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# Cloning and characterisation of a prophenoloxidase from the haemocytes of mud crab *Scylla serrata*

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**Abstract**

A prophenoloxidase (proPO) cDNA was cloned from the haemocytes of mud crab *Scylla serrata* using oligonucleotide primers and RT-PCR. Both 3′- and 5′-regions were isolated by rapid amplification of cDNA end (RACE) method. Analysis of the nucleotide sequence revealed that the cDNA clone has a full length of 2663 bp, with an open reading frame of 2019 bp, a 124-bp 5′-untranslated region, and a 520-bp 3′-untranslated region containing a poly A signal. It encodes a protein of 673 amino acids with a predicted molecular weight of 77.5 kDa and with an estimated p*I* of 5.96. It contains two putative tyrosinase copper-binding motifs with six histidine residues (copper A, 185, 189, 211, and copper B, 346, 350, 386). The proPO has thiol-ester-like motif (GCGWPQHM), which showed similar structural features of proPOs from other decapod crustaceans. It also contains five possible glycosylation sites, and a conserved C-terminal region common to all known proPOs. Sequence comparison showed that the proPO-deduced amino acid of mud crab *S. serrata* has an overall similarity of 78%, 57%, 56%, 51–55%, 54%, 53%, 52%, 52%, and 52% to that of Dungeness crab *Cancer magister*, American lobster *Homarus americanus*, European lobster *Homarus gammarus*, kuruma prawn *Marsupenaeus japonicus*, crayfish *Pacifastacus leniusculus*, white shrimp *Litopenaeus vannamei*, tiger shrimp *Penaeus monodon*, green tiger shrimp *Penaeus semisulcatus*, and giant freshwater prawn *Macrobrachium rosenbergii*, respectively. The proPO was strongly expressed in haemocytes, but not in heart, eyestalk, gill, muscle, ovary, hepatopancreas, stomach, and intestine. The proPO transcript of mud crab *S. serrata* increased significantly in 12 and 24 h post-lipopolysaccharide (LPS) injection, but returned to the original values in 72 h post injection.


*Keywords:* Mud crab; *Scylla serrata*; Prophenoloxidase; Immunity; LPS injection

## 1. Introduction

Mud crab also known as mangrove crab, belonging to the genus *Scylla* is distributed throughout the west Pacific and Indian Oceans. Among the four species of mud crab that have been described to date, *Scylla serrata* is the most common [1]. *S. serrata* spends its juvenile phase in brackish water, then moves to the sea during the adult period to spawn and subsequently releases zoeae into seawater [2]. This species has been cultured throughout the Indo-Pacific regions for more than 30 years [3].

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Several viral diseases and vibriosis caused by *Vibrio parahaemolyticus* have been reported to infect crab [4,5]. These pathogenic viruses and bacteria infected the haemocytes and epithelial cells, and are associated with mass mortalities during disease outbreaks.

It is known that invertebrates lack a true adaptive immune system, and rely instead on innate responses against invading pathogens. Once pathogens-like bacteria or virus enter the hemocoel of the host, they encounter a complex system of innate defense mechanisms. First, it initiates the prophenoloxidase (proPO) -activating system that leads proPO to active form phenoloxidase (PO) by an endogenous trypsin-like serine proteinase, so called prophenoloxidase-activating enzyme (ppA) resulting in melanisation through a complex enzymatic cascade in the presence of several microbial wall components like $\beta$-1,3-glucan, lipopolysaccharide (LPS) and peptidoglycan [6,7]. In addition, proteinase inhibitors like pacifastin and $\alpha$2-macroglobulin play an important role in controlling and regulating the proPO system to avoid the deleterious effects of its active component, PO, which can produce highly toxic intermediates like melanin [8]. Recently, serine proteinase and $\alpha$2-macroglobulin have been cloned from the mud crab *S. serrata* [9,10]. However, none is known on the cDNA cloning of other proteins involved in proPO system like proPO, peroxinectin, and pacifastin.

proPO has been isolated and purified from the freshwater crayfish *Pacifastacus leniusculus* [11], yellowleg shrimp *Farfantepenaeus californiensis* [12] and Japanese stone crab *Charybdis japonica* [13]. proPO has been cloned form the crayfish *P. leniusculus* [14], giant freshwater prawn *Macrobarchium rosenbergii* [15,16], giant tiger shrimp *Penaeus monodon* [17], white shrimp *Litopenaeus vannamei* [18], kuruma shrimp *Marsupenaeus japonicus* (AB065371, AB073223), green tiger shrimp *Penaeus semisculcatus* (AF521949), American lobster *Homarus americanus* (AY655139), and European lobster *H. gammarus* [19], and Dungeness crab *Cancer magister* [20].

The aim of the present study was (1) to present the nucleotide sequence of proPO from the haemocytes of *S. serrata*, and compare its sequence with other proPO, (2) to examine the expression of proPO from various tissues, and (3) to evaluate this proPO expression when *S. serrata* were injected with LPS.

## 2. Materials and methods

### 2.1. Collection and maintenance of mud crab S. serrata

*S. serrata* (200–250 g in weight) collected from a farm in Ilan, Taiwan, were acclimatised in plastic turf containing 35‰ seawater for 3 days. They were fed daily with fish or shrimp meat at 10% of body weight.

### 2.2. Degenerate primer design and strategy of propo cDNA cloning

Full-length proPO cDNA of *S. serrata* was obtained by the procedures of reverse transcription polymerase chain reaction (RT-PCR) and rapid amplification of cDNA ends (RACE) method. Multiple alignment and phylogenetic comparison of proPO amino acid sequences of other decapod crustaceans were performed using the Clustal W multiple sequence alignments program [21]. Degenerate primers were designed based on the highly conserved sequences of proPO of American lobster *H. americanus* (AY655139), European lobster *H. gammarus* (AJ581662) [19], white shrimp *L. vannamei* [18], kuruma shrimp *M. japonicus* (AB065371, AB073223), tiger shrimp *P. monodon* (AF099741) [17], green tiger shrimp *P. semisulcatus* (AF521949), crayfish *P. leniusculus* (X83494) [14], and giant freshwater prawn *M. rosenbergii* (AY947400) [15] in the GenBank database [22].

### 2.3. RNA isolation and RT-PCR

Haemolymph (50 ml) was withdrawn by inserting a syringe into the sinus at the base of *S. serrata* right chelate leg into a 125 ml polyethylene tube containing 50 ml of pre-cooled (4 °C) anticoagulant (10% trisodium citrate) [23]. The diluted haemolymph was centrifuged at 500*g* at 4 °C for 20 min. The resulting haemocyte pellet was used for total RNA isolation. Total RNA was extracted using TRIzol Reagent (Invitrogen, CA, USA) according to manufacturer's recommendations. The concentration of the total RNA was estimated by measuring the absorbance at 260 nm.

For the isolation of *S. serrata* proPO cDNA fragment, total RNA (5 μg) was reverse transcribed using Superscript$^{TM}$ III RNase H$^{-}$ reverse transcriptase (Invitrogen, CA, USA) and oligo $(T)_{18}$ as the primers to obtain first-strand cDNA. The