Mol Biol Rep (2009) 36:1159–1166
DOI 10.1007/s11033-008-9292-6

Molecular cloning and characterisation of prophenoloxidase (ProPO) cDNA from *Fenneropenaeus chinensis* and its transcription injected by *Vibrio anguillarum*

Hongwei Gao · Fuhua Li · Bo Dong · Qingli Zhang · Jianhai Xiang

Received: 26 March 2008 / Accepted: 11 June 2008 / Published online: 2 July 2008
© Springer Science+Business Media B.V. 2008

Abstract The prophenoloxidase(ProPO) gene was cloned from haemocytes of Chinese shrimp *Fenneropenaeus chinensis* by Rapid Amplification Complementary DNA Ends (RACE) method. The full-length cDNA of prophenoloxidase gene consists of 3040 bp with a 2061 bp Open Reading Frame (ORF), encoding 686 amino acids. Phylogenetic analysis revealed that it belongs to insect-type invertebrate prophenoloxidase gene family. To understand ProPO reaction for pathogeny's challenge in shrimp, the expressions of ProPO in different tissues were studied by real-time PCR after challenged by *Vibrio anguillarum*. The results showed that the expression level of ProPO gene in haemocytes was highest among three studied tissues including haemocytes, lymphoid organ and hepatopancreas. The time-course change of ProPO mRNA levels in challenge experiment showed that ProPO mRNA transcripts had the biggest change extent in lymphoid organ.

Keywords Cloning · Expression · Prophenoloxidase · *Fenneropenaeus chinensis*

H. Gao · F. Li · B. Dong · Q. Zhang · J. Xiang (✉)
Institute of Oceanology, Chinese Academy of Sciences, Qingdao, China
e-mail: jhxiang@ms.qdio.ac.cn

H. Gao
Shandong Entry-Exit Inspection and Quarantine Bureau of People's Republic of China, Qingdao, China

H. Gao
Graduate School of Chinese Academy of Sciences, Beijing, China

Introduction

The worldwide costs of disease control in the crustacean aquaculture industry are widely acknowledged [1–3]. To reduce the occurrence of disease in crustacean aquaculture, great efforts have been done to study the innate immune system of crustacean. According to previous studies, the primary immune response in crustacean is the melanization of pathogens and damaged tissues [4, 5]. This important process is controlled by the ProPO-activation pathway [4–6], which is a phenoloxidation cascade comprising of pattern recognition proteins, several serine proteases, their inhibitors and terminates with the zymogens, ProPO [4]. The ProPO activating enzyme or factor are serine proteases, which cleave ProPO to generate the active enzyme, phenoloxidase. Activation of ProPO is mediated by a proteinase cascade plus additional factors. The terminal serine proteinase that carries out the proteolysis of the ProPO precursor has been variously named the ProPO activating factor (PPA) [4].

Several arthropod haemocytes derived cell lines dispaly *ProPO* mRNA expression capability, and *ProPO* expression may also be used as a marker to follow haemocyte maturation; in *Drosophila melanogaster*, ProPO expression accompanies crystal cell maturation. Both cellular differentiation and ProPO expression can be annulled by interfering with gene coding for the transcription factors [7]. In *Pacifastacus leniusculus,* immature haemocytes within the hematopoietic organ do not express the ProPO, whereas mature haemocytes in circulation do [6]. It seems that ProPO expression level contains the information about ProPO function at different life phases [8]. ProPO are so important enzymes in crustaceans that they have been cloned from several species, including Pacific white shrimp *Litopenaeus vannamei* (GenBank accession number

EU373096 and AY723296 and EF565469 and EF115296), black tiger shrimp *Penaeus monodon* (AF099741.1, AF521948.1). Recently, the characterization of *ProPO* and its role in immunomodulation in white shrimp (*L. vannamei*) and giant freshwater prawn (*Macrobrachium rosenbergii*) were reported [9, 10].

Several immune parameters of the crustacean, including haemocyte counts, PO activity, respiratory bursts (production of superoxideanion), phagocytic activity and clearance efficiency, and the susceptibility to pathogens in relation with the molt cycle have been reported in crustaceans [11–13].The transcripts level change of *ProPO* in haemocytes and hepatopancreas after *V. anguillarum* injection has not been investigated while *V. anguillarum* was a significant pathogenic bacteria [14].

Materials and methods

Experimental shrimp and immune challenge

A batch of apparently healthy shrimp *F. chinensis,* with body length of 10.5 ± 0.5 cm were purchased from a local shrimp farm. They were acclimated in seawater for 7 days in the lab. Hemolymph was collected from the ventral sinus located at the first abdominal segment with an equal volume of anticoagulant-modified Alservier solution (27 mM sodium citrate, 336 mM NaCl, 115 mM glucose, 9 mM EDTA, pH 7) [15]. Haemocytes were isolated by centrifugation at 800*g*, at 4ºC for 10 minutes and immediately preserved in liquid nitrogen. Different shrimp tissues were dissected out at the same time, and preserved in liquid nitrogen for RNA extraction.

Challenged experiments were performed by injecting 20 μl suspension of inactivated *V. anguillarum* (10^8 CFU/ml) in physiological saline solution into the last abdominal segment of each shrimp. Shrimp injected with 20 μl of sterile physiological saline were maintained as controls. Haemocytes, hepatopancreas and lymphoid organ from six experimental and six control shrimps were collected at 6, 12, 24, and 72 h post injection (hpi) and preserved for real time reverse transcript polymerase chain reaction (RT-PCR).

Total RNA extraction and the cDNA synthesis

Total RNA of each sample was extracted using Unizol reagent (Boxing Co. Limited., Shanghai, P. R. China). RNA quality was assessed by electrophoresis on 1% agrose gel. Total RNA was treated with RQ1 RNase-Free DNase (Promega) to remove contaminated DNA. cDNA was synthesized from 5 μg total RNA by M-MLV reverse transcriptase (TaKaRa, Dalian, China) following the manufacturer's protocol with Hexadeoxyribonucleotide Mixture primer [16].

Cloning and sequencing of ProPO cDNA fragment

A pair of primers, ProPO f1 and ProPO rl, was designed according to the EST from haemocytes cDNA library. PCR amplification was performed using the template cDNA from haemocytes (one cycle of 94°C for 5 min; 35 cycles of 94°C for 45 s, 55°C for 45 s, and 72°C for 40 s; followed by one cycle of 72°C for 10 min). PCR products were isolated on 3% agarose gels, purified by PCR purification kit (Promega), and cloned into PMD 18-T vector (TaKaRa). The resultant recombinant plasmid was then transformed into TOP 10, and the positive transformants were screened by PCR.

3′ and 5′-RACE

Based on the partial sequence of *ProPO*, the 3′ and 5′ ends were obtained by the rapid amplification of cDNA ends (RACE) approaches, using gene-specific primers and adapter primers. For *ProPO* 3′-RACE, nested PCR was applied and the cDNA template used was from haemocyte RNA. PCR reaction was performed with gene-specific outer primer ProPO f2 and AP primer. The PCR was carried out according to the program of 94°C for 5 min, 35 cycles of 94°C for 50 s, 55°C for 50 s and 72°C for 1 min, and then an extension of 72°C for 10 min. Then, the inner primer ProPO f3 and AP primer were used to amplify the 3′ terminal sequence with the same conditions, except for the annealing temperature of 58°C [16].

For *ProPO* 5′-RACE PCR, nested PCR strategy was employed to increase specificity. The outer gene-specific primer ProPO r2 and the gene-specific inner primer ProPO r3 were designed from the 3′-RACE. The PCR conditions were the same as mentioned above. The specific amplicons obtained were cloned into the TOPO-TA cloning vector (Invitrogen), sequenced, and aligned with the original sequences. The continuity of the 3′- and 5′-RACE products was verified by contiguous overlapping regions. For both *ProPO* 3′ and 5′-RACE PCR, cDNA template used was from haemocytes RNA.

Sequence analysis of *ProPO*

The nucleotide and deduced amino acid sequences of *ProPO* cDNA were analyzed using BLAST algorithm (NCBI, http://www.ncbi.nlm.nih.gov/BLAST/). The signal peptide, extracellular domain, transmembrane, cytoplasmic domains and other characteristic structures of *ProPO* were predicted by the simple modular architecture research tool (SMART) program (http://smart.embl-heidelberg.de/).

Phylogenetic and molecular evolutionary analyses of the predicted amino acid sequences of different crustacean *ProPO* were conducted using neighbor-joining method with the software of molecular evolution genetics analyses (MEGA) version 3.1 [17].

Real time PCR analysis of gene expression and relative quantitative statistical analysis

Relative quantification of mRNA transcript abundance required a *Taqman* probe and a pair of primers for Prophenoloxidase (ProPO) and endogenous reference Triosephosphate isomerase (TPI). Primers and probes of TPI gene were the same as reported [18]. Primers and probe sets for *ProPO* were designed using the software package Primer Express (Applied Biosystems, USA). Each primer and probe set was designed using the default parameters optimized by ABI Prism 7900 HT (Applied Biosystems, USA). The sequences of all the primers and probes used are summarized in Table 1.

Before comparing control and stimulated samples, the overall variations of 18S rRNA expression were first examined. Real-time RT-PCR assays were carried out in a fluorometric thermal cycler (ABI PRISM 7900 HT Sequence Detection System, Applied Biosystems, New Jersey, USA) with a final volume of 25 μL. Each sample was run in triplicate and NTC (no template controls) were included for each primer and probe set. Fluorescence was monitored during every PCR cycle at the annealing step. The RT-PCR mixture contained the following: 1× *Taqman* One-Step RT-PCR Master Mix Reagents (Applied Biosystems, New Jersey, USA), 200 nM primers and 300 nM probes. Real-time RT-PCR were run with the following program: 30 min at 48°C, 10 min at 95°C, 45 cycles of 15 s at 95°C, 30 s at 55°C, 30 s at 72°C. All the primers and fluorescent probes were synthesized and purified by Shanjing Co. Ltd (Shanghai, China).

$2^{-\Delta\Delta Ct}$ method was used to analyze the quantitative expression after verifying the amplification efficiency of endogenous and target genes. The amplification efficiency of each gene was calculated from the slope of a line generated by plotting the Ct value against the $\log_{10}$ of the dilution factor. No significant difference was found for the *ProPO* and *TPI gene* (*t*-test, $P > 0.05$). A plot of the log cDNA dilution versus ΔCtTPI and ΔCt ProPO was made to compare their amplification efficiency. The slope of *TPI* was −3.4206, and *ProPO* was −3.4952, respectively. The absolute value of their dispersion was less than 0.1. Further, the amplification efficiency of *TPI* and *ProPO* were 1.9425 and 1.9324 respectively. The two genes' amplification values were very closed (dispersion between them < 0.04), so the assumption seems valid and the ΔΔCt method could be used to analyze the data [19].

Results

Full length cDNA of *ProPO*

The full-length nucleotide sequence and the deduced amino acids sequence of shown in Fig. 1. The complete sequence was 3040 base pairs and contained an open reading frame (GenBank accession number EU015060) beginning at the nucleotide 74 and ending at the nucleotide 2132. It yielded a deduced polypeptide of 686 amino acids. SignalP software (ExPASy) predicted that the first 12 amino acids form a signal peptide. The protein has a predicted molecular mass of 78,221 Da and a predicted isoelectric point of 5.86. In Fig. 2, the black parts of the alignment were the same amino acids in five shrimps. The deduced amino acid

Table 1 Oligonucleotide sequences of all used primers

Primer	Application	Sequences
ProPO f1	PCR	5′-AAGTGAACGAGACCCTGTTTGT-3′
ProPO rl	PCR	5′-CAGGAGTCGAGATCGCCATATT-3′
ProPO f2	3′race	5′-CTCGG CAAGCTTTCAT-3′
ProPO f3	3′race	5′-GCTGTGGTTCATATCTGTCC-3′
ProPO r2	5′race	5′-ATGCCATAGTCCTCTCGCCAGT-3′
ProPO r3	5′race	5′-AGGGTCTCGTTCACTTTGCCAT-3′
ProPO f4	Real-time PCR	5′-GGAGTCGTCGATCACCAACCT-3′
ProPO r4	Real-time PCR	5′-ACCTGTTCGCTCGTGTTTCC-3′
ProPO P	Probe	FAM 5′-TTCAAAGACCTGGAGAACT-3′ TAMARA
TPI f	Sense	5′-AGCCAGACTTTGTTCAGA-3′
TPI r	Antisence	5′-TACCCAAGTTCAAGCATCTG-3′
TPI P	probe	FAM 5′-TGTAGAAGCAAGTAAAGTC-3′ TAMARA
AP	3′race	5′-GGCCACGCGTCGACTAGTAC-3′

sequence of ProPO showed 93% identity with that of *P. monodon*, 92% with that of *P. semisulcatus*, 87% with that of *Litopenaeus vannamei*, 80% with that of *Marsupenaeus japonicus*.

Three Kinase binding sites (V51, V138, V300), one tyrosine kinase (399–423 amino acid), one NOZZLE site (598–617 amino acid) and α2-macroglobulin (GCGWPQHM) were also present in *F. Chinensis* ProPO (in Fig 1). Multiple alignments with other crustaceans *ProPO* showed high conservation in six histidines sites (H207, H211, H233, H366, H370 and H408) which marked with asterisks (in Fig. 2), which were putative copper-binding sites.

Phylogenetic analysis of *ProPO*

To examine the relationships among various prophenoloxidases (ProPOs), a phylogenetic tree was constructed using the overall amino acid sequences of 14 crustacean ProPOs (Fig. 3). *Branch 1* consisted of two subgroups. FcProPO

```
1    ACA CTC TTT CTC TCA CCT CCA CCC TGA GAG CTC TTC CTT TCC ACG TCT CGC TCT GAC ACC TTC TCC   66
67   AGA GCC ATG GCC AAT GAC CAG CAG CGT CTT CTG TAC CTG TTT GAA CTT CCT CAG GAG GAT ATT CAA   132
1            M   A   N   D   Q   Q   R   L   L   Y   L   F   E   L   P   Q   E   D   I   Q    20
133  GTG CCC AGG GGA GGT GGC TCT GTC CTC TTC AAG CTC GAG AAC GAG GAG ACG CCT CCT TCT GTG GCC   198
21    V   P   R   G   G   G   S   V   L   F   K   L   E   N   E   E   T   P   P   S   V   A    42
199  ACG AGA GTA GGA GTT TCC CCC TCT GTC AGC CTG CCA GTG CCA GAG CGA GAT GAC GTG GCA CTG CAG   264
43    T   R   V   G   V   S   P   S  (V)  S   L   P   V   P   E   R   D   D   V   A   L   Q    64
265  GCT CTC GGC ACT GCC ACC TCG ATC CCC AAA GGA TCA GCC TTC TCC TTC TTC CTC GCG TCT CAC CGT   330
65    A   L   G   T   A   T   S   I   P   K   G   S   A   F   S   F   F   L   A   S   H   R    86
331  AAA GCC GCC AAG GAT CTG TGT GAC TTC TTC ATG AAG ACG AGC GGG GCA GAA GAC CTG ATG CAG GTT   396
87    K   A   A   K   D   L   C   D   F   F   M   K   T   S   G   A   E   D   L   M   Q   V    108
397  GCA GCG CGC GTG CAT GGC AAA GTG AAC GAG ACC CTG TTT GTC TAT GCA ATT TCT TTT GTC ATC CTG   462
109   A   A   R   V   H   G   K   V   N   E   T   L   F   V   Y   A   I   S   F   V   I   L    130
463  AGA AAG AAG GAG CTC AGA TCT GTT CGC CTG CCG ACC ATG GTG GAG GTC TTT CCC TCC CGC TTC GTT   528
131   R   K   K   E   L   R   S  (V)  R   L   P   T   M   V   E   V   F   P   S   R   F   V    152
529  CCC CAG GAG GCG CTG GCG AAG GCA CAG CTG CAG ATA AAC AGA ATG GAT CCA AAT CAG ACT GAA CCT   594
153   P   Q   E   A   L   A   K   A   Q   L   Q   I   N   R   M   D   P   N   Q   T   E   P    174
595  GTG ATC ATA GAA CAC GGA CTG GAG TTC TCT GGC ACT CAT CTC AAG CCA GAA CAC AGA CTC TCT TAC   660
175   V   I   I   E   H   G   L   E   F   S   G   T   H   L   K   P   E   H   R   L   S   Y    196
661  TGG CGA GAG GAC TAT GGC ATC AAC GTC CAC CAC TGG CAC TGG CAC TTG ATC TAC CCG CCT GGC ATG   726
197   W   R   E   D   Y   G   I   N   V   H   H   W   H   W   H   L   I   Y   P   P   G   M    218
727  GGT TTT GAC CGT GAC AGG AAA GGG GAA TTG TTT TAC TAT ATG CAC CAG CAA GTG ATC GCC AGA TAC   792
219   G   F   D   R   D   R   K   G   E   L   F   Y   Y   M   H   Q   Q   V   I   A   R   Y    240
793  GAC ATA GAG CGT CTC TGT CTG GGT CTC CCG AGG GTG GAG AAG CTA GAC AAC TGG CGC ATT CCC ATC   858
241   D   I   E   R   L   C   L   G   L   P   R   V   E   K   L   D   N   W   R   I   P   I    262
859  AAG GAC GGT TAC TTC CCC AAA CTG ACT ATC AGC AAC TCC GGC AGG CAA TGG GGG TCG CGC CAG GAC   924
263   K   D   G   Y   F   P   K   L   T   I   S   N   S   G   R   Q   W   G   S   R   Q   D    284
925  AAC ACA CTG CCA AAG GAC CTC CGG CGC AGA GAA CTC GGA GAA TTT GTC GAC ATC ACT GAC ATG GAA   990
285   N   T   L   P   K   D   L   R   R   R   E   L   G   E   F  (V)  D   I   T   D   M   E    306
991  ATA TGG CGA TCT CGA CTC CTG GAT GCC ATA CAT CAG GGC TTC ATG ATT GAT TGC AAC GGC GAC AAA   1056
307   I   W   R   S   R   L   L   D   A   I   H   Q   G   F   M   I   D   C   N   G   D   K    328
1057 GTT CCT CTT CGC GAC GAC GTC ACT TCT GGC AAA AGA GGA ATC GAA ATC TTG TCC GAA GCT CTT GAG   1122
329   V   P   L   R   D   D   V   T   S   G   K   R   G   I   E   I   L   S   E   A   L   E    350
1123 GCC GAC GCC GAA CTC AGC GTC AAC TTC CCT TAT TAC GGC GAC CTG CAC AAC AGA GGC CAC GAC ATC   1188
351   A   D   A   E   L   S   V   N   F   P   Y   Y   G   D   L   H   N   R   G   H   D   I    372
1189 CTG GCC TTC TCG CAC GAC CCT GAC AAC GCT CAC AAG GAG GAG ATG GGT GTC GTC GGG GAC TTG GGG   1254
373   L   A   F   S   H   D   P   D   N   A   H   K   E   E   M   G   V   V   G   D   L   G    394
1255 ACG TCC CTG AGA GAC CCT GTG TTC TTC CTC CTG CAC AAG CTC GTG GAT GAC TTG TTC CAG GAG TAC   1320
395   T   S   L   R   D   P   V   F   F   L   L   H   K   L   V   D   D   L   F   Q   E   Y    416
1321 AAG GTT ACC CAG CCA CCG TAC ACA GAA GCG GAG CTG TTC CTG CCC GGC GTG AGG ATC GAG CGG GCT   1386
417   K   V   T   Q   P   P   Y   T   E   A   E   L   F   L   P   G   V   R   I   E   R   A    438
1387 GGG GTG GTG CGG GGT AAT GAG GCC GAC GTC CTC CTC ACC GGC TGG AAC ACG AGG GAG TTC GAA GCC   1452
439   G   V   V   R   G   N   E   A   D   V   L   L   T   G   W   N   T   R   E   F   E   A    460
1453 AGC CGC GGC ATC GAC TTC AAC GGC AAA CCC GTG ATT CTG CGC CTC ACG CAT CTC GAC CAC AAG CCC   1518
461   S   R   G   I   D   F   N   G   K   P   V   I   L   R   L   T   H   L   D   H   K   P    482
1519 TTC GAG TAC CAT GTG CAG ATA AAC AAC GAT CTC CGA GAG CCG AAG GAA GTG ACT GTG AGG ATA TTT   1584
483   F   E   Y   H   V   Q   I   N   N   D   L   R   E   P   K   E   V   T   V   R   I   F    504
1585 TGG GCT CCG AAG TTC AAT GGT CAA GAG GAA GAG ATG GGA TTC ATG GAA CAG CGG ATC CTC TGG TCT   1650
505   W   A   P   K   F   N   G   Q   E   E   E   M   G   F   M   E   Q   R   I   L   W   S    526
1651 GAA ATG GAC AAG TTC ACT GTT AAC TTA AAA CCC GGC AAG AAC CAC GTC GTC AGG TCG TCC AAG GAG   1716
527   E   M   D   K   F   T   V   N   L   K   P   G   K   N   H   V   V   R   S   S   K   E    548
1717 TCG TCG ATC ACC AAC CTC GAG GAA CTG ACC TTC AAA GAC CTG GAG AAC TCT GGG CCC GGA AAC ACG   1782
549   S   S   I   T   N   L   E   E   L   T   F   K   D   L   E   N   S   G   P   G   N   T    570
1783 AGC GAA CAG GTA GCA TTT AAC TTC TGC GGG TGT GGA TGG CCC CAG CAT ATG CTT CTC CCC CGC GGC   1848
571   S   E   Q   V   A   F   N   F   C   G   C   G   W   P   Q   H   M   L   L   P   R   G    592
1949 CGT CCA GAG GGA ATG GCC TTC CAG CTC TTC TTC ATG CTC ACC GAC TAC GCC CAG GAC AAA GTG TCG   1914
593   R   P   E   G [ M   A   F   Q   L   F   F   M   L   T   D   Y   A   Q   D   K   V   S ] 614
1915 AAC CCG GGG GGC GTG AGA CGG TGT GCC AAC GGT GTG TCC TTC TGC GGG ATG CAG GAC GCC AAA TAC   1980
615 [ N   P   G ] G   V   R   R   C   A   N   G   V   S   F   C   G   M   Q   D   A   K   Y    636
1981 CCC GAC GCC CGA CCG ATG GGC TTC CCG TTC GAC CGC AGC CCC GCC CCC TTG CTC CAA GGG CTC CCC   2046
637   P   D   A   R   P   M   G   F   P   F   D   R   S   P   A   P   L   L   Q   G   L   P    658
2047 GTC AAC ACG ACT TCC GAC TAC GCC CGC CTC GGC AAC GCT TTC ATA CAC GAC ATC ACG ATT AGG TTC   2112
659   V   N   T   T   S   D   Y   A   R   L   G   N   A   F   I   H   D   I   T   I   R   F    680
2113 CTG GGC GAG AAA TTG AAC TGA TAT GCG CAT CAC GTG ACG CTG TGG TTC ATA TCT GTC CAT GTT TTT   2178
681   L   G   E   K   L   N                                                                  686
2179 TGT TTC TTT TTT ATG TTA AGT GTC ACT TTC AAT CTA CAC ACA CAC TGT TTT AAT TCC CGG ACC GAG   2244
2245 AAA ATA GTA GGA AAA AAT GTA TGC CAG CCT TCG AGC TTC CCT GCC TTC AAG GTA TAG ATG ACC GTG   2310
2311 AAC TCT CTT CAG CCA GTG CAT GTG ACC TTA CCA CAG TGC ATA ACC AAG AAT AAT GGT ATC TCG CAG   2376
2377 GGA ATA GCA ATG CAT TCG ACA AAG TTA ACT CTG GAT TAA ATT TTT ACC ATG GGA TTA CAT TGA GAG   2442
2443 ATT GAA CAC AGG ACT ACA CTG AAC GGA GCT AAC ACA GGA ACA TAG AAC TAC ACA GGA CAG ATT TAT   2508
2509 CAT AGG ATT ACA TTA GGT AGA TTT ATC ACA GAA ACA TAT AGC TAT ATT GGA CAG ATT TAA CAC AGG   2574
2575 AGC ATA GGA CTA CAT GGG GCA GAT TTA ACA TAG AAC ACA AAA CTA CAA TGG ACA AAT TTA ACA CTG   2640
2641 GAA CAT AGG GCT TTA TTT TCA GTG AGG CGT TTC TCT AAT CTG TAA AAG CTG AGC CCA ACA ACA GAA   2706
2707 GCG GAT GTG AGT CCA GGA AAA TGT TGC ACA GTA TTG CAA CTC TTG CAT ATG ATT CTC CCT TAT TTC   2772
2773 CAC CTC TCC ACG TGC ATT TTC CCT GGA TCA TTT CCT TGG ATG GAA ACA ATA GAC CAG CTT GTT TAT   2838
2839 AAT ATT AAT TAT CTG TTT TGT CTT ATA ATC TAT GTT TTA TGA AGT GTA ACT GTA TAA AGA TGG CCT   2904
2905 TGC ATT TAT TCA TCA TAC ATT ATA CCA CCC TGC GGC AAT GAG AAT TTG CGC AGA GGA ACA GGT TTG   2970
2971 TTT CTT GAA TGT ATA TGT TAA ATA AAT TTG TGT TGC TCA AAA AAA AAA AAA AAA AAA AAA AAA AAA   3036
3037 AAA                                                                                      3039
```

Fig. 1 Complete nucleotide and deduced amino acid sequence of ProPO from *F. chinensis*. The letters in box indicated start codon (ATG), stop codon (TGA) and the polyadenylation signal sequence (AATAAA). The putative sequence of signal peptide is shaded by gray. Three Kinase binding sites (V51, V138, V300) are labeled as Ⓥ, one tyrosine kinase (399–423 amino acid) with gray, one NOZZLE site (598–617 amino acid) is framed α2-macroglobulin (GCGWPQHM) are labeled with underline

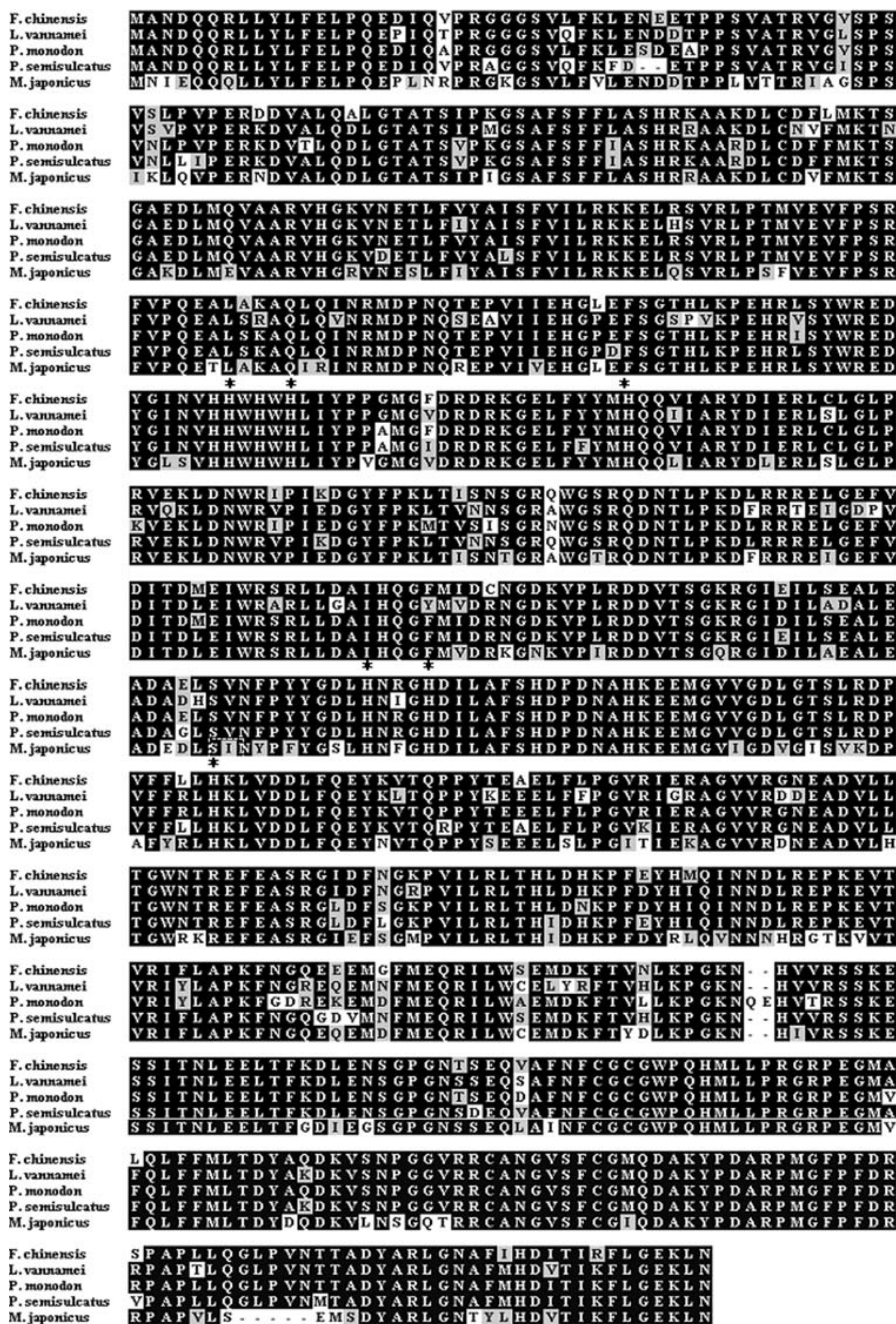

Fig. 2 Multiple alignment of amino acid sequences of clip domain ProPO homolog: *F.chinensis* (present study), *Penaeus monodon* (AAM77689), *Penaeus semisulcatus* (AAM77690), *Litopenaeus vannamei* (AAW51360), *Marsupenaeus japonicus* (BAB83773). Shaded areas indicate complete conservation in these five species. Six conserved histidines are marked with asterisks

together with other shrimp polyphenoloxidases (*P. semisulcatus*, *P. monodon*, *L. vannamei* and *M. japonicus*) was clustered into the same subgroup. ProPOs from lobsters (*H. americanu* and *H. gammarus*) and crayfish (*P. clarkia* and *P. leniusculus*) were placed into another subgroup. *Branch 2* was composed of ProPOs from two crabs (*S. serrata* and *C. magister*).

Expression of *ProPO* in haemocytes, hepatopancreas and lymphoid organ challenged by *V. anguillarum*

The results of relative *ProPO* expression in haemocytes, hepatopancreas and lymphoid organ were also calculated by $2^{-\Delta\Delta Ct}$ method. The information of *ProPO* expression was given after *t* testing. Before challenged by *V. anguillarum*,

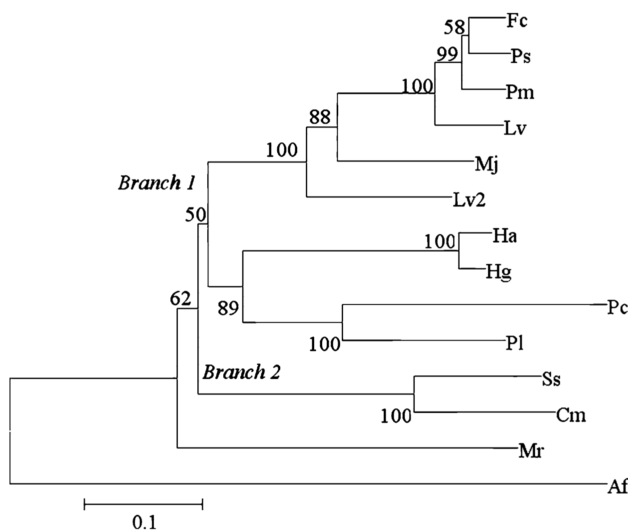

Fig. 3 A phylogenetic tree of 14 crustacean ProPOs. The reliability of each branch was tested by 1,000 bootstrap replications. Numbers at the nodes indicated bootstrap values. Sequences obtained from GenBank include: Fc (EU015060), Ps(AF521949_1), Pm (AF521948_1), Lv (AAW51360.1),Mj (BAB70485.1), Lv2 (ABY81277.1), Ha (AAT73 697.1), Hg (CAE46724.1), Pc (ABR12412.1), Pl (CAA58471.1), Ss (ABD90511.1), Cm (ABB59713.1), Mr (ABA60740.1), Af (CAO98 768.1). Abbreviations are: Fc, *F. chinensis*; Ps, *Penaeus semisulcatus;* Pm, *Penaeus monodon*; Lv, *Litopenaeus vannamei*; Mj, *Marsupenaeus japonicus*; Lv2, *Litopenaeus vannamei* prophenoloxidase *-2*; Ha, *Homarus americanus*; Hg, *Homarus gammarus*; Pc, *Procambarus clarkia*; Pl, *Pacifastacus leniusculus*; Ss, *Scylla serrata*; Cm, *Cancer magister*; Mr, *Macrobrachium rosenbergii*; Af, putative prophenoloxidase of *Artemia franciscana*

the highest level of mRNA was obtained in haemocytes and the lowest was got in hepatopancreas (Fig. 4).

After challenged by *V. anguillarum*, the expression of ProPO in haemocytes, increased significantly ($P < 0.05$) from 12 h to 72 h in the infection group, while the control group changed little. In lymphoid organ, ProPO expression level was up-regulated to its peak at 6 hpi, then decreased slowly to normal level ($P < 0.05$) (Fig. 5). There were remarkably significant difference between the control

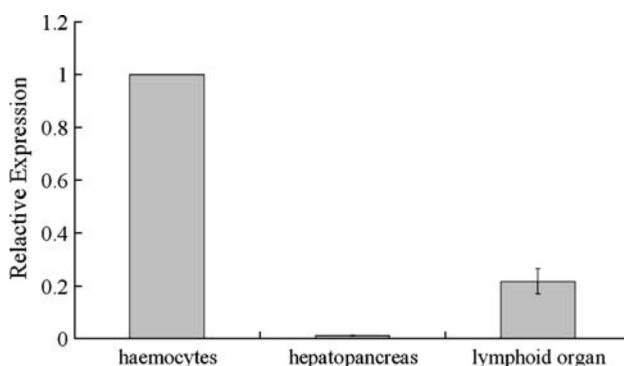

Fig. 4 Relative expression of *ProPO* in different tissues of *F. chinensis*

groups and the injection groups at 6 hpi and 12 hpi. In hepatopancreas, *ProPO* expression in infected group was sharply decreased at 6 hpi ($P < 0.05$) and showed little up-regulation compared with that of control group at 12 hpi and 24 hpi.

Discussion

A new full-length 3040 bp cDNA sequence with 686 amino acids of ProPO were cloned from *F. chinensis.* This sequence has a high identity to ProPO gene from other two penaeid shrimp, *P. monodon* and *P. semisulcatus.* Similarly *F. chinensis* ProPO has a high sequence homology to other invertebrate *ProPO*s. All of which contain at least one conserved putative cell adhesion integrin-binding motif [20] The ProPO of both insects and branchiopods are copper-binding molecules that play important role in sclerotization of cuticle and encapsulation of foreign particles [21]. The domains found in *F. chinensis* ProPO were Hemocyanin-N, Hemocyanin-M and Hemocynin-C, which suggested that crustacean and insect prophenoloxidases were potential members of hemocyanin gene family [21, 22]. The entire hemocyanin gene family—hemocyanin, cryptocyanin, prophenoloxidase and hexamerins—may participate at various degrees in these two vital functions of molting animals (oxygen binding and molting) [22]. We found that even if the conservative domain of ProPO and hemocyanin shared high identities, their amino acid sequences showed a big difference, such as Hemocyanin (GenBank accession number CAB85965.1, CAA51880.1) and ProPO of *L. vannamei* (GenBank accession number AAW51360.1, ABX76968.1, ABL10871.1). Therefore, even if the primers and probe designed using the sequence from the hemocyanin domain, ProPO primers and probe were still specific for ProPO and would not detect the hemocyanin cRNA in real time PCR.

Phylogenetic analysis suggested that *F. chinensis* ProPO together with other shrimp prophenoloxidases (*P. semisulcatus*, *P. monodon*, *L. vannamei* and *M. japonicus*) were clustered into the same subgroup. ProPOs from lobsters (*H. americanu* and *H. gammarus*) and crayfish (*P. clarkia* and *P. leniusculus*) were placed into another subgroup. *Branch 2* of ProPOs from two crabs (*S. serrata* and *C. magister*).

In this experiment, the mRNA expression analysis showed that the expression of ProPO in haemocytes was the highest among these three experimental tissues, which was consistent with previous reports [4, 9, 10, 23]. The distributions of ProPO were studied in three shrimp species [23–26] and two crab species [27, 28]. *P. monodon* was reported that ProPO mRNA is synthesized in the hemocytes and not in the hepatopancreas [23], so did the giant freshwater prawn [10]. In *L. vannamei,* ProPO expression were

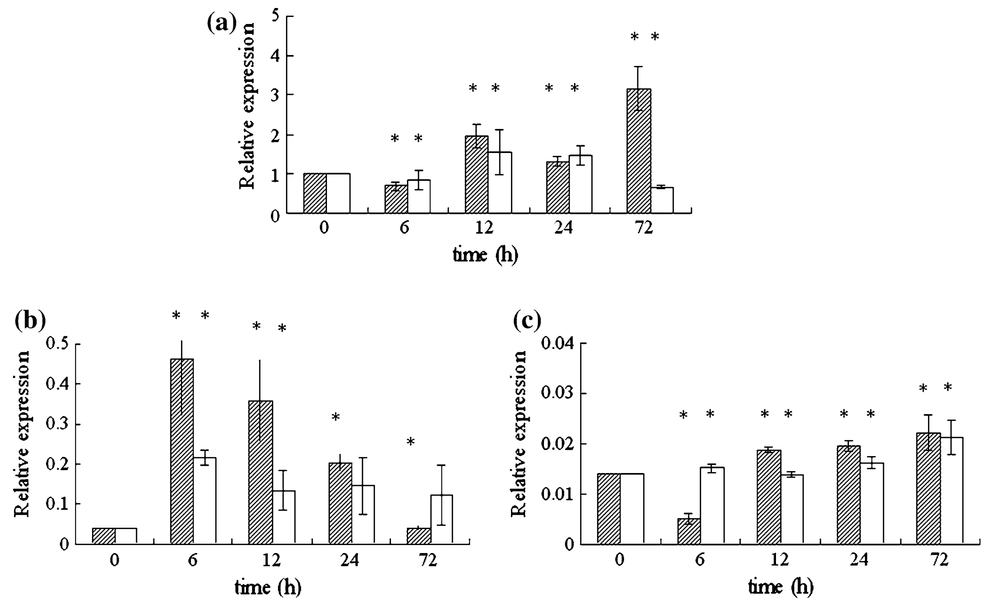

Fig. 5 The mRNA level expression of *ProPO* at different infection time in the three different tissues at 0, 6, 12, 24, 72 hpi. The columns with grids are the infection groups, the blank columns are control groups. (**a**) Expression of *ProPO* in haemocytes, (**b**) Expression of *ProPO* in lymphoid organ, and (**c**) Expression of *ProPO* in hepatopancreas

widely detected in haemocytes, gill, heart, lymphoid organ, stomach, midgut, anterior midgut caecum and ganglion. A lower expression level was found in hepatopancreas, muscle and cuticular epidermis. And ProPO transcripts, however, were also detected in non-haemocyte cells, including F and E cells of the hepatopancreas, epithelium of stomach and anterior midgut caecum [24–26]. The ProPO of mud crab *Scylla serrata* was strongly expressed in haemocytes, but not in heart, eyestalk, gill, muscle, ovary, hepatopancreas, stomach, and intestine [27]. But in Chinese mitten crab the mRNA transcripts of EsProPO and PO specific activities were detected in all the examined tissues with the highest level in hepatopancreas [28].

The mRNA levels of ProPO were detected as lower in haemocytes after *V. anguillarum* injection 6 hpi, then increased at 12 hpi and reached the highest level at 72 hpi. In the lymphoid organ, the ProPO level was up-regulated to fourfold of normal at 6 hpi and dropped slowly to normal level at 72 hpi. All the control groups in three tissues had slighter change than that in the infection groups at all infection time, which implied that *V. anguillarum* injection stimulated a remarkable increase of *ProPO* mRNA level in haemocytes and in lymphoid organ. Our observation is also to an earlier report in white shrimp *L. vannamei* [29] The *ProPO* level in the *V. anguillarum*-injected animals appeared that crustaceans rely primarily on their innate immune response to protect themselves from a variety of pathogens. Therefore, the data suggest that the animals try to clear the infected invader at early hours after injection and there is an up regulation of *ProPO* expression. As time progresses, the host defense and *ProPO* expression can be significantly down-regulated due to infection.

Further studies are currently underway to investigate *ProPO* protein expression in vivo, under stimulation by *V. anguillarum* and by chemically modified siRNA, to determine whether the proteins expression level is consistent with the mRNA level.

Acknowledgements This work was supported by grants from Major State Basic Research Development Program of China (973 program): 2006CB101804 and National High-Tech Research and Development Program of China (863 program) 2006AA09Z424.

References

1. Sindermann CJ (1984) Disease in marine aquaculture. Helgol Meeres 37:505–532
2. Flegel TW, Alday-Sanz V (1998) The crisis in Asian shrimp aquaculture. Current status and future needs. J Appl Ichthyol 14:269–273. doi:10.1111/j.1439-0426.1998.tb00654.x
3. Argue BJ, Arce SM, Lotz JM et al (2002) Selective breeding of pacific white shrimp (*Litopenaeus vannamei*) for growth and resistance to Taura Syndrome Virus. Aquaculture 204:447–460. doi:10.1016/S0044-8486(01)00830-4
4. Cerenius L, Söderhäl Kl (2004) The prophenoloxidase-activating system in invertebrates. Immunol Rev 198:116–126. doi:10.1111/j.0105-2896.2004.00116.x
5. Chuo CP, Liang SM, Sung HH (2005) Signal transduction of the prophenoloxidase activating system of prawn haemocytes triggered by CpG oligodeoxynucleotides. Fish Shellfish Immunol 18:149–162. doi:10.1016/j.fsi.2004.06.009
6. Wang R, Lee SY, Cerenius L et al (2001) Properties of the prophenoloxidase activating anzyme of the freshwater crayfish, *Pacifastacus leniusculus*. Eur J Biochem 268:895–902. doi:10.1046/j.1432-1327.2001.01945.x
7. Söderhäll I, Bangyeekhun E, Mayo S et al (2003) Hemocyte production and mature in an invertebrate animal; proliferation and gene exppression in hematopoietic stem cells of *Pacifastacus leniusculus*. Dev Comp Immunol 27:661–672. doi:10.1016/S0145-305X(03)00039-9
8. Sung HH, Yang YL, Song YL (1996) Enhancement of microbicidal activity in the tiger shrimp *Penaeus monodon* via immuno stimulation. J Crust Biol 16:278–284. doi:10.2307/1548883

9. Laia CY, Chenga W, Kuob CM (2005) Molecular cloning and characterisation of prophenoloxidase from haemocytes of the white shrimp. *Litopenaeus vannamei*. Fish Shellfish Immunol 18:417–430. doi:10.1016/j.fsi.2004.10.004
10. Liu CH, Tseng DY, Lai CY (2006) Molecular cloning and characterisation of prophenoloxidase cDNA from haemocytes of the giant freshwater prawn, *Macrobrachium rosenbergii*, and its transcription in relation with the moult stage. Fish Shellfish Immunol 21:60–69. doi:10.1016/j.fsi.2005.10.004
11. Cheng W, Chen JC (2001) Effects of intrinsic and extrinsic factors on the haemocyte profile of the prawn, *Macrobrachium rosenbergii*. Fish Shellfish Immunol 11:53–63. doi:10.1006/fsim.2000.0293
12. Cheng W, Juang FM, Li JT et al (2003) The immune response of the giant freshwater prawn *Macrobrachium rosenbergii* and its susceptibility to *Lactococcus garvieae* in relation to the moult stage. Aquaculture 218:33–45. doi:10.1016/S0044-8486(02)00415-5
13. Liu CH, Yeh ST, Cheng SY et al (2004) The immune response of the white shrimp *Litopenaeus vannamei* and its susceptibility to Vibrio infection in relation with the moult cycle. Fish Shellfish Immunol 16:151–161. doi:10.1016/S1050-4648(03)00058-5
14. Humberto MR, de Unidad PM (2004) A review of extracellular virulence product of *Vibrio* species important in diseases of cultivated shrimp. Aquac Res 35:1395. doi:10.1111/j.1365-2109.2004.01165.x
15. Liu FS, Liu YC, Li FH et al (2005) Molecular cloning and expression profile of putative antilipopolysaccharide factor in Chinese shrimp (*Fennerropenaeus chinensis*). Mar Biotechnol 7 (6):600–608. doi:10.1007/s10126-005-5006-4
16. Zhang QL, Li FH, Wang B et al (2007) The mitochondrial manganese superoxide dismutase gene in Chinese shrimp *Fenneropenaeus chinensis*: cloning, distribution and expression. Dev Comp Immunol 31:429–440. doi:10.1016/j.dci.2006.08.005
17. Yang CJ, Zhang JQ, Li FH et al (2008) A toll receptor from Chinese shrimp *Fenneropenaeus chinensis* is responsive to *Vibrio anguillarum* infection. Fish Shellfish Immunol. doi:10.1016/j.fsi.2007.12.012
18. Wang B, Li F, Dong B et al (2006) Discovery of the genes in response to white spot syndrome virus (WSSV) infection in *Fenneropenaeus chinensis* through cDNA microarray. Mar Biotechnol 8:491–500. doi:10.1007/s10126-005-6136-4
19. Dong B, Liu FS, Xiang JH et al (2005) Expression profile of penaeidins from *F. chinensis* in response to vibrio and WSSV infection by real time PCR. Acta Oceanol Sin 24(2):131–140
20. Westenberg M, Heinhuis B, Zuidema D et al (2005) siRNA injection induces sequence-independent protection in *Penaeus monodon* against white spot syndrome virus. Virus Res 114:133–139. doi:10.1016/j.virusres.2005.06.006
21. Hughes AL (1999) Evolution of the arthropod prophenoloxidase/hexamerin protein family. Immunogenetics 49(2):106–114. doi:10.1007/s002510050469
22. Terwilliger NB, Dangott L, Ryan M et al (1999) A crustacean molting protein: evolutionary link with arthropod hemocyanins and insect hexamerins. Proc Natl Acad Sci USA 96(5):2013–2018. doi:10.1073/pnas.96.5.2013
23. Sritunyalucksana K, Cerenius L, Söderhäll K (1999) Molecular cloning and characterization of prophenoloxidase in the black tiger shrimp, *Penaeus monodon*. Dev Comp Immunol 23(3):179–186. doi:10.1016/S0145-305X(99)00005-1
24. Wang YC, Chang PS, Chen HY (2006) Tissue distribution of prophenoloxidase transcript in the Pacific white shrimp *Litopenaeus vannamei*. Fish Shellfish Immunol 20(3):414–418. doi:10.1016/j.fsi.2005.05.003
25. Wang YC, Chang PS, Chen HY (2007) Tissue expressions of nine genes important to immune defence of the Pacific white shrimp *Litopenaeus vannamei*. Fish Shellfish Immunol 23 (6):1161–1177. doi:10.1016/j.fsi.2007.04.004
26. Lai CY, Cheng W, Kuo CM (2005) Molecular cloning and characterisation of prophenoloxidase from haemocytes of the white shrimp, *Litopenaeus vannamei*. Fish Shellfish Immunol 18 (5):417–430. doi:10.1016/j.fsi.2004.10.004
27. Ko CF, Chiou TT, Vaseeharan B et al (2007) Cloning and characterisation of a prophenoloxidase from the haemocytes of mud crab *Scylla serrata*. Dev Comp Immunol 31(1):12–22. doi:10.1016/j.dci.2006.05.002
28. Gai YC, Zhao JM, Song LS et al (2008) A prophenoloxidase from the Chinese mitten crab *Eriocheir sinensis*: gene cloning, expression and activity analysis. Fish Shellfish Immunol 24 (2):156–167. doi:10.1016/j.fsi.2007.08.006
29. Chiu CH, Guu YK, Liu CH et al (2007) Winton Cheng immune responses and gene expression in white shrimp, *Litopenaeus vannamei*, induced by *Lactobacillus plantarum*. Fish Shellfish Immunol 23(2):364–377. doi:10.1016/j.fsi.2006.11.010