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Cloning and characterization of tyrosine hydroxylase (TH) from the pacific white leg shrimp *Litopenaeus vannamei*, and its expression following pathogen challenge and hypothermal stress

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ABSTRACT

Tyrosine hydroxylase (TH) belongs to the biopterin-dependent aromatic amino acid hydroxylase enzyme family, and it represents the first and rate-limiting step in the synthesis of catecholamines that are required for physiological and immune process in invertebrates and vertebrates. Cloned *Litopenaeus vannamei* TH (LvTH), containing a short alpha helix domain, a catalytic core, a regulatory domain, a phosphorylation site and two potential N-linked glycosylation sites as presented in vertebrate and insect THs without acidic region and signal peptide cleavage sites at the amino-terminal, exhibited a similarity of 60.0–61.2% and 45.0–47.0% to that of invertebrate and vertebrate THs, respectively. Further, LvTH expression was abundant in gill and haemocytes determined by quantitative real-time PCR. *L. vannamei* challenged with *Vibrio alginolyticus* at 10^5 cfu shrimp^{-1} revealed significant increase of LvTH mRNA expression in haemocytes within 30–120 min and in brain within 15–30 min followed with recuperation. In addition, shrimps exposed to hypothermal stress at 18 °C significantly increased LvTH expression in haemocytes and brain within 30–60 and 15–60 min, respectively. The TH activity and haemolymph glucose level (haemocytes-free) significantly increased in pathogen challenged shrimp at 120 min and 60 min, and in hypothermal stressed shrimp at 30–60 and 30 min, respectively. These results affirm that stress response initiates in the brain while haemocytes display later response. Further, the significant elevation of TH activity in haemolymph is likely to confer by TH that released from haemocytes. In conclusion, the cloned LvTH in our current study is a neural TH enzyme appears to be involved in the physiological and immune responses of whiteleg shrimp, *L. vannamei* suffering stressful stimulation.

1. Introduction

Whiteleg shrimp, *Litopenaeus vannamei* (Boone), which is naturally spread along the Pacific coast of Central and South America, has been introduced to the Eastern hemisphere, and has become the dominant species presently being cultured in Asian countries. The shrimp culture has suffered plaguy problems linking to deteriorating cultured environments because of evolution intensification, which resulting in increased incidences of stress-induced diseases. The stressful environments induced physiological, immunological and behavioral responses to ensure the survival of living organisms, which are usually to depress the immunity and disease-resistance ability.

Neuroendocrine hormones play a major role in the regulation of homeostasis under stressful environments. Catecholamines (CAs), a class of biogenic amines, distribute both in crustacean central and in peripheral nervous systems [1,2], which function mainly as neuroregulators (i.e., neurotranmitters and neuromodulators). Its release is the first response to physiological stress, and while subsequent induction the hyperglycemia and suppression of immunity are secondary responses [3–5]. Involvement of biogenic amines in hyperglycemic response had been demonstrated in *Macrobrachium rosenbergii* and *L. vannamei* [4,6]. In *L. vannamei*, biogenic amines were also found to suppress the immune responses and increase the susceptibility to *Vibrio alginolyticus* infection [7,8].

Tyrosine hydroxylases (THs) are highly conserved enzymes found in both vertebrates and invertebrates, which belong to pterin-dependent monooxygenases that contain ferrous iron at the

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active site [9]. TH is a tetramer of four identical subunits (homotetramer). Each subunit consists of three domains including a short alpha helix domain [10], a catalytic core [11] and a regulatory domain [12]. In CA biosynthesis, TH is the first and rate-limiting enzyme, catalyzes the conversion of L-tyrosine to L-3,4-dihydroxyphenylalanine (L-DOPA) using molecular oxygen (O_2), as well as iron (Fe^{2+}) and tetrahydrobiopterin as cofactors [13,14]. L-DOPA is the precursor for the neurotransmitters dopamine, norepinephrine (NE, noradrenaline) and epinephrine (EP, adrenaline), which is converted to dopamine by DOPA decarboxylase (DDC, aromatic amino acid decarboxylase). Dopamine (DA) can be transformed to NE by the enzyme dopamine β-hydroxylase (DBH), which can be further modified by the enzyme phenylethanol N-methyltransferase to obtain EP [15]. The dopamine, serotonin and acetylcholine metabolism genes as well as their corresponding receptors are significantly expressed in neural tissues of lobster, *Homarus americanus*, and shrimp, *Penaeus monodon*, which were subjected to hypothermal stress [16].

Rat phagocytes contain mRNA for both TH and DBH, which clearly were inducible by cell contact with bacterial lipopolysaccharide (LPS) [17]. LPS also induce TH in a wax moth, *Galleria mellonella* [18]. A TH cDNA was cloned from the larval fat body of immunized *Samia cynthia ricini*, which was expressed only in the brain, but significantly induced in fat body and haemocytes of naïve larvae injected with UV-killed bacteria [19]. In *Manduca sexta*, TH mRNA and protein are present in eggs at the stage when the pharate larval cuticle begins to tan and in the integument of molting larvae also. In addition, TH is upregulated in haemocytes and the fat body in response to an immune challenge [20]. These results suggest that TH is required for cuticle tanning and immunity in *Manduca sexta* [20].

The aim of the present study was to isolate the full length cDNA clone of TH from the brain of *L. vannamei*, to determine its complete structure, and to compare it with known TH from various species of animals. Next, the study intended to evaluate the tissue specific expression and TH activity in *L. vannamei*, which were stressed by *Vibrio alginilyticus* challenge, and hypothermal exposure.

2. Materials and methods

2.1. L. vannamei

Whiteleg shrimp, *L. vannamei* used in the study were obtained from a commercial farm at Pingtung, Taiwan, and they were acclimated at 28 ± 1 °C and a salinity at 20‰ in tanks with recirculating water in the laboratory (Department of Aquaculture, National Pingtung University of Science and Technology) for 2 weeks prior to experiments. During the acclimation period, shrimp were fed a formulated shrimp diet (Shinta Feed Company, Pingtung, Taiwan) twice daily. For the study, tanks were provided with continuous aeration, and the water temperature was maintained at 28 ± 1 °C, pH at 7.9–8.3, salinity at 20‰, and the dissolved oxygen concentration at 5.5–6.5 mg L^{-1}. Shrimps at the intermolt stage weighing 15.5 ± 1.5 g (mean ± SD) were used.

2.2. V. alginolyticus

The bacterium *V. alginolyticus* (CH003) isolated from diseased *L. vannamei* collected from farms in Pingtung, Taiwan, which displayed symptoms of anorexia, inactivity, poor growth and necrotic musculature, was used in this study [21]. Stock cultures were first plated on tryptic soy agar (TSA supplemented with 2% NaCl, Difco) for 24 h at 28 °C, inoculated into 10 ml tryptic soy broth (TSB supplemented with 2% NaCl, Difco) and cultured for 24 h at 28 °C for the test sample. The broth culture was centrifuged at 7155 × g for 15 min at 4 °C and the supernatant was removed. Bacterial pellet was suspended in saline solution (0.85% NaCl) at concentrations of 5×10^4 and 5×10^6 colony-forming units (cfu) ml^{-1} and used for challenge stress.

2.3. Sampling treatments

Five acclimated shrimp were transferred from 28 °C to 20 °C for 30 min. After 30 min of this hypothermal exposure, the shrimp haemolymph was individually withdrawn and haemocytes were collected. In addition, hepatopancreas, eyestalk, gill, intestine, muscle, heart, haemocytes, sub-cuticular epithelium, antennal glands, abdominal ganglia, thoracic ganglia, circumoesphageal connective and brain were then excised according to the previously described method [22]. The haemocyte pellet and tissues were homogenised in liquid nitrogen, and suspended in Tris-EDTA buffer (50 mM Tris-HCl and 1 mM EDTA; pH 7.4) for total RNA isolation. The extraction and purification of total RNA was as described previously [22].

2.4. PCR and subcloning of LvTH cDNA

Full-length LvTH cDNA of *L. vannamei* was obtained by RT-PCR, as well as by 3′ and 5′ rapid amplification of cDNA ends (RACE) methods. Degenerate primers were designed based on the highly conserved TH amino acid sequences of *Samia cynthia ricini* (AB288228.1), *Papilio xuthus* (AB178006), *Mythimna separate* (AB274835.1), *Drosophila melanogester* (U14395.1), *Apis mellifera* (AY855337.1) and *Tribolium castaneum* (EF592178.1) from the GenBank database [23] using Clustal program [24]. Amplification primer pairs used for *L. vannamei* TH are as shown in Table 1. The primer pair TH-F/TH-R was used to amplify the TH cDNA fragment. The PCR reaction comprised of 10 mM Tris-HCl buffer (pH 8.3) containing 50 mM KCl, 5U Taq polymerase, 0.25 mM dNTPs, and 0.4 mM of each primer. PCR was performed as follows: 35 cycles of denaturation at 94 °C for 40 s, annealing at 54 °C for 40 s, and elongation at 72 °C for 1 min, followed by a 10-min extension at 72 °C and cooling to 4 °C.

The LvTH5′ primer was used for 5′-RACE, and the LvTH3′ primer were used for 3′-RACE. For 5′-RACE (SMART™ RACE cDNA amplification kit, Clontech, CA, USA), first-strand cDNA was prepared and terminated at the 5'-end by the terminal transferases, TdT and dCTP. A primer set combining LvTH5′ and UPM was then used, and PCR was performed as follows: 5 cycles of denaturation at 94 °C for 20 s, annealing at 56 °C for 20 s, and elongation at 72 °C for 2 min, and then 30 cycle of denaturation at 94 °C for 20 s, annealing at 54 °C for 20 s, and elongation at 72 °C for 2 min, followed by a 10-min extension at 72 °C and cooling to 4 °C. For the 3′-RACE, reverse-transcription was performed using the UPM primer, and then the first-strand cDNA was used as the template for amplification of the 3′ fragment of shrimp TH. PCR was performed as follows: 5 cycles of denaturation at 94 °C for 20 s, 62 °C for 20 s and 72 °C for 2 min, and

Table 1
Oligonucleotide degenerate and specific primers used in the LvTH cloning experiment.

Primer name	Primer sequence (5′ → 3′)
TH-F	GAGAAGCAGCAGAAGCAGACG
TH-R	ATGTCCAGGTCGGGCTCGTA
LvTH5′	GTAGCCATTCTCGATACTGTAAGA
LvTH3′	CCATTCCCCGCATCGAGTACCG
Q-TH-F	TCCATCGCCTCCGTCAA
Q-TH-R	TGATGTGCTTCGGGAACCA
Q-actin-F	CATCACCAACTGGGACGACATGGA
Q-actin-R	GAGCAACACGGAGTTCGTTGT