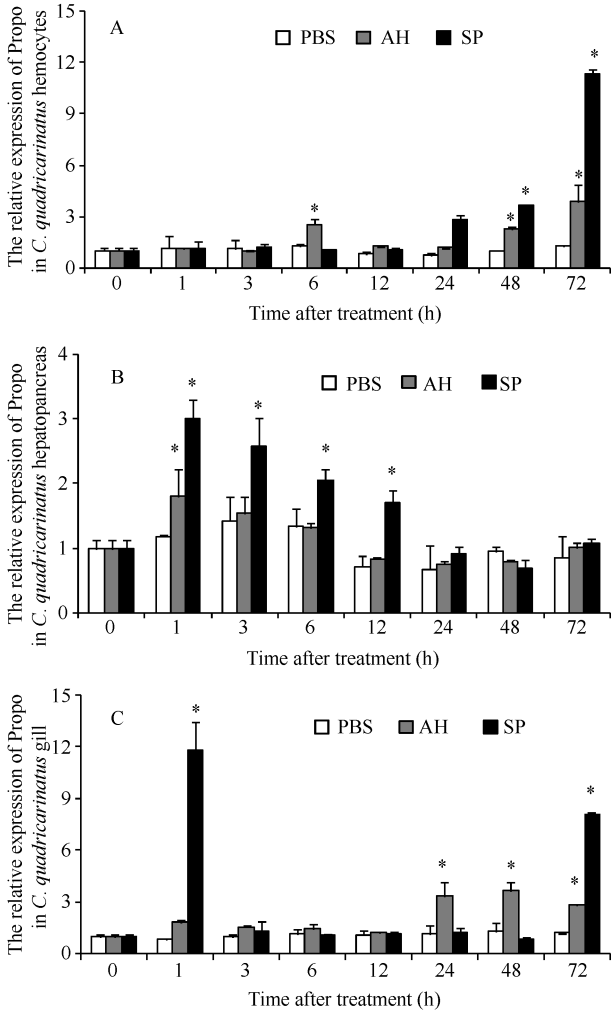

Fig. 5 The transcript levels of proPO in hemocytes (A), hepatopancreas (B) and gills (C) of *C. quadricarinatus* after *S. eriocheiris* or *A. hydrophila* challenge
The asterisk (*) indicates significant differences between PBS challenge (P<0.05)

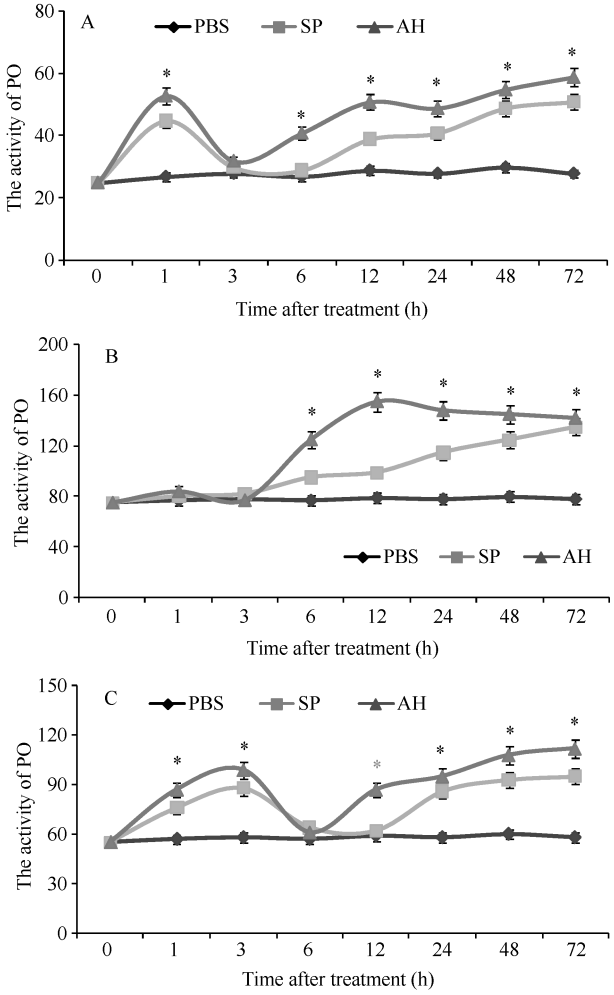

Fig. 6 The PO activity in hemocytes (A), hepatopancreas (B) and gills (C) of *C. quadricarinatus* after *S. eriocheiris* or *A. hydrophila* challenge.
The asterisk (*) indicates significant differences between PBS challenge (P<0.05)

PO activities was increased at 12, 24, 48 and 72h post pathogen stimulation (also at 6h post *A. hydrophila* stimulation) (Fig. 6A). The PO activities remained at normal level during the first 3h after pathogen stimulation, then increased from 6h (Fig. 6B). Similar to the PO activities in hemocytes, there were two peaks of the PO activities in gills post pathogen stimulation. The PO activities increased at 1 and 3h, and reached the first peak at 3h. After decrease to normal level at 6h (also at 12h post *S. eriocheiris* stimulation), the PO activities gradually increased after 12h post pathogenic stimulation (Fig. 6C).

3 Discussion

The first *proPO* gene was cloned from the freshwater crayfish *Pacifastacus leniusculus* in 1995 [10]. To date, more than 40 *proPO* genes have been obtained from about 30 species in crustaceans, such as *P. leniusculus*, *Macrobarchium rosenbergii* [11, 12], *Penaeus monodon* [13], *Litopenaeus vannamei* [14—16], *Fenneropenaeus chinensis* [17, 18], *Marsupenaeus japonicus* [19], *P. semisculcatus* (AAM77690), *P. clarkii* [14], *H. americanus* (AAT73697), *H. gammarus* [20], *Eriocheir sinensis* [21], *Portunus trituberculatus* [22], *Scylla serrata* [7], *Cancer pagurus* (CBW54878) and *C. magister* [23]. The *C. quadricarinatus proPO* has not cloned and studied its characteristic. Therefore the immune characteristic of *C. quadricarinatus* proPO was studied in this paper.

In the present study, the cDNA encoding prophenoloxidase was cloned from *C. quadricarinatus*. The 2951 bp full-length cDNA contained a 1995 bp open reading frame (ORF) encoding a putative proPO protein of 665 amino acids, a 5′-UTR of 75 bp and a long 3′-UTR (881 bp). The analysis of the amino acid sequence of this fragment showed that it contained six histidine residues which define two putative copper

binding motifs for tyrosinase and also contained a thiol ester-like motif (GCGWPEHL) just like the structural features of other proPOs from other crustacean. This sequence has similarity of 69%, 63%, 63% and 59% to proPO-deduced amino acid from *P. leniusculus*, *H. gammarus*, *H. americanus* and *P. clarkii*. As a unique defense system of invertebrate, proPO system is of pivotal importance in crustacean immune response. The MW of proPO from crustacean is almost about (75—80) kD. The calculated MW of the proPO was 75.7 kD which is bigger than expression in *E. coli* (66 kD) (Fig. 2). SDS-PAGE is a common method to determinate the MW of protein. But sometimes MW analyzed using SDS-PAGE is inconsistent with the calculated MW. This may be caused by inappropriate electrophoresis conditions, changes of protein structural, irregular electric charge distribution and post-translational modification such as phosphorylation [24]. But the reasons for the deviation have not been fully elucidated now. Phylogenetic analysis revealed that *C. quadricarinatus* proPO is distinct far away from proPOs of Insecta, and is also distinct from proPO of shrimps, crabs and prawn. Multiple alignments indicated that the proPO of *C. quadricarinatus* forms monophyletic subgroup with proPO of *P. Leniusculus* and *P. clarkii*. The present study also indicated that the decapod crustacean proPOs can be probably classified into five distinct branches: prawn, crayfish, lobster, shrimp, and crab.

The distribution of *proPO* in different tissues has been investigated in a variety of organisms. In the present study, the *proPO* of *C. quadricarinatus* were highly transcription in hemocytes, hepatopancreas and gills, lowly transcript levels were seen in other tissues (muscle, heart, intestine and nerve). The *proPO* mRNA transcripts were strongly expressed in hemocytes of *C. quadricarinatus*. The same situation appears in mud crab *S. serrata* [7], crayfish *P. Leniusculus* [25], white shrimp *L. vannamei* [14—16], freshwater prawn *M. rosenbergii* [11], *F. chinensis* [17] and *P. tritu-berculatus* [22]. The *proPO* transcript was located in hemocytes, and released into the circulation, but not transcription in other tissue of crayfish *P. leniusculus* [25]. But the PO activities were detectable in all examined tissues, including muscle, heart, intestine and nerve with the highest level of both in hepatopancreas. The possibility of the widely distributed presence of PO activities could be the result of the proPO released into the circulation and other different tissues. The high PO activity in hepatopancreas might be caused of the cooperation of multiple POs or the cross interference of hemocyanin, which had been demonstrated to possess PO activity in crayfish [26—28].

Both *proPO* mRNA transcription and protein activity levels post stimulation were constructive to understand its roles in the immune mechanisms. In the present study, *C. quadricarinatus proPO* mRNA transcriptions in hemocytes, hepatopancreas and gills were up-regulated after *S. eriocheiris* and *A. hydrophila* challenges. The mRNA transcriptions of *proPO* in hemocytes were up-regulated at 48h and 72h post *S. eriocheiris* or *A. hydrophila* stimulation (also up-regulated at 6h post *A. hydrophila* stimulation). The PO activity in hemocytes significantly increased and reached two peaks at 1h and 72h after pathogen stimulation. The up-regulation of *proPO* mRNA transcriptions and PO activity in response to different stimulations was reported in *S. serrata* [7], *Astacus astacus* [29], *H. gammarus* [20] and *E. sinensis* [21], which was in agreement with our results. The changing trends of *proPO* mRNA transcriptions and PO activity in hepatopancreas in response to stimulations were different. The mRNA transcriptions of *proPO* in hepatopancreas were up-regulated at the first 12h after pathogenic challenging. But the PO activity increased beginning at 6h. This case maybe result from that proPO system is an energy-consuming, multifunctional mechanism [12], it might function stepwise in different phases of the host-pathogen interaction. The mRNA transcriptions of *proPO* in gills were up-regulated at 1h and 72h post *S. eriocheiris* stimulation and at 24h, 48h and 72h post *A. hydrophila* stimulation. The PO activity in gills significantly increased and reached two peaks at 3h and 72h after pathogen stimulation. The increase of *proPO* transcription and PO activity at first several hours maybe cause by the stimulation of pathogen. And the *proPO* transcription and PO activity increased at 72h maybe cause by appeared of immune response. This result states the proPO may have relationship with the immune response of hosts to resist pathogen. But further investigations are required to better understand the regulation mechanism of proPO to resist pathogen stimulation.

In conclusion, *proPO* gene was cloned here for the first time from hemocytes of *C. quadricarinatus*. The proPO immune functions of *C. quadricarinatus* in hemocytes, hepatopancreas and gill were confirmed by real-time PCR, and PO activity was also analyzed in hemocytes, hepatopancreas and gill after *S. eriocheiris* and *A. hydrophila* challenges. The result will help us to understand the characteristic and function of proPO from *C. quadricarinatus*.

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红螯螯虾酚氧化酶原及其特性研究

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摘要: 采用同源克隆策略和 RACE 技术, 从红螯螯虾 *Cherax quadricarinatus* 血细胞中克隆得到酚氧化酶原基因的全长 cDNA 序列, 共 2951 bp, 开放读码框为 1995 bp, 编码 665 个氨基酸。预测的分子量和等电点分别为 75.7 kD 和 6.23。酚氧化酶原含有两个推测的 tyrosinase copper-binding motifs (带有六个组氨酸残基)和一个 thiol-ester-like motif, 这些特征和其他甲壳动物的酚氧化酶原特征相同。红螯螯虾酚氧化酶原氨基酸序列与通讯螯虾 *Pacifastacus leniusculus*、欧洲龙虾 *Homarus gammarus*、美洲龙虾 *Homarus americanus* 和克氏原螯虾 *Procambarus clarkii* 酚氧化酶原的相似率分别为 68%、63%、63%和 59%。酚氧化酶原基因双酶切后连接入 pET-28a 原核表达载体, 转化到大肠杆菌 BL21 后重组表达酚氧化酶原蛋白。在重组蛋白纯化后, 免疫新西兰大耳兔制备得到的酚氧化酶原多克隆抗体, 其效价大于 1∶12800。红螯螯虾血淋巴、肝和鳃组织中的酚氧化酶原 mRNA 表达和酚氧化酶活性较高, 而神经、心、肠和肌肉中较低。中华绒螯蟹螺原体和嗜水气单胞菌免疫红螯螯虾后, 血淋巴细胞、肝和鳃组织中的酚氧化酶原和酚氧化酶活性在免疫后的不同时间均出现了显著性的增加, 此结果表明酚氧化酶原和酚氧化酶在红螯螯虾对抗细菌感染的过程中起到重要的免疫作用。此结果为进一步深入研究酚氧化酶原基因和酚氧化酶的功能及其调控机理奠定基础。

关键词: 酚氧化酶原; 中华绒螯蟹螺原体; 嗜水气单胞菌; 红螯螯虾