Aquaculture 434 (2014) 385–396

Contents lists available at ScienceDirect

Aquaculture

journal homepage: www.elsevier.com/locate/aqua-online

Molecular cloning and characterization of three novel Hemocyanins from Chinese mitten crab, *Eriocheir sinensis*

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ARTICLE INFO

Article history:
Received 13 May 2014
Received in revised form 29 July 2014
Accepted 31 July 2014
Available online 8 August 2014

Keywords:
Eriocheir sinensis
Hemocyanin
Innate immunity
Bacteria challenge

ABSTRACT

Hemocyanin is a copper-binding protein and plays a crucial role in the physiological processes in crustaceans. However, little is known about the hemocyanin from the Chinese mitten crab, *Eriocheir sinensis*. In this study, three forms of hemocyanins designated as *EsHc1*, *EsHc2* and *EsHc3* were cloned from *E. sinensis* by using expressed sequence tag (EST) analysis and rapid amplification of cDNA ends (RACE) approach. The open reading frames (ORFs) of *EsHc1*, *EsHc2* and *EsHc3* genes were 2182, 2580 and 2220 bp encoding proteins with 678, 662 and 691 amino acids, respectively, and all contain three tandem hemocyanin domains: 1) hemocyanin N, 2) hemocyanin M or tyrosinase, and 3) hemocyanin C domain. BLASTP and phylogenetic tree analysis showed that EsHc1 was clustered together with cryptocyanin 1 from *Portunus pelagicus* (PpCc1) and cryptocyanin from *Metacarcinus magister* (MmCc), EsHc2 and EsHc3 with hemocyanin from *Pacifastacus leniusculus* (PlHc) and hemocyanin subunit 1 from *M. magister* (MmHc1) were gathered into one clade. *EsHc1* was mainly expressed in hepatopancreas and hemocytes with a lower level of expression in nerves, eyestalk, muscles, intestine, gills and heart; whereas *EsHc2* and *EsHc3* were mainly expressed in hepatopancreas and were also detected in hemocytes. Quantitative real-time RT-PCR analysis showed that *EsHcs* mRNA transcription in hepatopancreas was significantly expressed at various time points after infection with Lipopolysaccharide (LPS), peptidoglycan (PGN), *Staphylococcus aureus*, *Vibrio parahaemolyticus* and *Aeromonas hydrophila*. In summary, there is evidence that the three isoforms of hemocyanin genes participate in the innate immune response against bacteria infecting the Chinese mitten crab.

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1. Introduction

Chinese mitten crab *Eriocheir sinensis* is an economically important species and has been cultured commercially in China and other Asian countries (Li et al., 2007). With the development of intensive culture and environmental deterioration, this aquatic production frequently incurred serious infectious diseases caused by viruses, fungi, bacteria, spiroplasma and parasites, resulting in decreased growth in crab production and vast economic losses (Bonami and Zhang, 2011; Morado, 2011; Wang, 2011; Wang et al., 2002, 2004). As an invertebrate, crustaceans (including crabs) lack a true adaptive immune response system (Hoffmann and Reichhart, 2002). However, living in an aquatic environment rich in microorganisms, crabs have developed effective systems for detecting and eliminating noxious microorganisms, which depend entirely on a non-specific innate immune response (Mu et al., 2011). The defense mechanisms, largely based on the activity of blood cells, include hemolymph coagulation, a rapid and powerful system that prevents blood loss upon wounding and participates in the engulfment of invading microorganisms (Destoumieux et al., 1997). Therefore, studies on crustacean innate immunity are needed to provide new insights into the control of infectious diseases and the development of sustainable crab farming.

Hemocyanin (Hc) is a large, oxygen-transport protein, freely dissolved in the hemolymph of various arthropods and mollusks (Van Holde and Miller, 1995), and plays an essential role in transporting exogenous copper to accumulator sites in respiratory pigment (Rtal and Truchot, 1996). Copper is a structural component of hemocyanin in the respiratory protein of crustaceans (Engel and Brouwer, 1987; Rainer and Brouwer, 1993). It has been well documented that the level of dietary copper is essential to the normal function of the immune system in animals (Bala and Failla, 1992; Lall, 2002). However, copper is not only an essential trace element but also is potentially toxic to animals. So an adequate level of copper in crustacean hemolymph is required (Lee and Shiau, 2002; Sun et al., 2011). Besides traditional function of hemocyanin as the transport and storage of molecular oxygen for many arthropods, it has been demonstrated that hemocyanin is a multi-functional protein involved in several physiological processes such as protein storage, osmotic regulation, ecdysone transportation, molting regulation and exoskeleton formation (Adachi et al., 2005; Jaenicke et al., 1999; Paul and Pirow, 1998).

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http://dx.doi.org/10.1016/j.aquaculture.2014.07.033
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Table 1
Primers used in the current study.

Primers name	Sequences (5′-3′)
EsHc1-F	TCCTTTGGTCCACCGAGACATCA
EsHc1-R	AACCTGACGGTCGAGCATGGTGTAG
EsHc2-F	GCCCCCGCTCTACGAGGTCACGC
EsHc2-R	TCGGAGTGCGTGACGGCAATGTA
EsHc3-F	GCCACCCTACACCGCCGACGAAC
EsHc3-R	ATGCCGATGTCCTCGCCGAAGTAG
UPM	
Long	CTAATACGACTCACTATAGGGCAAGCAGTGGT ATCAACGCAGAGT
Short	CTAATACGACTCACTATAGGGC
5′-CDS Primer A	T_{25}VN
SMARTerIIA oligo	AAGCAGTGGTATCAACGCAGAGTACXXXXX
3′-CDS primer A	AAGCAGTGGTATCAACGCAGAGTAC(T)30VN
EsHc1-RT-F	TCACTGACGCTGCTGAGGACG
EsHc1-RT-R	CGATGAAGACGCCACGGTTGTA
EsHc2-RT-F	CAAGGGTAACGAGGAGGGTCT
EsHc2-RT-R	AAGTAGTAAAGGGGAGGGGGG
EsHc3-RT-F	TCGAGTTCTGGCTCAATGTGTA
EsHc3-RT-R	CTTAGTGCTGGTCTTTGTGTTT
Es-GAPDH-RT-F	CTGCCCAAAACATCATCCCATC
Es-GAPDH-RT-R	CTCTCATCCCCAGTGAAATCGC

X = undisclosed base in the proprietary SMARTer oligo sequence.
N = A, C, G, or T; V = A, G, or C.

Recently, more and more reports reveal that hemocyanin can provide an immediate and rapid immune response to invading microorganisms, and it has been reported as a novel and important defense molecule of the non-specific innate immune system (Decker and Jaenicke, 2004; Decker et al., 2001; Jiang et al., 2007; Lei et al., 2008; Nagai et al., 2001; Zhang et al., 2004, 2006). For example, hemocyanin of the horseshoe crab *Tachypleus tridentatus* could be functionally converted into a phenoloxidase-like enzyme by the clotting enzyme and by chitin-binding antimicrobial peptides (Decker and Jaenicke, 2004; Decker et al., 2001). Hemocyanin isolated from horseshoe crab *Carcinoscorpius rotundicauda* is activated by microbial proteases to produce reactive oxygen species (ROS), resulting in formation of a strong antimicrobial response (Jiang et al., 2007; Nagai et al., 2001). The black tiger shrimp *Penaeus monodon* hemocyanin is an antiviral agent against a variety of viruses including DNA and RNA viruses (Zhang et al., 2004). Two subunits of hemocyanin from the penaeid prawn *Penaeus japonicas* exhibit differences in antiviral defense (Lei et al., 2008). Moreover, hemocyanin from shrimp *Litopenaeus vannamei* reacts with anti-human Ig as an antigen, binds to bacteria as an agglutinin, binds to vertebrate erythrocytes as a hemolysin, and acts as an immune-enhancing protein (Zhang et al., 2006). However, presently, the biological functions of *E. sinensis* hemocyanin have not been well studied, thus there is a need for further examination.

In the present study, three novel hemocyanins were identified in the Chinese mitten crab *E. sinensis* (designated as *EsHc1*, *EsHc2 and EsHc3*). Their mRNA distributions in different tissues were studied, and the expression patterns were examined in hepatopancreas after crabs were challenged with lipopolysaccharide (LPS), peptidoglycan (PGN), *Staphyloccocus aureus*, *Vibrio parahaemolyticus* and *Aeromonas hydrophila*. This research contributes toward a better understanding of the innate immunity of *E. sinensis*.

2. Materials and methods

2.1. Experimental animals and microbes

Healthy *E. sinensis*, averaging 60 g in weight, were collected from a local market in Yangzhou, Jiangsu Province, China, and cultured in 200 L aquaculture tanks containing freshwater and an aeration system at 23 ± 2 °C for two weeks before processing.

Lipopolysaccharide (LPS) (*Escherichia coli* Serotype 055:B5) and peptidoglycan (PGN) (*Micrococcus luteus*) were purchased from Sigma (St. Louis, MO, USA). The Gram-positive bacterium *S. aureus* (obtained from the Shandong University), and Gram-negative bacterium *V. parahaemolyticus* (ATCC 17802, Microbial Culture Collection Center, Beijing, China) were grown in LB broth at 37 °C. Live *A. hydrophila* (ATCC 7966, Microbial Culture Collection Center, Beijing, China) were grown in LB broth at 28 °C.

2.2. Immune challenge in crabs

Two hundred crabs were employed for the immune challenge experiment. The crabs were randomly divided into 6 groups (LPS-, PGN-, *S. aureus*-, *A. hydrophila*-, *V. parahaemolyticus*- and PBS-challenged groups) and each group contained 30 individuals. Each of the challenged groups received an injection of one of the following infectious pathogens: 50 μl LPS (0.5 μg/μl), 50 μl PGN (0.5 μg/μl), 50 μl of

A

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agcacaagctccagcatgaaggtcctagttgtgttttctctggtggccctcagcgccgca
                M  K  V  L  V  V  F  S  L  V  A  L  S  A  A   15
gccgccgtgtccggctatatgtcggatgagccggacggcgtttccacacaacagaagcaa
 A  A  V  S  G  Y  M  S  D  E  P  D  G  V  S  T  Q  Q  K  Q   35
tatgacatgacctacgccttctacaagatcttcgagccaatccgcgacaacaacatgctt
 Y  D  M  T  Y  A  F  Y  K  I  F  E  P  I  R  D  N  N  M  L   55
gagaaggccatgaccttcaacccagtggcggatacctccatgtacaaggacggaggcgtc
 E  K  A  M  T  F  N  P  V  A  D  T  S  M  Y  K  D  G  G  V   75
gccgtccggaagctcatgtacgagttcacacacgaacgtctgctggagaagaagcactgg
 A  V  R  K  L  M  Y  E  F  T  H  E  R  L  L  E  K  K  H  W   95
gctgccgccaccaacaaacgtcacctggaggaggcaatcatgctgttcgaggtgttcatg
 A  A  A  T  N  K  R  H  L  E  E  A  I  M  L  F  E  V  F  M  115
cagtgcaaggactggaactgcttctccagcaacgccgcctacttcagggagcgagtcaac
 Q  C  K  D  W  N  C  F  S  S  N  A  A  Y  F  R  E  R  V  N  135
gaggaggagttcctctacgccgcctaccatgccatcaaacactccccttcacacagaac
 E  E  E  F  L  Y  A  A  Y  H  A  I  K  H  S  P  F  T  Q  N  155
gtcgtgctgccagccgtctacgaagtcaagccccactacttcaccaaggcccaggtcatc
 V  V  L  P  A  V  Y  E  V  K  P  H  Y  F  T  K  A  Q  V  I  175
gaccaagcctacgaggcccacgagatgaagctgaagaacatggtcttccagaacaacttc
 D  Q  A  Y  E  A  H  E  M  K  L  K  N  M  V  F  Q  N  N  F  195
actggtaatcccaacgacatggaacagagagtcgcctactaccgtgaggacctcggaatt
 T  G  N  P  N  D  M  E  Q  R  V  A  Y  Y  R  E  D  L  G  I  215
ggaactcaccacttcatgatccacctggaaaacccattctggtggaaagacacctacggc
 G  T  H  H  F  M  I  H  L  E  N  P  F  W  W  K  D  T  Y  G  235
taccgcattgacaggaagggagagaacttcttctacgcctaccaccagctcctcaaccgc
 Y  R  I  D  R  K  G  E  N  F  F  Y  A  Y  H  Q  L  L  N  R  255
tacgacgccgagcgcatctccaactacatgcccctcctcgaggagctgaaactggacgag
 Y  D  A  E  R  I  S  N  Y  M  P  L  L  E  E  L  K  L  D  E  275
cccctgcacgagggctttgctccccagaccacctacaagttcggcgctcccttccccatc
 P  L  H  E  G  F  A  P  Q  T  T  Y  K  F  G  A  P  F  P  I  295
cgcaacgacgacatcaagctgcaggacgtcgacagggtcggcaaaatccacgagcttgtc
 R  N  D  D  I  K  L  Q  D  V  D  R  V  G  K  I  H  E  L  V  315
cacatggaggaccgtatctacgacgccattgctcacggttatgtggagaacgagcaggga
 H  M  E  D  R  I  Y  D  A  I  A  H  G  Y  V  E  N  E  Q  G  335
aacaaaatcaacatcgaaaatgacaacggcatcgaaatcctgggtgacatcatccagtcg
 N  K  I  N  I  E  N  D  N  G  I  E  I  L  G  D  I  I  Q  S  355
tcttactacagccccaaccgcaagtactacggcaacctcaccaccctggcctacaccatg
 S  Y  Y  S  P  N  R  K  Y  Y  G  N  L  T  T  L  A  Y  T  M  375
ctcgatcgtcaggttgaccccaaggacaagtacgacctgccccctggcgtggtcgcccac
 L  D  R  Q  V  D  P  K  D  K  Y  D  L  P  P  G  V  V  A  H  395
atggagctgtacccccgcgaccccgccgcatggaggctgcaaaagcgcatcgacaacatc
 M  E  L  Y  P  R  D  P  A  A  W  R  L  Q  K  R  I  D  N  I  415
ttcagggagcatattgactctttcctccttacaccaaggaacagctcgagttcaccgga
 F  R  E  H  I  D  S  L  P  P  Y  T  K  E  Q  L  E  F  T  G  435
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Fig. 1. Nucleotide (above) and the deduced amino acid sequences (below) of the *EsHc1* to *EsHc3* cDNAs from *E. sinensis*. Deduced amino acid residues are numbered on the right. Start codon and stop codon are shown in bold type. Signal peptide sequences are labeled in italics. The hemocyanin N domains of *EsHc1* to *EsHc3*, located behind the signal peptide, are underlined; the hemocyanin M domain of *EsHc1* or the tyrosinase domains of *EsHc2* and *EsHc3* are shaded; the boxes denote the hemocyanin C domains of *EsHc1* to *EsHc3*.

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