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Cloning of prophenoloxidase from hemocytes of the blue crab, *Callinectes sapidus* and its expression and enzyme activity during the molt cycle

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ABSTRACT

The arthropods cuticle undergoes dramatic morphological and biochemical changes from being soft to hardness through each molting process. Prophenoloxidase (PPO) known as a key enzyme in the arthropod innate immune system involved in the melanization reaction, has been related with the initial shell-hardening process, specifically in the sclerotization of the protein matrix in the new cuticle. Since hemocytes have been reported as the main PPO source in arthropods, the transport of hemocyte PPO into the newly laid, soft cuticle has been proposed for shell-hardening occurring during and immediately after ecdysis. In order to define the role of hemocyte PPO in the shell-hardening of crustaceans, the full-length cDNA sequence (2806 nt) of hemocytes *PPO* of the blue crab *Callinectes sapidus* (*CasPPO-hemo*) is isolated using degenerate PCR and 5′-3′ RACE. *CasPPO-hemo* encodes a putative PPO (672 aa) showing three hemocyanin domains: N, M, and C in order and two copper binding sites (CuA & CuB). The sequence analysis identifies the putative CasPPO-hemo as zymogen which requires the cleavage at the *N*-terminus for its activation. Hemocyte extract (CasHLS) contains the PO, the activity of which depends on the *in vitro* activation of trypsin. The expression levels of *CasPPO-hemo* are kept constant during the molt cycle. The increase in the number of hemocytes at early premolt correlates with the elevated PO activity, while at late premolt, the increment in hemocyte numbers does not reflect on the PO activity. The functional importance of the changes in the levels of CasHLS-PO activity during molt cycle is discussed in relation to cuticle hardening process.

1. Introduction

As one of most important immune systems in invertebrates, prophenoloxidase (PPO) cascade plays a central role in melanization in response to the invasion of pathogens and wound sealings [1–4]. PPO is produced as an inactive form of zymogen that requires a serine protease, prophenoloxidase activating enzyme (PPAE) for its activation. Hemocytes have been known as the principal source of PPO in arthropods including crustaceans [3–8].

Together with the changes in the total number of hemocytes, the levels of *PPO* expression and enzyme phenoloxidase (PO) activity in hemocytes are often associated with physiological conditions of the crustaceans including stress by environmental factors, harvest, microbial and fungi infections [7,9–15]. Specifically, the molt cycle appears to influence not only the number but also the type of hemocytes together with PO activity, especially with respect to a molt stage dependent susceptibility to disease [13,16,17]. In these species, animals at post-molt stage had hemocyte numbers lower than those at intermolt, which also corresponded to levels of PO activity relevant to each of these molt stages.

Interestingly, the PO activity present in hemocytes may participate in the sclerotization of the initial shell-hardening process of the new cuticle of crustaceans [4,18]. This is due to the findings that the aggregation of hemocytes has been noted underneath the hypodermis of crustaceans at ecdysis and early postmolt [4,18,19]. Furthermore, bursicon, an arthropod tanning hormone, has been recently found to induce the recruitment and granulation of hemocytes beneath the hypodermis of the blue crab, *Callinectes sapidus* at ecdysis [19].

PO activities have also been measured in the cuticle of insects and crustaceans [20–24]. The cuticles undergo dramatic changes during molt cycle from softness during and after ecdysis to hardness most of the molt cycle and provide the first defensive barrier against invasive microorganisms [6,25]. More specifically,

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two insect studies show that PPOs present in the cuticle have been transported from hemolymph [26,27], while a laccase of *Tribolium castaneum*, a PO regulating the tanning of the new cuticle after ecdysis is not originated from insect hemocytes [28]. These reports suggest that POs either in hemocytes or cuticles may play a critical role in initial shell-hardening process of arthropods, the process of which resembles melanization reaction. We hypothesized that if a PO is involved in shell-hardening of crustaceans, hemocytes may be one of the sources.

To better understand the molecular mechanism(s) underlying the initial shell-hardening process after ecdysis of *C. sapidus*, we first isolated the cDNA sequence of *PPO* from hemocytes and characterized the PO activity. Herein, this paper reports the isolation of the full-length cDNA sequence of *PPO* from hemocytes of *C. sapidus* (*CasPPO-hemo*) using PCR with degenerate primers and 5′-3′ RACE and the levels of its expression during the molt cycle using a qPCR assay and of PO activity. With the changes in the levels of *CasPPO-hemo* expression, the PO activity in hemocytes and their numbers during the molt cycle, a functional role of PPO resided in hemocytes is discussed in relation to the sclerotization of the new cuticle.

Table 1
List of primer sequences that were used for the full-length cloning of *CasPPO-hemo* and qPCR assays.

Primers	5′ to 3′ nucleotide sequence
*CasPPO*dF1	TAYTGGMGNGARGAYTAYGG
*CasPPO*dF2	CAYCAYTGGCAYTGGCA
*CasPPO*dR1	CKRTCRAANGGRAANCCCAT
*CasPPO*dR2	GGCCANCCRCANCCRCA
*CasPPO*3F1	TCTTGTCTACCCTGTGGACCTTAGT
*CasPPO*5R1	TTCAGGAGCACCTGGGTCATCTGG
*CasPPO*3F2	AGCAGTGGCACAATAGGATCATGGA
*CasPPO*5R2	AGAAGGTCAATCCCACGCTTCTCAG
*CasPPO*3F3	ATTCTGCAGTCCTCCACCTCTTCATCC
*CasPPO*5R3	TTCTCCACCCTGTTCAGCCCGACACT
*CasPPO*3F4	ATGGAGAGCGAACAGAAGCAAGTG
*CasPPO*5R4	CACTTCACTGGGTGATCTGAGCAT
*CasPPO*5R5	GGTACCCACTTCAAACACAATGG
*CasPPO*3F6-QF	CACCTCTTCATCCATCACAAACTC
*CasPPO*5R6-QR	CAACCACACCCACAGAAGTTAAAG
CasAK-LF	GACTTCGGCGATGTCCACCA
CasAK-LR	CCACACCAGGAAGGTCTTGT
CasAK-QF	CTACCACAACGACAACAAGACCTTC
CasAK-QR	ACGCGCTTCTCAATCTCGTTA

QF and QR primers were used for qPCR assays.
"d" indicates the degenerate primers.

2. Materials and methods

2.1. Animals

Juvenile blue crabs, *C. sapidus* (15–30 mm carapace width, CW), were received from the blue crab hatchery [Aquaculture Research Center, the Institute of Marine and Environmental Technology (IMET), Baltimore, MD]. The animals were maintained in tanks with individual cages in aerated and recirculated artificial seawater at 22 °C and 20 ppt as described [19,30,31]. They were fed daily with a piece of squid and monitored their growth. The crabs with 75–85 mm CW were molt-staged as described [29].

2.2. Molecular cloning

The total RNA was isolated from hemocytes obtained from animals at various molt stages as describe Chung et al. and Chung & Zmora [30–32], using a QIAzol Lysis Reagent (Qiagen) following the manufacturer's protocol. The total RNA concentration and purity was estimated using a NanoDrop spectrophotometer (FisherSci). All RNA samples treated with DNase I (Fermentas) were subjected to syntheses of cDNAs using MMLV transcriptase (Fermentas) or 5′-3′ RACE cDNAs using an SMART™ cDNA synthesis kit (BD Biosciences).

Degenerate primers (Table 1) for *C. sapidus PPO* from hemocytes (*CasPPO-hemo*) were designed with the conserved regions based on the multiple sequence alignment of known crustacean PPOs listed in GenBank. A two-step PCR amplification was employed for degenerate PCR and 5′-3′ RACE as described [30,31,33–35]. In brief, the first touch-down PCR was performed using *CasPPO*d3F1 primer and universal primer (UPM, BD Biosciences) at the following PCR conditions: 94 °C, 2.5 min; 94 °C, 30 s, annealing temperature decreasing 1 °C/cycle starting at 47 °C and ending at 40 °C, 30 s (8 cycles), extension at 72 °C for 2 min; 27 cycles at 94 °C, 30 s, 48 °C, 30 s, 2 min extension, and final extension at 72 °C for 7 min. The first PCR product was used as the template for the nested PCR after being diluted 20 fold in sterilized water with a combination of *CasPPO*d3F2 and *CasPPO*d5R2 (Table 1) at PCR conditions: 94 °C, 2.5 min; 40 cycles at 94 °C, 30 s, 57 °C, 30 s, 72 °C, 1 min; final extension at 72 °C for 7 min. The nested product was electrophoresed on a 1.5% agarose gel. The band at the expected size of ~1100 bp was excised and used for cloning and sequencing as described [31]. Based on this initial sequence of *CasPPO-hemo*, gene specific primers (Table 1) for the cloning of the full-length cDNA sequence of *CasPPO-hemo* were designed.

For 5′ and 3′ RACE, a similar two-step PCR method was employed as above. Briefly, for 5′ RACE, 5′ RACE cDNA (50 ng total RNA eqv.) was amplified with *CasPPO*5R1 and UPM at the following PCR conditions: first denaturation 94 °C for 2.5 min; 94 °C, 30 s, annealing temperature decreasing 1 °C/cycle starting at 57 °C and ending at 49 °C, 30 s (8 cycles), extension at 72 °C for 2 min; 27 cycles at 94 °C, 30 s, 58 °C, 30 s, 2 min extension, and final extension at 72 °C for 7 min. This PCR product diluted 20 fold with sterilized water served as the template for the nested PCR with *CasPPO*5R2 primer and nested universal primer (NUP, BD Biosciences). The nested PCR products were treated similarly as above for cloning and sequencing. For 3′ RACE, the same procedures were carried out with 3′ RACE cDNA and specific primers for the touch-down PCR with *CasPPO*3F1 and UPM and for the nested PCR with *CasPPO*3F2 primer and NUP (Table 1).

The ORF sequence of *CasPPO-hemo* was confirmed with the amplification of cDNAs of three different crabs using a High Fidelity Tag polymerase (Bioline) and *CasPPO*3F4 and *CasPPO*5R4 (Table 1). The phylogenetic analysis was carried out with the putative amino acid sequence of 19 number of crustacean PPOs including CasPPO-hemo (MEGA 5.05) [38].

2.3. Expression analysis

The expression levels of *CasPPO-hemo* at different molt stages were estimated using a qPCR assay with Fast SYBR Green Master Mix (Applied Biosystems) on a 7500 Fast Real-Time PCR System (Applied Biosystems). The standards of *CasPPO-hemo* were prepared similarly as described in Chung et al. [35]. The primers for the qPCR assay were selected in the region specific to *CasPPO-hemo* that is located between the hemocyanin domain M and C. The data were normalized with the levels of arginine kinase (*CasAK*) expression in the same sample cDNAs [36]. The expression levels are presented as mean ± SE copies/μg total hemocyte RNA.

The spatial expression of *CasPPO-hemo* was examined using an end point PCR assay with 10 different tissue cDNAs prepared from an animal at intermolt stage and CasPPO3F4 and -5R4 (Table 1) that amplify its entire ORF (2019 nt).