Fish & Shellfish Immunology 35 (2013) 1483–1500

Contents lists available at ScienceDirect

Fish & Shellfish Immunology

journal homepage: www.elsevier.com/locate/fsi

Full length article

An evolutionarily ancient NO synthase (NOS) in shrimp

Chun-Hung Wu [a,1], Vinu S. Siva [a], Yen-Ling Song [a,b,*]

[a] Institute of Zoology, National Taiwan University, Taipei 10617, Taiwan, ROC
[b] Department of Life Science, National Taiwan University, Taipei 10617, Taiwan, ROC

ARTICLE INFO

Article history:
Received 15 October 2012
Received in revised form
11 July 2013
Accepted 18 August 2013
Available online 30 August 2013

Keywords:
Nitric oxide synthase
Penaeus monodon
PmNOS
Ancient

ABSTRACT

Nitric oxide (NO) is a well known essential molecule that is involved in multiple functions such as neuron transduction, cardiac disease, immune responses, etc.; nitric oxide synthase (NOS) is a critical enzyme that catalyzes the synthesis of it. A very few crustacean NOS molecules were biochemically characterized so far. In the present study, we cloned and characterized a NOS cDNA from haemocytes of tiger shrimp (*Penaeus monodon*) (PmNOS). The full-length of PmNOS cDNA contained 3997 bp, including a 5′UTR of 249 bp, ORF of 3582 bp and a 3′UTR of 166 bp. The putative peptide was 1193 amino acid residues in length, with an estimated molecular weight of 134.7 kDa and pI 6.7. Structurally, PmNOS contained oxygenase and reductase domains at N-terminal and C-terminal, respectively, and connected with a calmodulin binding motif. The deduced amino acid sequence of PmNOS shared 98% identical to the Chinese shrimp (*Fenneropenaeus chinensis*) NOS. Phylogenetically, PmNOS clustered with invertebrate NOS, but not clustered with iNOS, eNOS or nNOS found in vertebrates. PmNOS mRNA was expressed in many tissues or organs including thoracic and ventral nerves, midgut, gill, eyestalk, haemocytes, subcuticular epithelium and heart, but not found in hepatopancreas, muscle and lymphoid organ. But there was no significant difference in PmNOS mRNA expression after stimulation with LPS either by different concentration or time course or against CpG-ODN 2006. The enzyme activities of rPmNOS or crude homogenates from different tissues were detected, and were shown its highest activity in thoracic and ventral nerves, moderate in midgut and haemocytes but the lowest activity were seen in muscle. The addition of NOS antibody against NADPH binding domain leads to less activity which suggested that NADPH was an essential cofactor for PmNOS catalytic activity. The calcium dependency of PmNOS was ascertained using calmodulin inhibitor, Trifluroperazine. To confirm the population of haemocyte which produce NOS, the florescence test was assayed, and it implicated that the production of NO was catalyzed by subset of granulocytic NOS. Since the MW range, inducible/noninducible transcript, calcium-dependent activity and tissue distribution, we suggest that PmNOS may recognize as an ancient NOS evolutionarily.

1. Introduction

Nitric oxide (NO) is a molecule whose functions are reported to have long-term potentiation in neuron, to kill pathogens in macrophage and to regulate blood pressure in smooth muscle [1]. NO is formed by nitric oxide synthase (NOS) in the reaction from L-arginine to L-citrulline. This reaction requires cofactors such as NADPH, FAD, BH_4, calcium and calmodulin [2]. In vertebrates, there are three kinds of NOS isoform: neuronal NOS (nNOS) or NOS1; inducible NOS (iNOS) or NOS2 and endothelial NOS (eNOS) or NOS3. All three NOS have similar structural organization and functions.

The functions of nitric oxide have been reviewed in marine invertebrates [3]. In insects, NO acts as a vasodilator and avoids the platelet aggregation in the blood-sucking insect *Rhodnius prolixus* [4]. NO acts in osmo-regulation and excretory capacity in *Drosophila melanogaster* [5,6]. In molluscs, NO regulates the metabolic activity of ink gland and extent of melanin production [7]. In crustaceans, NO acts on potassium currents in muscle membrane of isopods, *Idotea baltica*, decreasing both the inotropic and chronotropic properties of heart beat [8]. The interaction between NO and serotonin controls total amount of bioluminescence in northern

* Corresponding author. Department of Life Science, National Taiwan University, Taipei 10617, Taiwan, ROC. Tel.: +886 2 33662455; fax: +886 2 33669499.
E-mail address: song@ntu.edu.tw (Y.-L. Song).
[1] Present address: Marine Bureau, Kaohsiung City Government, Kaohsiung City, Taiwan.

1050-4648/$ – see front matter © 2013 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.fsi.2013.08.016

Table 1
Sequences of oligonucleotides used in the present study.

Primer	Sequence (5′–3′)	Note
F1	$_{608}$TNG GNM GNA THC ART GG$_{624}$	N = A or T or C or G, M = A or C, H = A or T or C, R = A or G
F2	$_{3522}$TAC CAT GGC TGA GTG TGT GTA CCA GAA A$_{3549}$	
F3	$_{3548}$AAC TGA AGT CTA TTG TCC AGG AGC ATG$_{3574}$	
F4	AAG CAG TGG TAT CAA CGC AGA GTA CGC GGG	SMART II A Oligo; for 5′RACE
F5	0.4 μM: CTA ATA CGA CTC ACT ATA GGG CAA GCA GTG GTA TCA ACG CAG AGT; 2 μM: CTA ATA CGA CTC ACT ATA GGG C	Universal primer A mix; for 5′RACE
F6	AAG CAG TGG TAT CAA CGC AGA GT	Nested Universal Primer A; for 5′RACE
F7	$_{2395}$TTC TAG CAC GCC TGG ATA$_{2630}$	
F8	$_{250}$ATG AAG GAG GTG AAC AAA CCG CAG$_{273}$	
F9	*AGGCCT* AGG ATG AAG GAG GTG AAC AAA CCG CAG	Italic for *Stu*I restriction site
R1	$_{2882}$TCC TCA TAT TCA TGT GGG TC$_{2863}$	
R2	$_{2873}$TCA TGT GGG TCA GTT GCC AG$_{2854}$	
R3	GGC CAC GCG TCG ACT AGT ACT TTT TTT TTT TTT TTT TTT T	3′RACE adaptor primer
R4	GGC CAC GCG TCG ACT AGT AC	Abridged universal amplification primer
R5	(T)$_{25}$VN	5′CDS primer
R6	$_{1463}$TTG AGG TAG TAG AGC GAC ATC TCC TGG$_{1438}$	
R7	$_{1131}$CAC CAT GTA CCA GCC GTT GAA$_{1111}$	
R8	$_{3949}$CTA ATA TTT ATG TTG GCT TTA TGT TGT TTG CAA GAT$_{3914}$	
R9	$_{3831}$CTA TTC AAC AGG CGC TGC TGT GGA$_{3808}$	
R10	*TTA ATT AA* CTA GTG GTG GTG GTG GTG GTG TTC AAC AGG CGC TGC TGTGGA	Italic for *Pac*I restriction site Underline for 6×His tag

krill, *Meganyctiphanes norvegica* [9]. NO is used to regulate neurogenesis and morphogenesis in the development of *Homarus americanus* brain [10]. Furthermore, NOS activity implicated its role in the processing of visual information in the eyestalk of crayfish, *Procambarus clarkii* [11].

Due to high oxidative capability, NO can activate the immunity [12–14]. NO could defeat parasites and considerably reduce the infectious rate in many species [15–18]. Blowfly inoculated with yeast could produce a large amount of NO in the granular cells [19]. Silkworm (*Bombyx mori*) larva could significantly increase NOS activity once LPS was injected in the fat body [20].

In crustaceans, the spiny lobster *Panulirus argus* haemocytes disclosed for the ability to produce inducible nitric oxide synthase *in vitro*/*in vivo* LPS stimulation [21,22]. Likewise, the significant increase in haemocyte NOS activity after immune challenge with LPS was shown in mud crab *Syclla paramamosain* [23]. The giant freshwater prawn, *Macrobrachium resenbergii*, produced NO in haemocytes during phagocytosis [24]. Kuruma shrimp, *Marsupenaeus japonicus*, increased haemocytic NOS activity during White Spot Syndrome Virus (WSSV) infection [25]. Similar results revealed in crayfish, *P. clarkii*, that NOS activity associated with destroyed pathogen adhesion which was demonstrated using NOS inhibitor [26]. These observation indicated that NO and NOS activity has a pivotal role involved in shrimp defense system.

NOS cDNA has been cloned from several invertebrates, silkworm (*B. mori*) [20], blood-sucking insect (*R. prolixus*) [4], fruit fly (*D. melanogaster*) [6], Japanese fireflies (*Luciola lateralis*; *Luciola cruciata*) [27] and pond snail (*Lymnaea stagnalis*) [28,29]. Although the existence of NOS has been reported to be universal in a wide variety of organisms [30], there are four shrimp NOS cDNA were cloned, but a few biochemical, functional assays were disclosed so far.

In the present study, we cloned and characterized a NOS cDNA from tiger shrimp (*Penaeus monodon*) haemocytes. The recombinant PmNOS produced in a baculovirus expression system exhibits biochemical function.

2. Materials and methods

2.1. Shrimp cultivation

Healthy *P. monodon* weighing about 20 g each were purchased from Thailand and cultured in indoor tanks (75 × 30 × 40 cm^3, 26‰ salinity, 24 °C, 8 shrimps in each tank). Shrimps were fed artificial forage in 5% of shrimp weight and the remnant forage was cleansed once a day.

2.2. Haemocyte cDNA preparation

Shrimp haemolymph was withdrawn using 1 ml syringe with a 25 gauge-needle containing 0.3 ml of anticoagulant solution (0.1 M sodium citrate, 0.4 M sucrose, and 0.01 M Tris–HCl; pH 7.6; 780 mOsm/kg). The haemocytes were collected by centrifugation at 700*g* for 6.5 min. Total haemocyte RNA was extracted using Trizol reagent (Gibco BRL, USA), and cDNA synthesized using the superscript III First Strand Synthesis System for RT-PCR according to the manufacturer's instruction (Invitrogen Life Technologies).

2.3. Cloning of nitric oxide synthase

The forward F1 degenerate primer was directed to conserved region of variable species of NOS sequences from Genebank database (http://www.ncbi.nlm.nih.gov/), and alignment using clustal X software. One pair of reverse specific primer (R1 & R2) (Table 1) was designed based on *P. monodon* Functional Genomics Database (http://xbio.lifescience.ntu.edu.tw/pm/index.php). The PCR was started with an initial denaturation (95 °C for 2 min), then followed by 35 cycles of amplification (94 °C for 30 s, 60 °C for 30 s and 72 °C for 1.5 min) and final extension 72 °C for 10 min. Semi-nested PCR conditions were same as first reaction.

2.4. Rapid amplification of the 5′ and 3′ cDNA ends (5′ and 3′ RACEs)

To amplify the 3′ end of PmNOS, 5 μg of RNA was reversely transcribed by R3 primer to synthesize the first stand cDNA as template. First-round PCR used NOS specific F2 and R4 primer (Table 1) was run for 35 cycles of 96 °C for 30 s, 60 °C for 30 s and 72 °C for 2 min. The conditions of the second round PCR were same as those of the first round PCR except the primer being replaced with nested NOS specific F3 primer (Table 1). To amplify the 5′ end of PmNOS, the SMART™ RACE cDNA amplification kit (BD Biosciences, Inc., CA, USA) was used. Briefly, 3 μg of RNA, 1 μl R5 primer and 1 μl F4 primer were mixed and incubated at 68 °C for 2 min then on ice no less than 1 min. Then 2 μl 5× first strand buffer, 1 μl