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The phenoloxidase activity and antibacterial function of a tyrosinase from scallop *Chlamys farreri*

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ABSTRACT

Tyrosinase (TYR), also known as monophenol monooxygenase, is a ubiquitous binuclear copper-containing enzyme which catalyzes the hydroxylation of phenols to catechols and the oxidation of catechols to quinones. In the present study, the cDNA of a tyrosinase (CfTYR) was identified from scallop *Chlamys farreri*, which encoded a polypeptide of 486 amino acids. The CfTYR mRNA transcripts were expressed in all the tested tissues, including haemocytes, adductor muscle, kidney, hepatopancreas, gill, gonad and mantle, with the highest level in mantle. The expression level of CfTYR mRNA in haemocytes decreased significantly during 3–6 h after LPS stimulation, and reached the lowest level at 6 h (0.05-fold, $P < 0.05$). Then, it began to increase at 12 h (0.32-fold, $P > 0.05$), and reached the highest level at 24 h (2.91-fold, $P < 0.05$). At 3 h after LPS stimulation, the phenoloxidase activity catalyzing L-dopa and dopamine in haemolymph increased significantly to 53.13 and 40.36 U mg^{-1} respectively, but it decreased to 10.82 U mg^{-1} and even undetectable level after CfTYR activity was inhibited. Furthermore, the antibacterial activity of haemolymph against *Escherichia coli* was also increased significantly at 3 h after LPS stimulation, but it decreased significantly when the haemolymph was treated by TYR inhibitor. The recombinant protein of the mature CfTYR peptide expressed in the *in vitro* Glycoprotein Expression Kit displayed phenoloxidase activity of 64.36 ± 5.51 U mg^{-1} in the present of trypsinase and Cu^{2+}. These results collectively suggested that CfTYR was a homologue of tyrosinase in scallop *C. farreri* with the copper-dependence phenoloxidase activity, and it could be induced after immune stimulation and mediate immune response for the elimination of invasive pathogens in scallop.

1. Introduction

Tyrosinase (TYR; monophenol monooxygenase; EC 1.14.18.1) is an important bifunctional copper-dependent enzyme, which can utilize molecular oxygen to catalyze the oxidation of monophenols to their corresponding o-diphenols (monophenolase activity) and their subsequent oxidation to o-quinones (catecholase activity) [1]. It is responsible for the production of melanin and other pigments, and known as phenoloxidase in arthropods owing to its crucial role in the immunity, therefore the function catalyzing the conversion of phenols to unstable quinones is also considered to be phenoloxidase activity in other invertebrates [2].

Three enzymes including TYR, hemocyanin and laccase have all been reported to possess phenoloxidase activity in invertebrate. In arthropod, the TYR-mediated phenoloxidase activity takes up the majority of the total phenoloxidase activity, and it is distributed mainly in the haemocytes in the form of proenzyme with lower activity, which would be released into haemolymph with higher activity after a proteolytic cleavage [3–5]. In more primitive mollusc, phenoloxidase activities can also be detected in different forms [6–9]. For example, TYR-mediated phenoloxidase activity was reported in clam *Ruditapes philippinarum* [10], and laccase-mediated phenoloxidase activity was observed in Pacific oyster *Crassostrea gigas* [11,12]. Some hemocyanin genes have been characterized in mollusc [13,14], and hemocyanin have also been revealed to have phenoloxidase activity in the marine snails *Rapana venosa*, garden snails *Helix vulgaris*, gastropod *Helix pomatia* and cephalopod *Sepia officinalis* [15,16]. Although the three type phenoloxidase activities have all been detected in mollusc, the knowledge about their functions and detailed mechanism are still far from well understood.

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TYR-mediated phenoloxidase activity is important for the immunity of arthropod by catalyzing the melanization reaction to produce some reactive intermediate substances with broad-spectrum antimicrobial activity such as o-quinones and 5, 6-dihydroxyindole (DHI) [17,18], and end product melanin to play important roles in wound healing, phagocytosis and encapsulation of haemocytes to invasive pathogens in arthropod [19]. Although there is no apparent melanization observed in mollusc, some studies have focused on the immune role of phenoloxidase-associated reaction in recent years. It has been found that the outbreak of 'Queensland unknown' or 'QX' disease in summer was associated with lower phenoloxidase activity in Sydney rock oyster *Saccostrea glomerata* [20], and the phenoloxidase activity in the haemolymph could be induced and repressed by the immune stimulation and environmental stress respectively [21,22]. The phenoloxidase-associated cellular defense provided resistance of Sydney rock oyster to against QX disease infections [23]. Furthermore, the laccase-mediated phenoloxidase activity was found to involve in the immune response of pacific oyster *C. gigas* [11,12,24]. TYR-mediated phenoloxidase activity was considered to be implicated in the periostracum formation in mollusc [25,26]. Although TYR-mediated phenoloxidase activity has been confirmed to be important for the immunity of arthropod, the knowledge of its relative immune functions is still very limited in mollusc.

The scallop *Chlamys farreri* is a dioecious bivalve native to the coast of China, Korea and Japan, and contributes weightily to the aquaculture industry of northern China. In recent years, the outbreak of disease has resulted in severe mortality of scallops and threatened the development of aquaculture industry. Investigations of the TYR-mediated phenoloxidase activity in the scallop *C. farreri* will contribute to further understanding of the immune defense mechanism of scallops and offer enlightenment to its disease control. The purposes of this study were to (1) identify TYR from *C. farreri* (designated as CfTYR) and verify its phenoloxidase activity, (2) investigate tissues distribution of CfTYR transcripts, (3) survey the temporal expression of CfTYR mRNA and the response of CfTYR-mediated phenoloxidase activity after LPS stimulation, and (4) examine the CfTYR-mediated antibacterial activity in the haemolymph of scallops.

2. Materials and methods

2.1. Scallops, tissue collection, LPS stimulation

Healthy scallop *C. farreri* were collected from a local farm in Qingdao, Shandong Province, China, and maintained in the aerated seawater at 15 °C for two weeks before processing.

For the tissue distribution analysis of CfTYR mRNA, six tissues including hepatopancreas, adductor muscle, kidney, gonad, gill and mantle from six healthy adult scallops were collected as parallel samples. Haemolymph from these six scallops was also collected from the adductor muscle and then immediately centrifuged at 800 × g, 4 °C for 10 min to harvest the haemocytes. All these samples were stored at −80 °C after addition of 1 mL TRIzol reagent (Invitrogen) for subsequent RNA extraction.

In the LPS stimulation experiment, totally eighty scallops were employed and divided into three groups. Thirty scallops in the first group were employed as control group which received an injection of 50 μL phosphate buffered saline (PBS, 377 mmol L^{-1} NaCl, 2.7 mmol L^{-1} KCl, 8.09 mmol L^{-1} Na_2HPO_4, 1.47 mmol L^{-1} KH_2PO_4, pH 7.4). The second group with 30 scallops received an injection of 50 μL LPS from *Escherichia coli* 0111:B4 (Sigma Aldrich, 0.5 mg mL^{-1} in PBS), and was employed as stimulation group. These scallops were returned to water tanks after treatment, and 5 individuals were randomly sampled at 3, 6, 12, 24 and 48 h post-injection from the stimulation and control group. The rest 20 untreated scallops were employed as blank group, and 5 individuals were randomly sampled at 0 h. Haemolymph was collected from the adductor muscle and then immediately centrifuged at 800 × g, 4 °C for 10 min. The haemocytes were harvested and stored for subsequent RNA extraction, while the haemolymph supernate in the control and stimulation group at 3 h, and the blank group was collected for the determination of phenoloxidase and antibacterial activity.

2.2. RNA isolation and cDNA synthesis

Total RNA was isolated from the tissues of scallops using Trizol reagent (Invitrogen) according to its protocol. The first-strand synthesis was carried out based on Promega M-MLV RT Usage information using the DNase I (Promega)-treated total RNA as template and oligo (dT)-adaptor as primer (Table 1). The synthesis reaction was performed at 42 °C for 1 h, terminated by heating at 95 °C for 5 min. The cDNA mix was diluted to 1:100 and stored at −80 °C for subsequent SYBR Green fluorescent quantitative real-time PCR.

2.3. EST analysis and cloning of the full-length CfTYR cDNA

A cDNA library was constructed with the whole body of a scallop challenged by *Vibrio anguillarum*, and random sequencing of the library using T3 primer yielded 6935 successful sequencing reactions [27]. BLAST analysis of all the EST sequences revealed that one EST (no. rscag3283) was homologous to the TYRs identified previously in other animals.

Four specific primers (Table 1) were designed based on the sequence of EST to clone the full-length cDNA of CfTYR by rapid amplification of cDNA ends (RACE) approach. PCR amplification to obtain the 3′ end of CfTYR was carried out using sense primer P1 or P2 and antisense primer Oligo (dT)-adaptor, while sense primer Oligo (dG)-adaptor and antisense primer P3 or P4 were used to get the 5′ end according to the Usage Information of 5′ RACE system (Invitrogen). All PCR amplification was performed in a PCR Thermal Cycle (TAKARA, GRADIENT PCR).

The specific PCR products were gel-purified and cloned into pMD18-T simple vector (Takara, Japan). After being transformed into the competent cells of *E. coli* Top10F′, the positive recombinants were identified through anti-ampicillin selection and PCR screening. Three of the positive clones were sequenced on an ABI 3730 XL Automated Sequencer (Applied Biosystems). The sequencing results were verified and subjected to cluster analysis.

Table 1
Sequence of the primers used in the experiment.

Primer	Sequence (5′–3′)	Sequence information
P1 (forward)	TCACCGATGGGTAGCAG	3′ RACE primer
P2 (forward)	CCCATCTTCTTTCTTCATCAC	3′ RACE primer
P3 (reverse)	CGATTTCACTTCACCAATACCTG	5′ RACE primer
P4 (reverse)	ATCGCTGCCTCCATTACAAG	5′ RACE primer
P5 (forward)	CAACGCATTCTTACAAATCCTGC	CfTYR recombinant primer
P6 (reverse)	ACCAAGTGTATTGTTTGCTAACTGTA	CfTYR recombinant primer
P7 (forward)	ATCCCATCTTCTTTCTTCATCACAG	Real-time CfTYR primer
P8 (reverse)	GGTCTATGTTTCCAGTTTCCCTC	Real-time CfTYR primer
P9 (forward)	CAAACAGCAGCCTCCTCGTCAT	Real-time actin primer
P10 (reverse)	CTGGGCACCTGAACCTTTCGTT	Real-time actin primer
Oligo (dT)-adaptor	$GGCCACGCGTCGACTAGTACT_{17}$	
Oligo (dG)-adaptor	$GGCCACGCGTCGACTAGTACG_{10}$	