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Scallop phenylalanine hydroxylase implicates in immune response and can be induced by human TNF-α

Zhi Zhou [a,b], Lingling Wang [a,*], Mengqiang Wang [a,b], Huan Zhang [a], Tiantian Wu [a,b], Limei Qiu [a], Linsheng Song [a,*]

[a] Key Laboratory of Experimental Marine Biology, Institute of Oceanology, Chinese Academy of Sciences, Qingdao 266071, China
[b] Graduate school, Chinese Academy of Sciences, Beijing 100049, China

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ABSTRACT

Phenylalanine hydroxylase (PAH) is an important metabolic enzyme of aromatic amino acids, which is responsible for the irreversible oxidation of phenylalanine to tyrosine. In the present study, the full-length cDNA encoding PAH from *Chlamys farreri* (designated CfPAH) was cloned by using rapid amplification of cDNA ends (RACE) approaches and expression sequence tag (EST) analysis. The open reading frame of CfPAH encoded a polypeptide of 460 amino acids, and its sequence shared 64.4–74.2% similarity with those of PAHs from other animals. There were an N-terminal regulatory ACT domain and a C-terminal catalytic Biopterin_H domain in the deduced CfPAH protein. The mRNA transcripts of CfPAH could be detected in all the tested tissues, including adductor muscle, mantle, gill, gonad, haemocytes and hepatopancreas. And its expression level in haemocytes was increased significantly during 3–48 h after bacteria *Vibrio anguillarum* challenge with the highest level (9.1-fold, $P < 0.05$) at 24 h. Furthermore, the mRNA expression of CfPAH in haemocytes also increased significantly to 2.6-fold ($P < 0.05$) at 4 h and 3.3-fold ($P < 0.05$) at 6 h after the stimulation of 50.0 ng mL^{-1} human TNF-α. The cDNA fragment encoding the mature peptide of CfPAH was recombined and expressed in the prokaryotic expression system, and 1 mg recombinant CfPAH protein (rCfPAH) could catalyze the conversion of 192.23 ± 32.35 nmol phenylalanine to tyrosine within 1 min (nmol min^{-1} mg^{-1} protein) *in vitro*. These results indicated collectively that CfPAH, as a homologue of phenylalanine hydroxylase in scallop *C. farreri*, could be induced by cytokine and involved in the immunomodulation of scallops by supplying the starting material tyrosine for the synthesis of melanin and catecholamines.

1. Introduction

Phenylalanine hydroxylase (PAH; phenylalanine monooxygenase; EC 1.14.16.1) is an iron-dependent enzyme that catalyses the C-oxidation of phenylalanine in the presence of 6R-tetrahydrobiopterin (BH_4) utilizing molecular oxygen as an additional substrate [1–3]. PAH is an essential enzyme in the metabolism of aromatic amino acids, and it is also the rate limiting enzyme for the irreversible oxidation of the essential amino acid phenylalanine to tyrosine [4].

In eukaryotes, all PAHs share the similar structural feature of a homotetramer with subunits consisting of two domains, an N-terminal regulatory CAT domain and a C-terminal catalytic Biopterin_H domain. The regulatory CAT domains share low sequence identity in different animals, and it is devoid in procaryotes such as soil bacterium *Chromobacterium violaceum* [5,6]. The conformation of the regulatory CAT domain will change after the binding of phenylalanine to alter the interaction between the regulatory and catalytic domain and promote the hydroxylation reaction [7,8]. The homologous catalytic Biopterin_H domains contain an iron-bound motif and a tetramerization motif, and all the residues are required for the determination of substrate specificity and catalytic function [9–11]. For example, the iron is bound to the 2-His-1-carboxylate facial triad constituted by two histidines and an acidic residue in catalytic domain of PAH [9]. PAHs catalyze the reaction from phenylalanine to tyrosine mainly in the liver [12]. The catalytic activity is strictly regulated to control the concentration of phenylalanine and tyrosine, and the impairment of its activity will result in the disease named phenylketonuria (PKU) [13–15].

Because tyrosine can be further converted to melanin and catecholamines which function as important immunomodulators, the catalytic function of PAH is demonstrated recently to be

* Corresponding authors. Tel.: +86 532 82898552; fax: +86 532 82898578.
E-mail addresses: wanglingling@ms.qdio.ac.cn (L. Wang), lshsong@ms.qdio.ac.cn (L. Song).

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implicated in the immune response of animals. For example, human PAH activity induced by hydrogen peroxide has been reported to supply additional tyrosine for melanogenesis in melanocytes [16]. It has been observed that the expression level of PAH in haemocytes of *Aedes aegypti* was up-regulated after the immune challenge with *Dirofilaria immitis* microfilaria or bacteria [17], and its melanization was reduced after parasite challenge when PAH was knocked down by double-stranded RNA [18]. PAH knockout nematode *Caenorhabditis elegans* has manifested the increase of superoxide dismutase activity and the lack of melanin in the cuticle. Melanin is a widespread pigment present in all living organisms and important both for the adaptation to the variable environmental conditions and the protection against external insults [19]. Although these abnormal melanization in immune response of invertebrates has been proven to be resulted from the alteration of PAH in transcriptional level, the underlying mechanism of PAH activation is still unknown, and it is required to better understand the potential immunomodulation of PAH by influencing the synthesis of melanin and catecholamines.

Cytokines are important modulators or inducers for numerous physiological activities, which are also coordinated to help optimizing immune response [20–22]. Cytokines regulate the metabolism of amino acids in vertebrate, whereas it is still unknown whether cytokines are involved in the metabolism of phenylalanine and the corresponding enzymes in invertebrate even some cytokine like molecules have been identified [23–25]. In the present study, a PAH was identified and characterized from scallop *Chlamys farreri* to gain preliminary insight into the modulation mechanism of PAH in invertebrate immune response. The purposes of this study were (1) to investigate the mRNA expression pattern of PAH from *C. farreri* (designated as CfPAH) in different tissues (2) to detect its temporal expression in haemocytes against bacteria *Vibrio anguillarum* challenge and human TNF-α stimulation, (3) to examine the catalytic activity of recombinant CfPAH protein to better understand the function of CfPAH in the immune response.

2. Materials and methods

2.1. Scallops, tissue collection, bacteria challenge and TNF-α treatment

Healthy scallops *C. farreri* with an average 53 mm of shell length and about 1 year old, 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 CfPAH mRNA, five tissues including hepatopancreas, adductor muscle, 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.

Two hundred and eighty scallops were employed for the bacteria challenge experiment, and divided into three groups. One hundred and twenty scallops in the first group receiving 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) were employed as control group, and other one hundred and twenty scallops in the second group receiving an injection of 50 μL alive *V. anguillarum* suspended in PBS (8×10^6 CFU mL^{-1}) were employed as challenge group. These treated scallops were return to water tanks, and fifteen individuals were randomly sampled at 3, 6, 12, 24, 48 and 96 h post-injection from control and challenge groups. The rest forty untreated scallops were employed as blank group, and fifteen individuals were randomly sampled at 0 h. Haemolymph collected from three individuals were pooled together as one sample. The haemocytes were harvested and stored as described above.

Another one hundred and twenty scallops were employed for the TNF-α stimulation experiment, and divided averagely into four groups, TNF-α stimulation group 1, TNF-α stimulation group 2, control group and blank group. The scallops in the first three groups received an injection of 50 μL of 5.0 ng mL^{-1} human TNF-α (Invitrogen, in PBS), 50.0 ng mL^{-1} TNF-α and PBS, respectively. These treated scallops were return to water tanks, and six individuals were randomly sampled at 2, 4 and 6 h post-injection from control and stimulation groups. Simultaneously, six individuals were randomly sampled at 0 h in the blank group containing untreated scallops. The haemolymph were collected and stored as described above.

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 (RT-PCR).

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

A cDNA library was constructed with the whole body of a scallop challenged by *V. anguillarum*, and random sequencing of the library using T3 primer yielded 6935 successful sequencing reactions [26]. BLAST analysis of all the EST sequences revealed that one EST (no. rscag0_006331, 595 bp) was homologous to the PAHs identified previously in other animals.

Six specific primers (Table 1) were designed based on the sequence of EST to clone the full-length cDNA of CfPAH by rapid amplification of cDNA ends (RACE) approach. PCR amplification to obtain the 3′ end of CfPAH was carried out using sense primer P1, P2 or P3 and antisense primer Oligo(dT)-adaptor P7, while sense primer Oligo(dG)-adaptor P8 and antisense primer P4, P5 or P6 were used to get the 5′ end according to the Usage Information of 5′

Table 1
Sequence of the primers used in the experiment.

Primer	Sequence (5′–3′)	Sequence information
P1 (forward)	GGATTCAGTGTCCGACCAGTTG	CfPAH specific primer
P2 (forward)	CGTGTTACTGGTTTACGGTGGAG	CfPAH specific primer
P3 (forward)	AGTTTGAACCTTCCAAGACAGCAG	CfPAH specific primer
P4 (reverse)	CTATGGCGAATGTACTGTGTTGC	CfPAH specific primer
P5 (reverse)	AACTCCACCGTAAACCAGTAACAC	CfPAH specific primer
P6 (reverse)	CAGATCGGGTTTATCAGTCAAGC	CfPAH specific primer
P7 (forward)	GGCCACGCGTCGACTAGTACT$_{17}$	Oligo(dT)-adaptor
P8 (reverse)	GGCCACGCGTCGACTAGTACG$_{10}$	Oligo(dG)-adaptor
P9 (forward)	TACGGTGGAGTTTGGACTGTGC	Real-time CfPAH primer
P10 (reverse)	GCTCTCATCTTTTCCTTGGCTTC	Real-time CfPAH primer
P11 (forward)	CAAACAGCAGCCTCCTCGTCAT	Real-time actin primer
P12 (reverse)	CTGGGCACCTGAACCTTTCGTT	Real-time actin primer
P13 (forward)	ATGGATGCGGGGATTTCTCC	CfPAH recombinant primer
P14 (reverse)	TCATAATTTCCCAACTTTCTTCAGTGC	CfPAH recombinant primer
M13-47	CGCCAGGGTTTTCCCAGTCACGAC	pMD18-T vector primer
RV-M	GAGCGGATAACAATTTCACACAGG	pMD18-T vector primer
T7	TAATACGACTCACTATAGGG	pEASY-E1 vector primer
T7t	GCTAGTTATTGCTCAGCGG	pEASY-E1 vector primer