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

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Abstract

Expression of prophenoloxidase (proPO) cDNA was determined from haemocytes of the giant freshwater prawn *Macrobrachium rosenbergii* by a reverse-transcription polymerase chain reaction (RT-PCR) and rapid amplification of cDNA using oligonucleotide primers based on the proPO sequence of tiger shrimp *Penaeus monodon*, freshwater crayfish *Pacifastacus leniusculus*, green tiger shrimp *Penaeus semisulcatus*, kuruma shrimp *Marsupenaeus japonicus*, and white shrimp *Litopenaeus vannamei*. The proPO of *M. rosenbergii* was constitutively expressed. The 2547-bp cDNA contained an open reading frame (ORF) of 2013 bp, a 96-bp 5' untranslated region, and a 438-bp 3' untranslated region containing the poly A tail. The molecular mass of the deduced amino acid (aa) sequence (671 aa) was 76.7 kDa with an estimated pI of 7.05. It contained putative copper-binding sites, a complement-like motif (GCGWPRHM), a proteolytic activation site, and a conserved C-terminal region common to all known proPOs. However, no signal peptide sequence was detected in giant freshwater prawn proPO. Comparison of amino acid sequences showed that prawn proPO is similar to the proPO of penaeid, crayfish and lobster. Prawn proPO was only synthesised in haemocytes. The proPO transcript was significantly increased in the A stage and achieved the highest level in the B stage, and then declined sharply in the C stage and reached the lowest level in the D2/D3 stage.

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Keywords: Prophenoloxidase; *Macrobrachium rosenbergii*; Nucleotide sequence; Copper-binding motifs; Moult stages; Transcription
1. Introduction

The giant freshwater prawn *Macrobrachium rosenbergii* is commercially important as a cultured species in Taiwan where it is intensively farmed [1]. Outbreaks of diseases caused by yeast infections in the cool season [2] and *Lactococcus garvieae* in the hot season have dramatically reduced production of farmed prawns in Taiwan [3,4]. The farmed production of freshwater prawn reached a maximum of 16,196 tons in 1991, but decreased thereafter, and was 10,045 tons in 2003 in Taiwan.

Invertebrates, including crustaceans, do not have acquired immunity; instead they have an innate immune system, which includes melanisation by activation of the prophenoloxidase-activating (ppA) system, a clotting process, phagocytosis, encapsulation of foreign material, antimicrobial action, and cell agglutination [5]. The prophenoloxidase (proPO) system is considered a constituent of the immune system and forms an important part of an immunorecognition process of the defence mechanism, and can specifically be activated by extremely low quantities of microbial cell components, such as β-1,3-glucans, lipopolysaccharide (LPS), and peptidoglycan (PG) [6–8]. In crustaceans, proPOs have been demonstrated to be confined to haemocyte granules [9]. In addition, Ca²⁺ is required for the conversion of the proPO-activating enzyme (ppAE) to an active proteinase that transforms proPO to active phenoloxidase (PO) [10]. The major enzyme produced during proPO system activation is PO, which is necessary for the melanisation process observed in response to infection, and which occurs in cuticular wounds or nodules and capsule formation around invading parasites [11–13]. Melanisation is induced by a complex enzymatic cascade which involves activation of proPO to PO [13,14]. PO is a copper-containing oxidase that catalyses the oxidation of phenolic substances such as L-3,4-dihydroxyphenylalanine (L-DOPA) to quinones which are then further polymerised non-enzymatically to the black pigment, melanin [15].

Biochemical studies on the crustacean proPO system have been carried out in *Farfantepenaeus californiensis* [10,16,17], *Farfantepenaeus paulensis* [18], *Litopenaeus stylirostris* [19], and *Penaeus monodon* [20]. The proPO has been purified and characterised from *Pacificastacus leniusculus* [21] and *F. californiensis* [10] haemocytes, and the molecular masses were 76 and 114 kDa, respectively. Recently, full-length proPO cDNA has also been cloned and characterised in the tiger shrimp *P. monodon* [22], green tiger shrimp *Penaeus semisulcatus* (accession no.: AF521949), kuruma shrimp *Marsupenaeus japonicus* (accession no.: AB065371), and white shrimp *Litopenaeus vannamei* [23]. All of these proPOs contain two functional copper-binding sites, a complement-like motif, a proteolytic activation site, and lack a signal peptide.

Several immune parameters including haemocyte counts, PO activity, respiratory bursts (production of superoxide anion), phagocytic activity and clearance efficiency, and the susceptibility to pathogens in relation with the moult cycle have been reported in crustaceans [24–26]. The total haemocyte count (THC) measured at the D3 stage was significantly lower than those observed at the C and B stages [24], whereas PO activity was significantly higher in the C stage than among the A, B, D1/D2, and D3 stages for *M. rosenbergii* [25].

The aim of the present study is to present the cDNA cloning data of proPO isolated from the haemocytes of *M. rosenbergii*, to compare its sequence with other known decapod proPOs, establish the site of proPO synthesis in prawn, and evaluate proPO expression in haemocytes of *M. rosenbergii* in relation to moult stage.

2. Materials and methods

2.1. Experimental design

There are three moult stages — postmoult, intermoult, and premoult — which can be distinguished by the degree of hardness of the exoskeleton. Each moult stage is divided into several substages according to the retraction of the epithelium within setae of the antennal scale [27]. They are the following: (1) A and B for the postmoult; (2) C for the intermoult; and (3) D₀, D₁, D₂, and D₃ for the premoult. Five moult stages (A, B, C, D₀/D₁, and D₂/D₃) were used to examine proPO expression in prawns in this study.

About 300 prawns of *M. rosenbergii* obtained from a commercial farm in Pingtung, Taiwan, were acclimated in running-water tanks at 27 ± 1 °C and fed with a commercial prawn diet (Shinta Feed, Pingtung, Taiwan) daily for 2 weeks before experimentation. Only prawns in the intermoult stage were used for the study of the proPO sequence, and those weighed 24.2–29.5 g. For the study of proPO expression in relation to moult stage, six prawns from each stage were sampled, and those weighed 14.3–17.6 g.
2.2. Haemocyte collection

Haemocyte was individually withdrawn according to the method of previous description [23] except using glucose to adjust the osmolality to 490 mOsm kg⁻¹.

2.3. Total RNA isolation and reverse transcription (RT)

Total RNA was isolated and further purified using the guanidinium thiocyanate method [28] as described previously [23].

2.4. PCR and subcloning of proPO cDNA

Full-length proPO cDNA of *M. rosenbergii* was obtained by the procedures of reverse-transcription polymerase chain reaction (RT-PCR) and rapid amplification of cDNA (RACE). Amplification primer pairs for *M. rosenbergii* proPO cDNA were designed based on the highly conserved nucleotides of proPO of the known crustaceans and are shown in Table 1. The relative position and direction of each primer are shown in Fig. 1. The details of the procedures were described previously [23].

2.5. Nucleotide sequence analysis

The methods for the nucleotide sequence analysis and comparison were described previously [23].

2.6. Phylogenetic analysis

Phylogenetic trees were constructed on the basis of the proportion of aa differences (p-distance) by the Neighbour-joining method [29] using MEGA 2 software [30]. For the construction of the phylogenetic tree, indels were removed from the multiple alignments. The reliability of the obtained tree was assessed by bootstrapping, using 1000 bootstrap replications [31].

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5’ → 3’)</th>
</tr>
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<tbody>
<tr>
<td>proPO1F</td>
<td>CACCAYTGCCAYTGCCAYTGYTSRTNTAYCC</td>
</tr>
<tr>
<td>proPO1R</td>
<td>WRGNYGTGCMRGCGGTTAGAASTRNGGGTC</td>
</tr>
<tr>
<td>proPO2F</td>
<td>GTGCATCTCTCTCTCTCTCT</td>
</tr>
<tr>
<td>proPO2R</td>
<td>CATGTCATACCTGGCCAGCAC</td>
</tr>
<tr>
<td>proPO3F</td>
<td>CCTCATCTCTCTAGCCACGA</td>
</tr>
<tr>
<td>proPO3R</td>
<td>NTTRARCCCATVGCGWGBYRTCBG</td>
</tr>
<tr>
<td>proPO5’1R</td>
<td>GGTTCGGAATGATGACGA</td>
</tr>
<tr>
<td>proPO5’2R</td>
<td>TTGCGTGCTCTAGTGAAGTAT</td>
</tr>
<tr>
<td>AAP</td>
<td>GGCCACCGCTCGACTAGTAC</td>
</tr>
<tr>
<td>AUAP</td>
<td>GGCGGACGACGGAGGTAC</td>
</tr>
<tr>
<td>proPO3’1F</td>
<td>CACCAGTGGAATTCTAGTAAT</td>
</tr>
<tr>
<td>proPO3’2F</td>
<td>CAAGTCATCCGACGGACGGCT</td>
</tr>
<tr>
<td>PT1</td>
<td>AGTCAACTTTACACTTTTTTTTTT</td>
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<tr>
<td>PT2</td>
<td>CGTTCACCATTTTCAGCT</td>
</tr>
<tr>
<td>Q-PO-F</td>
<td>ACCGTGAAGGACATAAGGGCGAGAT</td>
</tr>
<tr>
<td>Q-PO-R</td>
<td>AGTACGTTCCAAGTCGAGATGCT</td>
</tr>
<tr>
<td>GSPPOF</td>
<td>GGAAGGTTTTTCTCCGT</td>
</tr>
<tr>
<td>GSPPOR</td>
<td>GGAAGGTTTTTCTCCGT</td>
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<tr>
<td>β-ActinF</td>
<td>TAGTGGTGCTCTCGTGAGTCG</td>
</tr>
<tr>
<td>β-ActinR</td>
<td>GAGACCTTACACACCCCGC</td>
</tr>
</tbody>
</table>

Y, C/T; S, G/C; R, A/G; N, A/T/C/G; W, A/T; M, A/C; K, G/T; V, A/C/G; and B, G/T/C.
The following proteins were used in the alignment: *P. monodon* proPO [22], *P. leniusculus* proPO [32], *L. vannamei* proPO [23], *P. semisulcatus* proPO (accession no.: AF521949), *M. japonicus* proPO (accession no.: AB0733223), *H. gammarus* proPO (accession no.: AJ581662), *H. americanus* proPO (accession no.: AY655139), *L. vannamei* haemocyanin (accession no.: X82502) and *P. leniusculus* haemocyanin (accession no.: AF522504).

2.7. Tissue expression analysis

Expression of proPO mRNA in haemocytes, the hepatopancreas, and muscle was demonstrated by RT-PCR as described previously [23]. Two specific primers of GSPPOF and GSPPOR were used, and the primers, β-actinF and β-actinR (Table 1), were used to amplify the β-actin fragment that was used as a positive control. The PCR reaction was the same as that described previously [23].

2.8. Preparation of a standard curve for proPO mRNA

The absolute real-time standard curve of the proPO gene was prepared according to a previous study [33]. Briefly, using the plasmid vector containing *M. rosenbergii* proPO cDNA as a template with the addition of SP6 RNA polymerase (Promega), proPO RNA was transcribed. After transcription, the sample was digested by the addition of RNase-free DNase, and the RNA was accurately quantified with a spectrophotometer at 260 nm. RNA dilutions in DEPC—water were used as RNA standards. The RNA was reverse-transcribed as described above and stored at −20 °C until used for real-time RT-PCR.

2.9. Quantification of proPO gene expression by real-time RT-PCR

The mRNA expression of proPO in haemocytes of prawn from different moult stages was measured by real-time RT-PCR. The cDNA and proPO standards were used for the assay of real-time RT-PCR. The SYBR Green I real-time RT-PCR assay was carried out in an ABI PRISM™ 7900 Sequence Detection System (Perkin—Elmer Applied Biosystems). The amplifications were performed in a 96-well plate in a 25-μl reaction volume containing 12.5 μl of 2× SYBR Green Master Mix (PE Applied Biosoysts), 2.5 μl (each) Q-PO-F and Q-PO-R primers (10 μM).
(Table 1), 1 µl of the template (1 µg cDNA of haemocytes or different concentrations of the proPO RNA standard), and 9 µl of DEPC—water. The thermal profile for the SYBR Green I real-time RT-PCR was 50 °C for 2 min and 95 °C for 10 min followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. In a 96-well plate, each sample was analysed in duplicate. DEPC—water replaced the template as the negative control.

Data analysis of RT-PCR was performed with the SDS software version 2.0 (Perkin—Elmer Applied Biosystems). Linear relationships among Ct, the threshold PCR cycle, and different log concentrations of the proPO RNA standard were tested using the General Linear Model Procedure and the Regression Procedure, version 6.03, SAS (Statistical Analysis System, Cary NC, USA) computer software. Different ΔCt values of proPO were calculated to the actual concentrations of proPO mRNA in total RNA based on the proPO standard curve.

2.10. Statistical analysis

A multiple comparison (Tukey) test was conducted to compare the significant differences of proPO gene expression among different moult stages. A significance level of \( p = 0.05 \) was chosen.

3. Results

3.1. RT-PCR and cDNA cloning

Using RT-PCR and RACE, a full-length 2547 bp of proPO was obtained from the haemocytes of *M. rosenbergii*. It contained an open reading frame (ORF) of 2013 bp, 96 bp of the 5’-untranslated region, 438 bp of the 3’-untranslated region, and coded a protein of 671 aa with the putative initiation methionine codon (ATG) beginning at nucleotide 97 and the stop codon beginning at nucleotide 2109. The calculated molecular mass was 76.7 kDa, and the estimated pl of this protein was 7.05. No hydrophobic signal was present in the proPO sequence. The putative trypsin cleavage sites were detected close to the N-terminus of prawn proPO, and Arg70—Ser71 was suggested to be the cleavage site for activation of prawn proPO. A thiol ester region-like motif (GCGWPRHM) and five potential N-linked glycosylation sites were also present in *M. rosenbergii* proPO (GenBank accession no.: DQ182596).

Sequence analysis with the BLAST algorithm showed that the deduced aa sequence of *M. rosenbergii* proPO exhibited similarities with those of proPO of *P. monodon* (55%), *P. semisulcatus* (56%), *M. japonicus* (55%), *L. vannamei* (55%), *P. leniusculus* (55%), *H. americanus* (56%), and *H. gammarus* (56%), but had lower similarities with the haemocyanin of *L. vannamei* (29%) and *P. leniusculus* (29%). Comparison of the deduced amino acid sequence of copper-binding sites of *M. rosenbergii* proPO with penaeid and crayfish proPOs and haemocyanin showed that the putative copper-binding domain containing six histidines was also observed in *M. rosenbergii* proPO (Fig. 2).

A molecular phylogenetic tree was constructed to further analyse the evolutionary relationships among crustacean proPOs and haemocyanin (Fig. 3). Based on the MEGA 2 analysis, members of the proPO and haemocyanin protein form a statistically supported parallel evolution between proPOs and haemocyanin of crustaceans. Crustacean proPOs can be classified into two subgroups: one containing *P. monodon*, *P. semisulcatus*, *M. japonicus*, and *L. vannamei* proPO; and the other containing *P. leniusculus*, *H. gammarus*, and *H. americanus* proPO. The genetic distances among the penaeid proPO clade were rather short. However, the proPO of *M. rosenbergii* was isolated from the two subgroups.

3.2. Localisation of proPO

Tissue expression of proPO in haemocytes, the hepatopancreas, and muscle of *M. rosenbergii* is shown in Fig. 4. The proPO cDNA was only detected in haemocytes.

3.3. Expression of prawn proPO at different moult stages

The proPO mRNA transcripts were measured in haemocytes of prawns from different moult stages using real-time RT-PCR. The proPO mRNA expression of prawns is significantly upregulated in the A stage, and achieved the highest level in the B stage, and was then sharply downregulated in the C stage and reached the lowest in the D2/D3 stage.
However, no significant difference was observed in proPO mRNA transcription for prawns between the D0/D1 and D2/D3 stages (Fig. 5A, B).

4. Discussion

Localisation of proPO in different species has been controversial. In crustaceans, proPO exists in the haemocytes in an inactive form and is activated by microbial cell wall constituents [18,21,23]. The monomer proPO has a mass of about 70–80 kDa, and phenoloxidase (PO) has a mass of 60–70 kDa [32,34–36]. Lai et al. [23] indicated that the deduced L. vannamei proPO aa sequence showed that white shrimp proPO is more closely related to the proPO of known penaeid (with similarities of 77%–88%) than to that of a freshwater crayfish (with a similarity of 60%).

Fig. 2. Alignment of two copper-binding sites (A and B) amino acid sequences of crustacean proPO and shrimp haemocyanin. Six highly conserved histidine residues are shown in black reverse print and italicised letters (H). MRPP0, Macrobrachium rosenbergii proPO; LVPP0, Litopenaeus vannamei proPO; PLPP0, Pacifastacus leniusculus proPO; PMPP0, Penaeus monodon proPO; PSPP0, Penaeus semisulcatus proPO; MJPP0, Marsupenaeus japonicus proPO; and LVHC, L. vannamei haemocyanin.

However, no significant difference was observed in proPO mRNA transcription for prawns between the D0/D1 and D2/D3 stages (Fig. 5A, B).

Fig. 3. A molecular phylogenetic tree of crustacean proPOs and haemocyanin based on the Neighbour-joining method with values for each internal branch determined by bootstrap analysis with 1000 replications. Values indicate percentages along the branch. HGPPO, Homarus gammarus proPO; HAPPO, H. americanus proPO; and PLHC, P. leniusculus haemocyanin.
In the present study, 2547-bp cDNA from haemocytes of *M. rosenbergii* was obtained, and the calculated molecular mass was 76.6 kDa, and the cloned proPO cDNA showed a similarity with those of the penaeids *P. monodon* (55%), *P. semisulcatus* (56%), *M. japonicus* (55%) and *L. vannamei* (55%), and to the crayfish *P. leniusculus* (55%), as well as to the lobsters *H. americanus* (56%) and *H. gammarus* (56%). Cloned prawn proPO has only 29% and 29% similarities with *P. leniusculus* and *L. vannamei* haemocyanin sequences, respectively. These results clearly associate the proPO genes isolated here with other crustacean proPOs.

All arthropod proPOs being closely related to arthropod haemocyanin have been well reviewed, according to their similar sequences, which contain two copper-binding motifs [37]. The two copper ions of haemocyanin are coordinated by six histidines and reversibly bind a dioxygen molecule in a side-on co-ordination [38,39]. The six-histidine residues within the two copper-binding motifs of proPO are highly conserved in all arthropod proPOs, including shrimp proPO [23,37]. The putative trypsin cleavage site was detected close to the N-terminus of *M. rosenbergii* proPO, and Arg70—Ser71 is suggested to be a cleavage site for activation of *M. rosenbergii* proPO, since it can be aligned to the corresponding cleavage site of other arthropod proPOs [23,37].

In recent years, haemocyanin was demonstrated to convert its function to phenoloxidase [40,41]; this suggests that the origin of arthropod haemocyanins is from ancient prophenoloxidase-like proteins [42]. In the present study, members of the proPO and haemocyanin protein form a statistically supported parallel evolution between proPOs and haemocyanin, and appear to have evolved from a common ancestor in crustaceans. When haemocyanin was omitted from the analysis, *M. rosenbergii* proPO lays at the root of the proPO cluster, suggesting that the primordial proPO of *M. rosenbergii* was a founding member of the proPO family in crustaceans. The present study indicated that the proPO of *M. rosenbergii* was isolated from penaeid, and crayfish and lobster subgroups. The fact suggests that crustacean proPOs may be classified into at least three subgroups. Further research on the other *Macrobrachium* species proPOs and haemocyanin gene sequences is required.

In α2-macroglobulin and the complement proteins of C3 and C4, the thiol ester-like motif (GCGEQNM) is responsible for binding to other macromolecules, and the site (GCCWPQHM) was observed in proPO of *P. monodon* [22], *P. leniusculus* [32], and *L. vannamei* [23], and the site was also observed in proPO of *M. rosenbergii* (GCCWPRHM) in this study. The thiol group of cysteine displaces the amide group on glutamine at this site in native proteins. Upon proteolytic cleavage, the site is exposed, and the exposed thiol ester of the vertebrate complement becomes highly

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active in order to attack the hydroxyl or amino group resulting in the formation of amides and esters. In general, this process may react with biological surfaces to form ester or amide bonds causing the immobilisation of these molecules onto foreign cell surfaces [22,32].

Sritunyalucksana and Söderhäll [37] indicated that results of Northern blot analysis with a $^{32}$P-labelled cDNA probe, which was designed to cover the N'-terminal part and the first copper-binding site, showed that proPO was synthesised only in haemocytes and not in the hepatopancreas. Using RT-PCR with the specific primer pair, the proPO was synthesised only in haemocytes, and it was not present in the muscle or hepatopancreas of $L.\ vannamei$ [23] or $M.\ rosenbergii$ in this study.

The moulting cycle is one of the main intrinsic factors affecting the immune ability of crustaceans [19,25,26]. Among the A, B, C, D$_1$/D$_2$, and D$_3$ stages of $M.\ rosenbergii$, the THC was highest in the C stage and lowest in the
D_3 stage [24], while the PO activity was significantly higher in the C stage and lower in the A, B, D_0/D_1, and D_3 stages [25]. For patterns of haemocyte production and release throughout the moult cycle in the penaeid shrimp *Sicyonia ingentis*, Hose et al. [43] indicated that large number of maturing haemocytes including hyaline and granular haemocytes were channelled into the vessel lumens immediately after molting. Recently, the mRNA of proPO of *P. leniusculus* was detected by in situ hybridisation with a dioxigenin-labelled cDNA probe, and it was found that proPO was not expressed in haematopoietic tissue, but haemocytes released into the circulation express proPO [44].

The present study indicated that proPO mRNA expression of *M. rosenbergii* is significantly increased in the A stage, and achieved the highest level in the B stage, and was then sharply decreased in the C stage, and reached the lowest in the D_2/D_3 stage. The facts suggested that the proPO mRNA expression was dramatically upregulated resulting from an increased release of newly synthesised haemocytes from the haematopoietic tissue, and an increase of proPO mRNA transcription and translation during the postmoult (A and B stages) [43,44]. In addition, the PO activity increased significantly in the intermoult stage of prawn [25] resulting from proPO of translation stored in haemocytes reached the highest levels. However, the mechanism of higher proPO expression at B stage and lower expression at C, D_0/D_1 and D_2/D_3 stages is still undetermined.

In conclusion, a 2547-bp proPO cDNA was cloned from haemocytes of *M. rosenbergii*. It encodes a protein of 671 aa. The deduced aa of proPO of *M. rosenbergii* had similarities with those of penaeid, crayfish and lobster (55%–56%), but low similarities with the haemocyanin of *L. vannamei* and *P. leniusculus* (29%). Prawn proPO mRNA expression occurred in all moult stages, and increased significantly during the postmoult (A and B stages), and decreased sharply during the intermoult (C stage) and premoult (D stage), and is thus considered to be immunomodulatory.

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