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Phenoloxidase from the sea cucumber *Apostichopus japonicus*: cDNA cloning, expression and substrate specificity analysis

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ABSTRACT

Phenoloxidase (PO) is a crucial component of the immune system of echinoderms. In the present study, the full-length cDNA of PO (AjPO) was cloned from coelomocytes of the sea cucumber *Apostichopus japonicus* using 3′- and 5′-rapid amplification of cDNA ends (RACE) PCR method, which is 2508 bp, with an open reading frame (ORF) of 2040 bp encoding 679 amino acids. AjPO contains a transmembrane domain, and three Cu-oxidase domains with copper binding centers formed by 10 histidines, one cysteine and one methionine respectively. Phylogenetic analysis revealed that AjPO was clustered with laccase-type POs of invertebrates. Using the isolated membrane proteins as crude AjPO, the enzyme could catalyze the substrates catechol, L-3,4-dihydroxyphenylalanine (L-DOPA), dopamine and hydroquinone, but failed to oxidize tyrosine. The results described above collectively proved that AjPO was a membrane-binding laccase-type PO. The quantitative real-time PCR (qRT-PCR) analysis revealed that AjPO mRNA was expressed in muscle, body wall, coelomocytes, tube feet, respiratory tree and intestine with the highest expression level in coelomocytes. AjPO could be significantly induced by lipopolysaccharide (LPS), peptidoglycan (PGN), Zymosan A and polyinosinic-polycytidylic acid (PolyI:C), suggesting AjPO is closely involved in the defense against the infection of bacteria, fungi and double-stranded RNA viruses.

1. Introduction

Phenoloxidase (PO), a key component of the innate immunity of invertebrates, was mainly found in the humoral fluid and responsible for the conversion of phenol substrates to unstable quinones, which afterwards were transformed into melanin following a non-enzymatic pathway [1–3]. The melanin and a number of intermediate metabolites generated during melanization were involved in pathogen extermination, wound healing, phagocytosis, and encapsulation [4–6]. The melanization may be initiated by three different types of POs, which were laccase type, catechol oxidase type and tyrosinase type respectively [7]. The three different types of POs showed remarkable difference in substrate specificity, the laccase-type was capable of oxidizing both *p*-diphenols and *o*-diphenols, the catechol oxidase-type could just oxidize *o*-diphenols, and the tyrosinase-type showed high affinity to both monophenols and *o*-diphenols [8,9].

Since the first genetic study of PO in the freshwater crayfish *Pacifastacus leniusculus* in 1995, the gene cloning of PO has been performed in a variety of invertebrates, notably in insects and crustaceans [10,11]. These studies showed that most gene sequences of invertebrate POs had an open reading frame (ORF) of approximately 2000 bp in length, and the deduced amino acid sequences contained two conserved copper centers [1,12,13]. In addition, the functional analysis of PO indicated that the PO mRNA expression had a close relationship to the challenge of microbial polysaccharide or pathogens [14–16]. For example, the stimulation of lipopolysaccharide (LPS) caused a remarkable decrease in the mRNA expression of PO in hemocytes (REPH) in the scallop *Chlamys farreri* during 3–6 h after injection [17]; in contrast, the stimulation of LPS induced an increase in REPH in the crab *Scylla serrata* [18]. Furthermore, the challenges by *Vibrio anguillarum* in the crab *Eriocheir sinensis* and *Vibrio alginolyticus* in the crab *Portunus trituberculatus* both resulted in a dramatic enhancement of REPH at 12 and 48 h post infection [19,20], while

Abbreviations: PO, phenoloxidase; AjPO, *Apostichopus japonicus* phenoloxidase; ORF, open reading frame; RACE, rapid amplification of cDNA ends; L-DOPA, L-3,4-dihydroxyphenylalanine; PBS, phosphate buffered saline; qRT-PCR, quantitative real-time PCR; LPS, lipopolysaccharide; PGN, peptidoglycan; PolyI:C, polyinosinic-polycytidylic acid; dsRNA, double-stranded RNA; REPH, mRNA expression of PO in hemocytes; Cytb, cytochrome b; SE, standard error; UTR, untranslated region.

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the challenges by *Aeromonas hydrophila* and *Vibrio harveyi* in the prawn *Macrobrachium rosenbergii* caused a significant increase in REPH at 24 h and subsequent a decrease at 48 h post infection [21]. As far as virus challenge is concerned, the white spot syndrome virus infection induced an up-regulation of REPH in the crayfish *Procambarus clarkii* and a down-regulation in the shrimp *Litopenaeus vannamei* respectively at 48 h post infection [22,23]. All these studies suggested that invertebrate PO was closely involved in the immune response against pathogens infection. In addition, the temporal variation of REPH showed great difference in one species with different challenges or different species with the same challenge, suggesting that the immune action of PO had specificities to both immune challenge and species.

Sea cucumber *Apostichopus japonicus* is one of the most important commercial species in North China, and its culture is susceptible to the diseases caused by pathogens like bacteria and viruses [24–26]. The present study reports the cloning of full-length cDNA, the determination of substrate specificity, and the transcripts expression analysis of PO (AjPO) from *A. japonicus*. We aimed to provide primary data on PO as a potential immune index in this sea cucumber.

2. Materials and methods

2.1. Experimental animals and immune challenges

One-year old healthy sea cucumbers (with average body weight 13.2 ± 2.4 g) were collected from Dalian in China and kept in seawater aquaria at 15–18 °C, pH 8.1–8.3 and salinity of 31‰ for one week before use.

The immune challenges were conducted by coelomic injection using LPS (Sigma), peptidoglycan (PGN, Sigma), Zymosan A (Sigma) and polyinosinic-polycytidylic acid (PolyI:C, Sigma) as stimulants, which were dissolved in phosphate buffered saline (PBS, pH = 7.4) at a concentration of 1 mg/ml, 100 μg/ml, 300 μg/ml and 100 μg/ml respectively. Tested animals were injected with 500 μl of each stimulant solution respectively, and controls were injected with 500 μl PBS instead of stimulants, then 15 animals in each group were sampled randomly at 4 h, 12 h, 24 h, 48 h and 72 h post-injection. Tissues including body wall, muscle, coelomocytes, tube feet, intestine and respiratory tree were isolated and collected into 3 pools of 5 individuals each. Successively, the mixed tissues were preserved in RNAlater (Invitrogen) and stored at −80 °C for RNA extraction.

2.2. Total RNA extraction and cDNA synthesis

The total RNA of different tissues was extracted using the RNAprep pure Tissue Kit (TIANGEN) following the manufacturer's instructions. The concentration and quality of total RNA were detected by NanoPhotometer (Implen GmbH) and agarose gel electrophoresis. To synthesize first strand cDNA, 25 pmol Oligo dT Primer, 50 pmol random 6 mers, 4 μl 5 × PrimeScript™ buffer, 900 ng total RNA and 1 μl PrimeScript™ RT enzyme Mix I (PrimeScript™ RT reagent Kit, TaKaRa) were mixed in a 20 μl reaction system and incubated at 37 °C for 15 min, and then at 85 °C for 5 s, the products were used as cDNA samples and stored at −80 °C for the quantification of gene expression.

2.3. Full-length cDNA cloning

Based on the partial sequence of AjPO identified from the transcriptome sequencing analysis of *A. japonicus* [27], the primers for 3′- and 5′-rapid amplification of cDNA ends (RACE) PCR were designed (Table 1). The cDNA templates for 3′- and 5′-RACE PCR were synthesized respectively by employment of the total RNA from coelomocytes using the SMART PCR cDNA Synthesis Kit (Clontech) following the manufacturer's instructions. The 3′- and 5′-RACE PCR were performed in a hot-lid thermocycler using SMART RACE cDNA Amplification Kit (Clontech) according to the instructions, and a touch-down PCR was employed with the following thermal cycling profiles: one cycle of initial denaturation at 94 °C for 3 min; followed by 5 cycles of 94 °C for 30 s, 72 °C for 3 min; next 5 cycles of 94 °C for 30 s, 70 °C for 30 s, 72 °C for 3 min; next 25 cycles of 94 °C for 30 s, 68 °C for 30 s, 72 °C for 3 min; and a final extension of 72 °C for 10 min. The specific PCR products were purified by Gel Extraction Kit (Omega), and then cloned into PMD-19T vector (TaKaRa). After transforming into the competent cells of *Escherichia coli* JM109, the recombinants were spread on the LB-agar Petri dish containing 100 mg/ml ampicillin. Positive clones containing the expected-size inserts were screened by colony PCR and then subjected for DNA sequencing.

Table 1
The primers used in this study.

Primer name	Sequence (5′-3′)	Purpose
PO-3	CGTTGTTCTGTGATGCTAGAGTTGCG	3′-RACE PCR
PO-5	AAGCCGGTCCTTTGCCCTTTCCAT	5′-RACE PCR
UPM long	CTAATACGACTCACTATAGGGCA AGCAGTGGTATCAACGCAGAGT	5′- & 3′-RACE PCR
UPM short	CTAATACGACTCACTATAGGGC	5′- & 3′-RACE PCR
PO-rt-1s	CAGCAGTTACAAGTGGGATG	Quantitative real-time PCR
PO-rt-1a	CCAGTCACGAAGACCAGAAT	Quantitative real-time PCR
CytbQ-F	TGAGCCGCAACAGTAATC	Reference gene
CytbQ-R	AAGGGAAAAGGAAGTGAAAG	Reference gene

2.4. Sequence analysis

The similarity searches were performed using the BLASTX program at the National Center for Biotechnology Information (http://ncbi.nlm.nih.gov/blast/). ORF was predicted using Open Reading Frame Finder (http://www.ncbi.nlm.nih.gov/gorf/gorf.html). Translation and protein analysis were performed by ExPaSy tools (http://www.expasy.org/tools/). The protein motifs' features were predicted by Simple Modular Architecture Research Tool (SMART, http://smart.emblheidelberg.de/) [28]. Multiple sequences alignments were generated by ClustalW2 (http://www.ebi.ac.uk/Tools/msa/clustalw2/). An NJ phylogenic tree based on the deduced amino acid sequences was constructed using MEGA 4.0 program, and the reliability of the branching was tested by bootstrap resampling with 1000 pseudo-replicates [29].

2.5. Substrate specificity analysis

Using ProteoExtract® Native Membrane Protein Extraction Kit (Merck), the membrane proteins were isolated from coelomocytes and then used as crude AjPO for the determination of substrate specificity. Briefly, 10 μl of the crude AjPO solution was added to 190 μl of 15 mmol/L catechol, L-3, 4-dihydroxyphenylalanine (L-DOPA), dopamine, hydroquinone and tyrosine that were dissolved in PBS respectively, and controls were performed by replacement of crude AjPO with the same volume of PBS. Subsequently, melanochrome formation was determined spectrophotometrically at 492 nm every 3 min for 27 min using a Sunrise microplate reader (Tecan) [30]. The measurement for each substrate was performed in triplicate.