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Short communication

Molecular cloning, characterization and mRNA expression of copper-binding protein hemocyanin subunit in Chinese mitten crab, *Eriocheir sinensis*

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ABSTRACT

Hemocyanin is a copper-binding protein and plays a crucial role in the physiological processes in crustacean. In this study, the cDNA encoding hemocyanin subunit from Chinese mitten crab *Eriocheir sinensis* (EsHc) was cloned by using EST analysis and rapid amplification of cDNA ends (RACE) approach. The full-length cDNA of EsHc was 2573 bp, consisting of a 5′ untranslated region of 51 bp, a 3′ untranslated region of 458 bp, and an open reading frame of 2064 bp. The deduced protein had 688 amino acid residues with molecular mass of 77,997.31 Da. Quantitative real-time RT-PCR analysis showed that the EsHc gene was expressed in haemocytes, hepatopancreas, muscles, gills, and intestines with the highest level of expression in the hepatopancreas and the lowest in the muscles. After *Aeromonas hydrophila* challenge, the relative expression level of EsHc in hemolymph was up-regulated at 3 h post-injection of bacteria followed by a gradual recovery from 12 to 24 h. In the second set of transcriptional studies, the mRNA expression patterns of EsHc in haemocytes and hepatopancreas were measured by quantitative real-time RT-PCR after the Chinese mitten crab were fed six diets containing different levels of copper (0, 10, 20, 40, 80 and 400 mg kg^{-1}) for 8 weeks, respectively. The feeding trial showed that the expression levels of EsHc mRNA significantly increased at the copper levels of 20 −40 mg kg^{-1}. This study implies that the expression levels of EsHc could be affected by dietary copper in the hepatopancreas and haemocytes, and hemocyanin may be potentially involved in the immune responses of the Chinese mitten crab.

1. Introduction

Chinese mitten crab *Eriocheir sinensis* is an economic species for aquaculture in China and other Asian countries [1]. Due to high stocking density in intensive culture and deterioration of environmental conditions, various diseases caused by bacteria, fungi, viruses and rickettsia-like organisms have frequently occurred in the population of cultured *E. sinensis* [2,3]. Therefore, a better understanding of the innate immunity of *E. sinensis* through nutritional modulation will help disease control through dietary management in crab farming. It has been well documented that the level of dietary copper is essential to the normal function of the immune system in animals [4,5]. At least 21 copper-containing enzymes are known, functioning as redox catalysts (e.g., cytochrome oxidase, monoamine oxidase) or dioxygen carriers (e.g., hemocyanin) [6]. However, copper is not only an essential trace element but also has potential toxicity to animals. The most studied and best explained copper toxicity mechanisms involve inhibition of key enzymes, disruption of osmoregulation in the gill and reactive oxygen species generation [7–10]. Therefore, to alleviate the potential deleterious effects of copper, hemolymph has evolved many proteins such as hemocyanin that regulate the transport and storage of this free copper in tissues [11].

As an invertebrate, crab has no adaptive immunity [12] and largely relies on innate immunity including cellular and humoral immunity to defend invading microbes [13]. During this process, hemocyanin can provide an immediate and rapid immune response to invading microorganisms and is essential to innate immune defense molecules [14–22]. Hemocyanin, like hemoglobin and many other proteins, is a multisubunit molecule [23], and plays a prominent role in transporting exogenous copper to accumulatory sites in respiratory pigment. Since copper is a structural component of hemocyanin in the respiratory protein of crustacean [24,25], an adequate level of copper in crustacean hemolymph is required [26,27]. Furthermore, previous studies on the effect of dietary copper

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on non-specific immunity enzymes in *E. sinensis* have mainly focused on the post-translational level [26]. However, there have been no published data available on the possible interaction between differential hemocyanin mRNA expressions and dietary copper levels in crustacean. Although some hemocyanin subunits have been found in invertebrate animals [28–30], its biological functions have not been well studied yet. We hypothesize that hemocyanin plays an essential role in innate immunity in cooperation with the dietary copper, so identification and cloning of the hemocyanin genes involved in immune response will help understand the molecular basis of the innate immune response to copper deficiency in diet.

The objectives of this study were: (1) to clone the full-length cDNAs of hemocyanin subunit from the crab *E. sinensis* (designated as EsHc), (2) to investigate the distribution of EsHc mRNA expression in crab tissues, (3) to examine the expression of EsHc in immune defense after crab receiving bacterial challenge, (4) to clarify the level of EsHc expression in the hepatopancreas and haemocytes of *E. sinensis* fed different levels of copper.

2. Materials and methods

2.1. Animals, experimental designs and diets

Juvenile *E. sinensis* were obtained from Chongming Fisheries Co., Shanghai. Six artificial diets with different levels of copper supplement were formulated to feed crab. The graded levels of dietary Cu ($CuSO_4 \cdot 5H_2O$) were supplemented with 0, 10, 20, 40, 80 and 400 mg Cu kg^{-1} diet (Analytical Reagent, Shanghai Chemical Co., Shanghai, China), respectively. The compositions of the experimental diets are presented in Table 1. Procedures of diet preparation were adapted from a previous study on *E. sinensis* [31], and the resulting Cu concentrations in the diets were measured as 1.88, 11.85, 20.78, 40.34, 79.56 and 381.2 mg kg^{-1} diet, respectively. Dietary Cu concentrations were analyzed by flame atomic absorption photometry [32]. Prior to feeding, crabs were acclimated to the laboratory condition for 1 week. During the acclimatization period, crabs were fed with Cu-deficient diet (Table 1). Crabs (0.45 ± 0.01 g) were assigned to 18 tanks using a completely randomized design including six treatments in triplicates. Each tank was stocked with 25 crabs and contained black tiles as shelters. Crabs were fed to satiation twice daily (800 and 1600 h) for 8 weeks. Every morning, fecal pellets and excess diets in the water were removed, and 33% of the water was exchanged daily to maintain water quality. During the experimental period, water temperature was controlled at 27 ± 1 °C. Other water quality parameters including pH, dissolved oxygen and total ammonia–nitrogen in the rearing tanks were 7.9–8.2, 6.4–7.8 mg L^{-1} and 0.08–0.09 mg L^{-1}, respectively. After 1-day food deprivation at the end of trial, target tissues were separately extracted, frozen in liquid nitrogen, and stored at −80 °C until RNA extraction and analysis.

Table 1
Ingredients and approximate compositions of experimental diets (g kg^{-1}).

Item	Percentage dry weight
Casein vitamin-free[a]	300
Fish meal	200
Corn starch	300
Cellulose[b]	60
Lecithin[b]	5
Cholesterol[b]	5
SO/FO[c]	50
Vitamin mix[d]	20
Cu-free mineral mix[e]	40
Carboxymethylcellulose[b]	20
Proximate analysis ($n = 3$)	
Crude protein	380.3
Crude lipid	60.2
Crude ash	116.5

[a] Sigma Chemical, St. Louis, MO, USA.
[b] China National Medicine Corporation Co., Ltd, Beijing, China.
[c] Soybean oil:fish oil = 1:1. Fish oil supplied by Xiamen Xinsha Pharmaceutical Co. Ltd, Xiamen, China. Soybean oil was supplied by National Golden Dragon Fish Co. Ltd, Shanghai, China.
[d] Vitamin mixture, each 100 g of diet contained: vitamin E, 2160 IU; vitamin C, 60 mg; pantothenic acid, 240 mg; pyridoxine, 60 mg; riboflavin, 120 mg; thiamin, 60 mg; biotin, 60 mg; niacin, 600 mg; folic acid, 60 mg; inositol, 600 mg.
[e] Composition of mineral mixture (%): NaH_2PO_4, 10; KH_2PO_4, 21.5; $Ca(H_2PO_4)_2 \cdot 2H_2O$, 26.5; $CaCO_3$, 10.5; Ca-lactate, 16.5; $MgSO_4 \cdot 7H_2O$, 10; $AlCl_3 \cdot 2H_2O$, 1.2; $ZnSO_4 \cdot 7H_2O$, 0.511; Fe-citrate, 0.061; $MnSO_4 \cdot 4H_2O$, 0.143; KI, 0.058; $CoCl_2 \cdot 6H_2O$, 0.176; KCl, 2.8.

2.2. Bacterial challenge

Crabs (100 g) were obtained from a commercial crab farm, Shanghai, and acclimatized at 23 ± 1 °C for 1 week prior to the experiment. In this trial, 72 crabs were used, randomly divided into two groups with triplicate each, and kept in filtered fresh water. The Gram-negative bacterium *Aeromonas hydrophila* obtained from Guangzhou Microbiology Research Institute was used to challenge the crabs. Crabs receiving an injection of 100 μL live *A. hydrophila* in saline suspension (7.2×10^8 CFU mL^{-1}) were used as the challenged group, while animals in the control group were injected with the same amount of saline water. After injections, the crabs were returned to the rearing tanks and six individuals from each tank were randomly sampled at 0, 1.5, 3, 6, 12, 24 h post-injection, respectively. Haemolymph was collected from the base of the third pereiopod, diluted in an equal volume of anti-coagulant solution [33], and centrifuged at 4 °C for 10 min at 800 *g*. The haemocyte pellets were preserved in liquid nitrogen immediately for RNA extraction. At the same time, different crab tissues were extracted and preserved in liquid nitrogen for RNA extraction.

2.3. RNA extraction, reverse transcript and part of hemocyanin cDNA cloning

Total RNA was extracted using a Unizol reagent kit (Biostar, Shanghai, China) according to the manufacturer's protocol. The synthesis of the first strand cDNA was performed by using superscript™ III RNAse H^- reverse transcriptase (Invitrogen, USA) to transcribe poly $(A)^+$ RNA with oligo-d (T) 18 as the primers. Reaction conditions were complied with the manufacturer's instruction.

Immune-related genes in the haemocytes of Chinese mitten crab *E. sinensis* have been discovered by the expressed sequence tag (EST) and annotation analysis [34]. One EST (hem_0028_C03.ab1) in the library was homologous to the hemocyanin subunit 6 of *Cancer magister* (U48881.2). The sequences were chosen for further amplification of the hemocyanin gene from the mitten crab. The primers EsHc 01 and EsHc 02 (Table 2) designed from hem_0028_C03.ab1 were used to amplify the partial cDNA hemocyanin of the *E. sinensis* haemocytes. The PCR reactions were performed as follows: 30 cycles of denaturation at 94 °C for 1 min, annealing at 60 °C for 30 s, and elongation at 72 °C for 3 min, followed by a 10 min extension at 72 °C and cooling to 4 °C. The PCR fragments were subjected to electrophoresis on a 1% agarose gel for length difference and cloned into the pMD-18T vector (Takara, Japan). After transforming into the competent cells of *Escherichia coli* DH5a, recombinant bacteria were identified by blue/white screening and confirmed by PCR. Three of the positive clones were sequenced in both directions and these resulting sequences were verified and subjected to cluster analysis in NCBI.

2.4. Cloning and characterization of hemocyanin

Three gene-specific primer sets (Table 2) were designed based on the expressed sequence tag (EST) of the haemocytes of the Chinese