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Molecular cloning, mRNA expression and characterization of membrane-bound hemoglobin in oriental river prawn *Macrobrachium nipponense*

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ABSTRACT

Most hemoglobins are respiratory proteins and are ubiquitous in animals, bacteria, fungi, protists, and plants. In this study, we describe a membrane-bound hemoglobin in the oriental river prawn *Macrobrachium nipponense* (MnHb), which also expresses hemocyanin. MnHb cDNA was cloned using the rapid amplification of cDNA ends (RACE) approach, which afforded a 1201 bp gene encoding a 193 amino acid polypeptide. Bioinformatic evaluation suggested MnHb is membrane anchored by N-myristoylation, and immunofluorescence confirmed its location in the membrane of chief cells in the gill. The effect of hypoxia on MnHb expression was investigated, and reverse transcription PCR (RT-PCR) and Western blotting showed that MnHb was expressed almost exclusively in the gill. Quantitative RT-PCR revealed a significant increase in expression after 6 h of hypoxia, and levels peaked at 24 h due to oxidative stress. Exposure of cultured prawns to the stress inducer H_2O_2 significantly up-regulated the expression of MnHb in a dose-dependent manner. MnHb may have a role in protecting cell membrane lipids from damage by reactive oxygen species.

1. Introduction

There are three kinds of respiratory proteins in animals, hemocyanins (Hcs), hemoglobins (Hbs) and hemerythrin. Hcs are copper-containing respiratory proteins not only been found in crustaceans but also in other arthropods (Rainer and Brouwer, 1993; Terwilliger et al., 2006; Zhang et al., 2009). Recently, the function of Hb in crustaceans has attracted increased attention from scholars (Terwilliger, 2015). Interestingly, Hbs are present in the coelomic fluid, hemolymph and cellular components of invertebrates, which indicates diversity of structures and functions of Hb (Vinogradov, 1985).

It is widely believed that all Hbs have a respiratory function and transport O_2 in body fluids or for intracellular O_2 supply. However, previous studies have shown that some Hbs may function as antioxidants (Flögel et al., 2001; Lai et al., 2007) or nitric oxide dioxygenases (Gardner et al., 1998), and they are involved in the immune response (Bao et al., 2011) and oxidative metabolism (Hankeln et al., 2002; Gleixner et al., 2008). There are no published data on the exact functions of membrane-bound Hbs in crustaceans (Ertas et al., 2011; Blank et al., 2011).

Although most arthropod animals possess only hemocyanins (Ertas et al., 2009; Marxen et al., 2014; Pinnow et al., 2016), several entomostracan Crustacea have an extracellular Hb that is involved in oxygen transport in the hemolymph (Miller, 1964; Terwilliger, 1998; Weber and Vinogradov, 2001). Even more amazing was that the co-occurrence of hemoglobin and hemocyanin has been reported in the amphipod *Cyamus scammoni* (Terwilliger, 2008; Terwilliger and Ryan, 2006) and the shore crab *Carcinus maenas* (Ertas et al., 2011). Herein, we also found a membrane-bound Hb (MnHb) in the oriental river prawn *Macrobrachium nipponense* (Decapoda, Palaemonidae); this is a commercially important species and an attractive model organism for the study of hypoxia because it is susceptible to hypoxia and has a higher oxygen consumption rate than many other farmed shrimp (Li et al., 2004). To investigate whether MnHb is involved in the molecular mechanisms of hypoxic stress in *M. nipponense*, we isolated the full-length MnHb cDNA, and analyzed its expression pattern in different tissues and protein localization in the gill in the presence and absence of hypoxic stress.

2. Materials and methods

2.1. Experimental animals and hypoxia treatment

Wild, healthy *M. nipponense* (wet mass 1.26–4.25 g) were collected from Tai Lake, Wuxi Province, China (120°13′44″E, 31°28′22″N).

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Table 1
Primers used in this study.

Primer	Primer sequence (5′-3′)
MnHb-F1 (5′ RACE out primer)	CCGAACGGCCATGATAAGGA
MnHb-F2 (5′ RACE in primer)	AGGAGCAGCTCAGAACGAAC
MnHb-R1 (3′ RACE out primer)	TCTCTTGCAGTGGTTCTCGC
MnHb-R2 (3′ RACE in primer)	CGGTGCCTTAGAGTGAGACC
MnHb-F (Real-time primer)	GGAGCGGGTCTTCAAAGGTT
MnHb-R (Real-time primer)	CGGTGCCTTAGAGTGAGACC
MnHb-F CDS amplification (*Nde*I)	AAGATGGGGTCCATGCTAA
MnHb-R CDS amplification (*Xho*I)	TCAAGAATCACTCGGCTCG
β-Actin F (Real-time primer)	TATGCACTTCCTCATGCCATC
β-Actin R (Real-time primer)	AGGAGGCGGCAGTGGTCAT

Samples were transferred to the laboratory and maintained in six 300-L tanks containing aerated freshwater. Prawns were allowed to acclimate to their new environment for one week. For the hypoxia challenge experiment, some prawns were randomly divided into two groups and maintained in filtered fresh water. The control group were maintained under normoxia (6.5 ± 0.2 mg O_2 L^{-1}), and the hypoxia group were subjected to 2.0 ± 0.1 mg O_2 L^{-1} for 1, 3 or 24 h by bubbling nitrogen into the tank as described previously (Sun et al., 2014). All treatments were performed in triplicate for control and treatment groups. For each time point, gill tissue was removed from three prawns and then stored at −80 °C until use. Negative control samples were from air-saturated water (normoxia). This study was approved by the Institutional Animal Care and Use Ethics Committee of the Freshwater Fisheries Research Center, Chinese Academy of Fishery Sciences (Wuxi, China).

2.2. Cloning of the MnHb cDNA

Total RNA was extracted using TRIzol reagent according to the manufacturer's instructions. First strand cDNA was synthesized using a reverse transcriptase M-MLV kit (TaKaRa, Japan). Gene-specific primers (Table 1) were designed to obtain the 5′- and 3′-ends of cDNA on the basis of the sequences of partial fragments, following the manufacturer's instructions in the 3′-RACE and 5′-RACE kits (TaKaRa). The partial cDNA sequences were obtained from an RNA-Seq database based on the transcriptome assembly of expressed short reads of *M. nipponense* (Sun et al., 2015). PCR products were sequenced using an ABI3730 DNA Analyzer after insertion into vector pMD-19T.

2.3. Nucleotide sequence and bioinformatics analyses

Sequences were analyzed using BLASTX and BLASTN (http://www.ncbi.nlm.nih.gov/BLAST/). Multiple sequence alignment was carried out using ClustalW 1.81. Phylogenetic trees were generated by the neighbor-joining method in Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0.

2.4. Analysis of MnHb expression

Gene-specific primers (Table 1) were designed for analysis of the expression of MnHb in various tissues including muscle, heart, gill, hepatopancreas, and hemocytes. cDNAs from different tissues and treatments were synthesized from total DNA-free RNA (1 μg) using a Prime Script RT reagent kit (TaKaRa) according to the manufacturer's protocols. The RT-PCR reaction conditions were as previously described (Hu et al., 2015). All PCR products were electrophoresed on 1% (*w*/*v*) agarose gels. The stability of reference genes was analyzed in our previous study (Sun et al., 2016). qRT-PCR was performed in a Bio-Rad iCycler iQ5 Real-Time PCR system (Bio-Rad, USA) in conditions previously described (Qiao et al., 2015). Three replicate qPCRs were performed per sample, and samples from three prawns were analyzed each time. The expression levels of MnHb mRNA were calculated using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001).

2.5. Biochemical analysis

Antioxidant enzymes defend against reactive oxygen species (ROS); they include superoxide dismutase (SOD), which converts the superoxide radical to peroxide (H_2O_2), and glutathione peroxidase (GPx), which detoxifies H_2O_2 and organic hydroperoxides (Halliwell and Gutteridge, 2001). Malondialdehyde (MDA) is a marker of lipid peroxidation (Draper and Hadley, 1990). Each gill sample was homogenized in nine volumes of normal saline using an Ultrasonic Cell Disruption System (Sonics) and centrifuged at 5000*g* for 20 min at 4 °C. Supernatants were used for analysis of SOD and GPx activities, and determination of MDA and total protein concentration; these assays used kits from

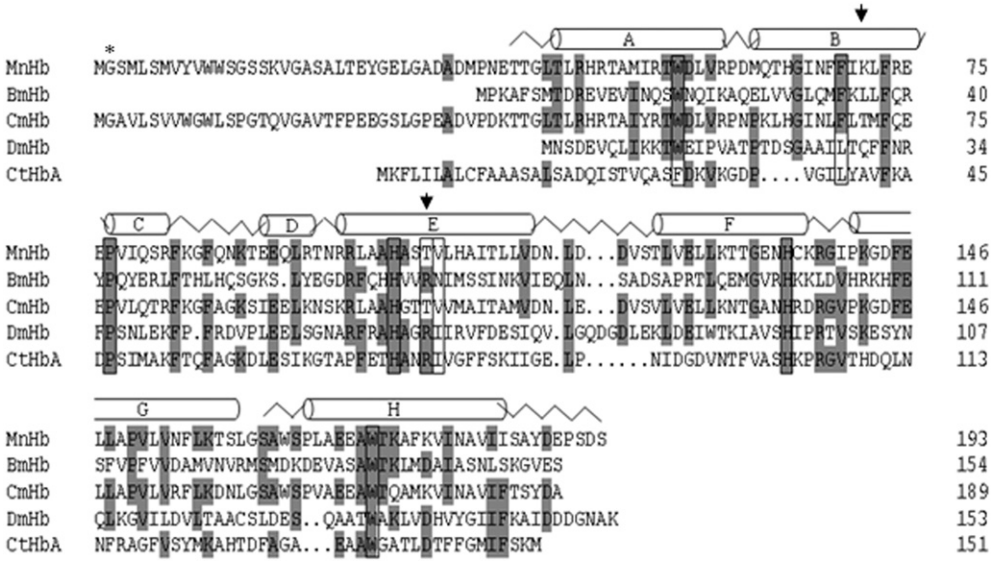

Fig. 1. Secondary structure of *M. nipponense* hemoglobin (MnHb, upper row) superimposed on an amino acid sequence alignment of related Hbs. The two intron positions B12.2 and E11.0 in the MnHb gene are indicated by black arrows, and the N-terminal glycine predicted for protein N-myristoylation is marked by an asterisk. BmHb, *Buenoa macrotibialis* hemoglobin; CmHb, *Carcinus maenas* hemoglobin; DmHb, *Daphnia magna* hemoglobin domain 1; CttHbVI, *Chironomus thummi* hemoglobin VI.