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Cloning and characterization of the hemocyanin gene of prawn *Macrobrachium nipponense*

[Karides *Macrobrachium nipponense* hemosiyanin gen klonlanması ve karakterizasyonu]*

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ABSTRACT

Aim: To clone the hemocyanin gene from *Macrobrachium nipponense*, and to do the bioinformatics and spatial and temporal expressions analysis of the gene.

Material and methods: The hemocyanin gene (MNHc) was cloned from hepatopancreas of *Macrobrachium nipponense* using the rapid amplification of cDNA ends method and its sequence was analyzed with biological software. Spatial and temporal expressions of the gene were detected by real-time PCR.

Results: The full-length cDNA of hemocyanin was 2151 bp, containing a 14 bp of 5′-untranslated region, a 148 bp of 3′-untranslated region and a 1989 bp of open reading frame. This gene encoded a protein of 663 amino acids with a calculated molecular mass of 76.65 kDa and isoelectric point of 5.42. The deduced amino acid sequence contained three hemocyanin domains and two conserved copper-binding sites, and exhibited higher similarity with that of *Pacifastacus leniusculus* (70%) and *Litopenaeus vannamei* (63%). This gene was expressed highly in the hepatopancreas, weakly in the muscles and haemocytes, and hardly in the mandibular organ, epidermis and ovarian. Its transcript derived from hepatopancreas was varied at different stages during the molt cycle. The transcript level of gene increased significantly 3 h after injection of pathogenic bacteria *Aeromonas hydrophila*.

Conclusion: Like other crustaceans, the hemocyanin gene of *Macrobrachium nipponense* contained the typical hemocyanin domains and conserved copper-binding sites, and was highly expressed in hepatopancreas during stage C, as well as acted as an important molecule involved in immune defence of *Aeromonas hydrophila*.

Key Words: *Macrobrachium nipponense*, hemocyanin, cloning, real-time PCR, molt stage.

Conflict of Interest: There is no conflict of interest in respect of this manuscript.

ÖZET

Amaç: Hemosiyanin geninin *Macrobrachium nipponense*'den klonlanması, biyoinformatik çalışmalarının tamamlanması ve genin zamansal ve mekansal ekspresyonunun analizi.

Gereç ve Yöntemler: Hemosiyanin geni (MNHc) *Macrobrachium nipponense* hepatopankreasından cDNA sonlarının hızlı amplifikasyon metodu kullanılarak klonlandı. Sekans analizi biyolojik yazılımlar ile ve genin zamansal ve mekansal ekspresyonu real-time PCR ile saptandı.

Bulgular: Tüm hemosiyanin cDNA'sı, 5'-kopyalanmamış bölgeye ait 14 baz çifti, 3'-kopyalanmamış bölgeye ait 148 baz çifti ve 1989 baz çifti içeren açık okunabilir bölge ile toplam 2151 baz çiftidir. Bu gen 663 amino asit içeren, hesaplanmış molekül ağırlığı 76.65 kDa ve izoelektrik noktası 5.42 olan bir proteini kodlar. Bilinen amino asit sekansı üç hemosiyanin domeyni ve korunmuş iki bakır bağlayan bölge içerir. *Pacifastacus leniusculus* (%70) ve *Litopenaeus vannamei* (%63) ile çok fazla benzerdir. Bu gen çoğunlukla hepatopankreasda eksprese edilir. Kas ve hemositte çok az, mandibular organ, epidermis ve ovaryumlarda ise eksprese edilmez. Hepatopankreasdan kaynaklanan kopyası kabuk değişim döngüsünün farklı basamaklarında çeşitlilik gösterir. Patojenik bakteri olan *Aeromonas hydrophila* enjeksiyonundan 3 saat sonra genin kopyalanma düzeyi belirgin olarak artar.

Sonuç: Diğer kabuklulara benzer şekilde, *Macrobrachium nipponense* ait hemosiyanin genide tipik hemosiyanin domeynleri ve korunmuş bakır bağlayan bölgeleri içerir. Hepatopankreasda C evresinde fazlaca eksprese edilirken, *Aeromonas hydrophila* infeksiyonunda immün korunmada önemli bir molekül olarak davranmaktadır.

Anahtar Kelimeler: *Macrobrachium nipponense*, hemosiyanin, klonlama; real-time PCR; kabuk değişim evresi

Çıkar Çatışması: Yazarlar herhangi bir çıkar çatışması bildirmemişlerdir.

Introduction

The copper-containing protein, hemocyanin, is present in the hemolymph of both mollusks and arthropods as high-molecular weight oligomers [1]. It is colorless in the deoxy form and blue in the oxygenated form. Hemocyanin, together with hemerythrin and hemoglobin, has been ranked as three key respiratory proteins. As the main protein component of haemolymph, hemocyanin typically represents up to 90% of total amount of protein [1]. Except for its primary function as an oxygen-carrier, hemocyanin is a multi-functional protein involved in several physiological processes and immue defence such as phenoloxidase activity [2, 3], hemolytic activity [4], anti-virus [5] and anti-bacteria [6].

The freshwater prawn *Macrobrachium nipponense (M. nipponense)* an economically important species, is a primary inland cultured species in China, Japan and Annam. However, recently, *M. nipponense* production has suffered great losses due to the outbreaks of disease caused by various pathogenic bacteria. Among them, *Aeromonas hydrophila* is considered to be the major threat to the commercial cultivation of *M. nipponense* aquaculture in China. Therefore, prevention of disease outbreak and enhancement of immunity of the prawn are of primary concern. Prawns, like other invertebrates, lack adaptive immunity and they have to rely on innate immune systems for defensing and eliminating microorganisms [7, 8]. Many non-specific molecules, including phenoloxidase, antimicrobial peptides, lysozyme and lectins, specifically hemocyanin, have been cloned and characterized in succession [1, 5, 6, 9]. Up to date, however, there is no report about hemocyanin gene from *M. nipponense*.

In this study we cloned for the first time the full length cDNA of hemocyanin from hepatopancreas of *M. nipponense,* compared its sequence with other crustacean hemocyanin, predicted its structure, investigated its tissue distribution, and evaluated its expression in hepatopancreas of prawn in relation to different molt stages, challenge by *A. hydrophila*. This study may further facilitate the understanding of hemocyanin structure, function and the resistant mechanism of prawn.

Materials and methods

Animal and experimental design

M. nipponense, used in the study were obtained from a Fishing Ground in Yuanyang, Henan Province, China, and acclimated at 27±1^{0}C in running-water tanks in the laboratory and fed twice daily for 2 weeks before the experiments. Only healthy prawns with body length of 4.5±0.5 cm in the inter-molt stage were used for the study, except for the molt cycle test.

The molt cycle is divided into several sub-stages according to the degree of hardness of the exoskeleton and the retraction of the epithelium within the setae of antennal scales [10] . They are post-molt (A, B), inter-molt (C) and pre-molt (D0, D1, D2, and D3) stages. Five molt stages (A, B, C, D0/1, and D2/3) were used for this study and prawns from each stage were taken to examine hemocyanin expression in the hepatopancreas. Each example was performed in triplicates with 3 prawns each.

M. nipponense in the inter-molt stage were injected with 20μL bacterial suspension of *A. hydrophila* into the ventral sinus, resulting in 1.0×10^5 cfu prawn^{-1}. After injection, the prawns were returned to the PVC tanks. Before the injection and at 3, 6, 12, and 24 h after the injection, respectively, three prawns were randomly sampled from the tanks, hepatopancreas were sampled for hemocyanin expression. Each treatment above was performed in three replicates.

Total RNA isolation and reverse transcription (RT)

Total RNA was extracted from *M. nipponense* in various tissues (hepatopancreas, muscles, haemocytes, mandibular organ, epidermis and ovarian) using TRIzol (Invitrogen Life Technologies, USA) according to the manufacturer's protocol. The quality of RNA was monitored by 1.2% agarose gel electrophoresis (Invitrogen). First-strand cDNAs were synthesized using M-MLV First-Strand cDNA synthesis Kit (Takara, Japan) according to the manufacturer′s instructions.

Polymerase chain reaction (PCR) and subcloning of hemocyanin cDNA

Total RNA and cDNA were prepared from the hepatopancreas of healthy shrimps as described above. The hepatopancreas cDNA was used as the template for PCR reaction. Degenerate primers (P1 and P2) were designed based on the highly conserved nucleotides of known hemocyanin of arthropods. The PCR product was subcloned into the pMDT-19 (Takara, Japan) and sequenced from both directions by commercial sequencing company (Invitrogen).

RACE-PCR was performed to obtain the full-length cDNA sequence of hemocyanin. From the partial cDNA sequences, specific primers designed were used to characterize the 5′ and 3′ regions of the hemocyanin cDNAs by RACE-PCR (Takara, Japan) according to the manufacturer′s protocol. 5′ Race outer primer and 5′ GSP1 were used for the first-round PCR of 5′ RACE, 3′ Race outer primer and 3′ GSP1 for the first-round PCR of 3′ RACE, respectively. Subsequently, the first-round PCR products were used as the template to perform the nested PCR using Race inner primers (5′ iner and 3′ iner) included in the kit, and gene specific primers (5′ GSP2 and 3′ GSP2). The 5′ RACE and 3′ RACE PCR products were gel purified, cloned, and sequenced as above described. The sequences of primers used above were listed in Table 1.

Table 1. Primers used in the experiments

Name of the primer	Sequences (5′-3′)
P1	GGARCAGMANCAYTGGTTCTC
P2	GAMCAGGWCGAAYTCCAKACC
5′ GSP1	CACCAGAAGGGGAAGTCCATA
5′ GSP2	ATAAAGTGGGGGGAGAACAATG
5′Race Outer primer	CATGGCTACATGCTGACAGCCTA
5′Race Inner primer	CGCGGATCCACAGCCTACTGATGATCAGTCGATG
3′ GSP1	GGCACTGCATTGAAATGGACAAA
3′ GSP2	CACGATTTTGAGAGGTCTTGCG
3′Race Outer primer	CCGACCTCAGGGAAATGAAG
3′Race Inner primer	TACCGTCGTTCCACTAGTGATTT
P-F	CGCGGATCCTCCACTAGTGATTTCACTATAGG
P-R	CTGAAGGGAACATACGCATAAGG
18SRNA-F	TGTTACGGGTGACGGAGAA
18SRNA-R	CATTCCAATTACGCAGACTCGG

Sequence and phylogenetic analysis

The hemocyanin gene sequence was analyzed and compared using the BLASTX and BLASTP programs with a GenBank database search. The signal peptide was predicted by SignalP program (http://www.cbs.dtu.dk/services/SignalP). The multiple sequence alignment of the prawn hemocyanin gene was created using the the CLUSTAL W analysis program. The domain architecture of hemocyanin was predicted by SMART (http://smart.embl-heidelberg.de/). The 3-D structure modeling of hemocyanin was predicted by Swiss-model(http://swissmodel.expasy.org/).

Phylogenetic trees were constructed by the Neighbor-joining method using MEGA4.0 software. The reliability of the tree obtained was assessed by bootstrapping, using 1000 bootstrap replications.

Quantification of hemocyanin expression by real-time PCR

The mRNA expression of *M. nipponense* hemocyanin in various prawn tissues, and that in hepatopancreas from *A. hydrophila* injected prawns and different molt-staged prawns was measured by quantitative real-time PCR following the manufacture instruction of SYBR *Premix Ex Taq*™ (TaKaRa, Japan). The hemocyanin primers P-F and P-R (Table 1), and the internal standard 18S rRNA primers 18SRNA-F and 18SRNA-R (Table 1) were designed based on the sequences of the prawn (*M. nipponense*) (GenBank accession No. JF683437, DQ531769.1). The PCR was performed using an ABI 7500 Real-Time Detection System (Applied Biosystems, USA) under the following conditions: initial denaturation at 95°C for 30 sec and amplifying for 40 cycles (95°C, 5 sec; 60°C, 34 sec). The amplifications were carried out in a 96-well plate in a 20 μL of reaction volume containing 10 μL of SYBR *Premix Ex Taq*™, 0.4 μL of ROX reference dye II, 0.4 μL each of forward and reverse primers 10 μM, 2 μL of diluted cDNA and sterile-water to adjust the reaction volume. Each sample had three replicates in each plate. The expression level of hemocyanin was calculated by $2^{-\Delta\Delta CT}$, and the data obtained were subjected to the statistical analysis followed by an unpaired sample *T*-test. Significant difference was accepted at $P<0.05$.

Results

Cloning and characterization of hemocyanin gene from M. nipponense

The full-length cDNA of hemocyanin gene from *M. nipponense* was 2151 bp and contained an open reading frame (ORF) of 1989 bp, a 145 bp of 5′UTR and a 14 bp of 3′UTR which contained a stop codon, a polyadenylation signal site (AATAAA) and a poly(A) tail. This sequence shared homologies of 72%, 70% and 65% with hemocyanin gene of *Pacifastacus leniusculus*(AY193781.1), *Homarus americanus* (AJ272095.1) and *Fenneropenaeus chinensis* (FJ594414.1), respectively. It has been submitted to the NCBI GenBank as accession No. JF683437 and named as MNHc.

The deduced 1989 bp ORF of MNHc encoded a protein of 663 amino acids with a calculated molecular mass of 76.65 kDa, a theoretical pI of 5.42 and a 15 amino acid-signal peptide at N-terminus. The predicted protein domain of MNHc, as identified by the SMART program, consisted of a hemocyanin-N, hemocyanin-M and hemocyanin-C domain (Fig. 1 and Fig. 2). Hemocyanin-M has two copper-binding sites containing six conserved histone residues (H206, H210, H237, H356, H360 and H396) (Fig. 1). Prediction of hemocyanin 3-D structure showed that there existed an α-helix and a β-sheet, similar with that of *Panulirus interruptus* HC1(Fig. 3).

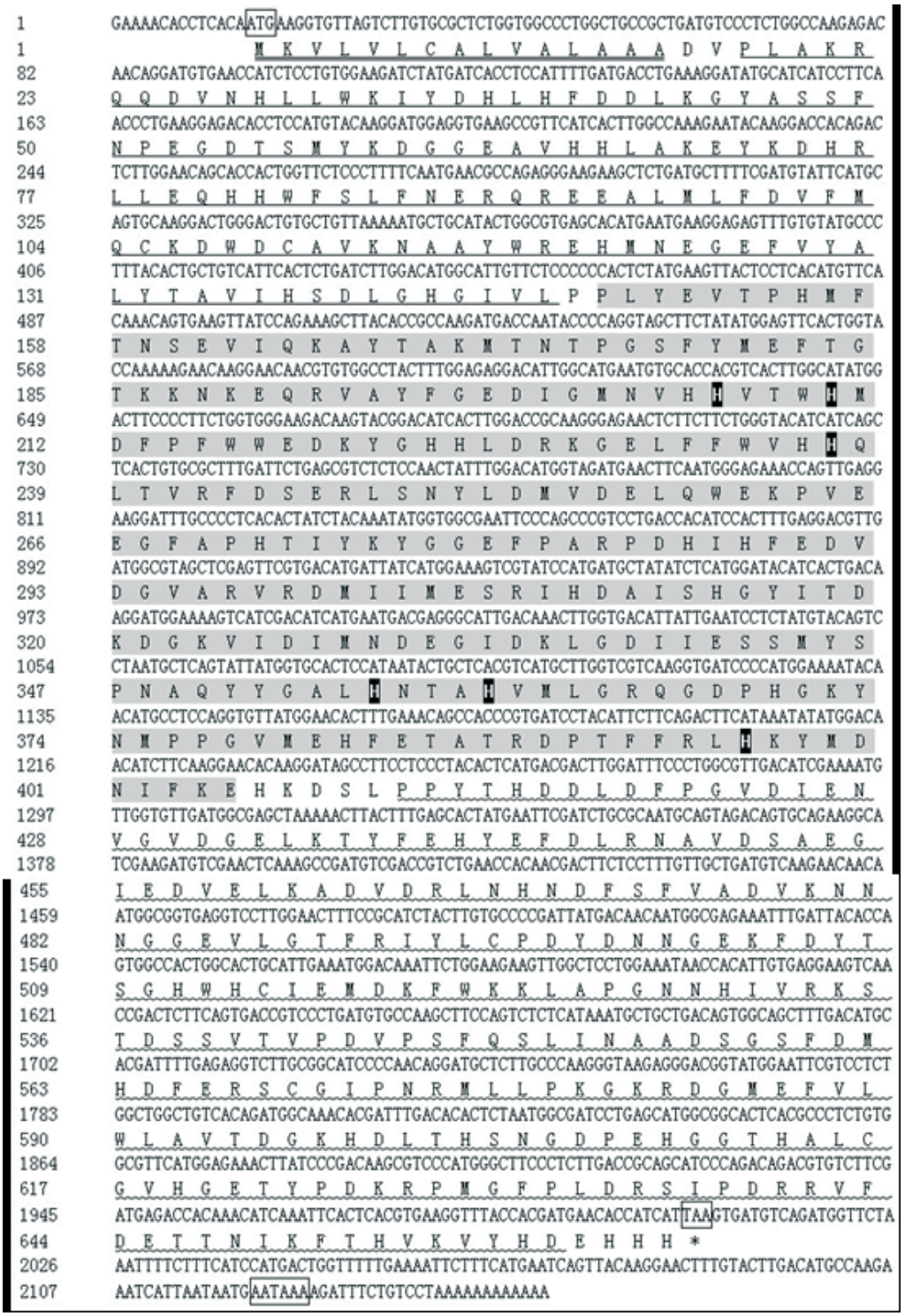

Figure. 1. Nucleotide and deduced amino acid sequence of hemocyanin from *M. nipponense*. The letter in the shadow indicated the start codon (ATG), the stop codon (TAA) and the polyadenylation signal sequence (AATAAA). The putative sequence of signal peptide was underlined with double line. The three hemocyanin domains were shown in single underline, the shadow and wavy underline, respectively. Six highly conserved histidine residues were shown in black reverse printing and boldfaced letters (**H**).

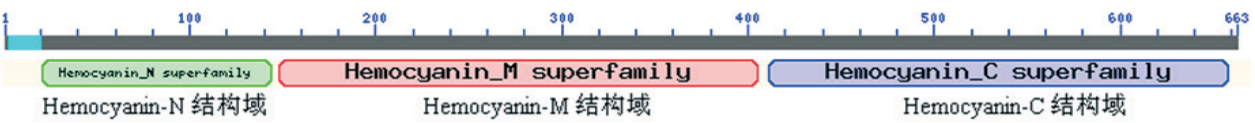

Figure 2. Distribution of three tandem hemocyanin domain of hemocyanin in *M. nipponense*.

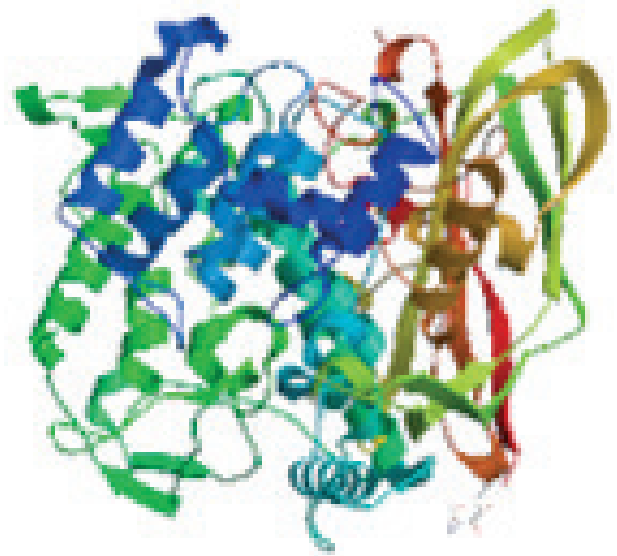

Figure 3. Predicted 3D structure of hemocyanin from *M. nipponense*.

MNHc shared the highest similarity (70%) with hemocyanin subunit 2 of crayfish *P. leniusculus* (AAO47336.1), and shared similarities of 68%, 63% and 62% with hemocyanin α subunit of *H. americanus*, hemocyanin subunit 4 of *Palinurus elephas* and hemocyanin subunit L of *Marsupenaeus japonicus*, respectively. Multiple amino acid sequence alignment of the MNHc with the other arthropod hemocyanin showed that MNHc had the common structural features of two conserved copper-binding sites with the highly conserved six histidine residues in the hmocyanin-M domain (Fig. 4). The deduced amino acids of hemocyanin-M domain of *M. nipponense* have high identities (45.9-46.0%) with those of other crustaceans.

Phylogenetic trees were constructed using MEGA4.0 for further examination of the evolutionary relationship among 15 arthropods hemocyanin (Fig. 5).

Tissue distribution of MNHc

MNHc mRNA transcripts were measured in the hepatopancreas, haemocytes, muscles, ovarian, mandibular organ and epidermis of prawns using real-time PCR (Fig. 6). The 18S rRNA gene was used as an internal control.

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L.vannamei      PLYEVTPHLFTNSEVIEEAYRAKQKQTPGKFKSSFTGTKKNPEQRVAYFGEDIGLNTHHVTWHME   65
P.elephas       PLYEVTPHMFTNSEVIDKAYSAKMTHKEGTFNMSFTGTKKNKEQRVAYFGEDIGMNIHHVTWHMD   65
P.leniusculus   PLYEVTPHLFTNSEVINKAYSAKMTQTPGKFNMDFTGTKKNKEQRVAYFGEDIGMNIHHVTWHMD   65
H.americanus    PLYEITPHMFTNSEIIHKAYTAKMTQTPGRFEMKFTGTKKNKEQRVAYFGEDIGLNIHHVTWHMD   65
M.japonicus     PLYEVTPHLFTNSEVIEAAYRAKQTQKPGKFKSSFTGTKKNPEQRVAYFGEDIGMNTHHVTWHME   65
M.nipponense    PLYEVTPHMFTNSEVIQKAYTAKMTNTPGSFYMEFTGTKKNKEQRVAYFGEDIGMNVHHVTWHMD   65

L.vannamei      FPFWWNDAYGHHLDRKGENFFWIHHQLTVRFDAERLSNYLDPVGELQWNKPIVDGFAPHTTYKYG   130
P.elephas       FPFWWDDSYGYHLDRKGELFFWVHHQLTARFDAERLSNWMEPVDELHWDDIIHEGFAPHTSYKYG   130
P.leniusculus   FPFWWKDSYGYHLDRKGELFFWVHHQLTARFDSERLSNWLDVVDELHWEDVIHEGFAPHTSYKFG   130
H.americanus    FPFWWKDSYGYHLDRKGELFFWAHHQLTVRFDAERLSNWLDPVDELHWERIIHEGFAPHTSYKYG   130
M.japonicus     FPFWWQDKYSHHLDRKGENFFWVHHQLTVRFDAERLSNYLDPVDELHWEKPIVQGFAPHTTYKYG   130
M.nipponense    FPFWWEDKYGHHLDRKGELFFWVHHQLTVRFDSERLSNYLDMVDELQWEKPVEEGFAPHTIYKYG   130

L.vannamei      GQFPARPDNVKFEDVDDVARIRDMVIVESRIRDAIAHGYIVDSEGKHIDISNEKGIDILGDIIES   195
P.elephas       GEFPVRPDNIRFENVDGVAHVHDMEITENRIRDAIAHGYITATDGHTIDIRQPNGIELLGDIIES   195
P.leniusculus   GEFPARPDNVHFEDVDGVARVRDMVILESRIRDAIAHGYIIDHSGNKIDIKNEHGIDTLGDIIES   195
H.americanus    GEFPARPDNVHFEDVDGVAHVRDMIIIESRIRDAIAHGYVTDNHGDNINIRNDHGIDVLGDIIES   195
M.japonicus     GQFPSRPDNARFEDVDGVARIRDLLIVESRIRDAIAHGYIVDREGKHIDIMNERGIDVLGDIIES   195
M.nipponense    GEFPARPDHIHFEDVDGVARVRDMIIMESRIHDAISHGYITDKDGKVIDIMNDEGIDKLGDIIES   195

L.vannamei      SLYSPNVQYYGALHNTAHIVLGRQGDPHGKFDLPPGVLEHFETATRDPSFFRLHKYMDNIFK     257
P.elephas       SMYSSNPHYYGSLHNTAHMMLGRQGDPHGKFDMPPGVMEHFETATRDPSFFRLHRYMDNIFK     257
P.leniusculus   SVYSPNVQYYGALHNTAHIMLGRQGDPHGKFDMPPGVMEHFETATRDPSFFRLHKYMDNIFK     257
H.americanus    SVYSPNAQYYGALHNTAHIMLGRQGDPHGKFNMPPGVMEHFETATRDPSFFRLHKYMDNIFK     257
M.japonicus     SLYSPNVQYYGALHNTAHIVLGRQSDPHGKYDLPPGVLEHFETATRDPSFFRLHKYMDNIFK     257
M.nipponense    SMYSPNAQYYGALHNTAHVMLGRQGDPHGKYNMPPGVMEHFETATRDPTFFRLHKYMDNIFK     257
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Figure 4. Alignment of the amino acid sequence of hemocyanin domains hemocyanin-M of *M. nipponense* and other animals hemocyanin. Six highly conserved histidine residues were indicated by an asterisk (*). *Litopenaeus vannamei*: (CAA57880.1); *Palinurus elephas:* (CAD56697.1); *Pacifastacus leniusculus*: (AAO47336.1); *Homarus americanus*: (CAB75960.1); *Marsupenaeus japonicus*: (ABR14693.1); *Macrobrachium nipponense*: (AEC46861.1).

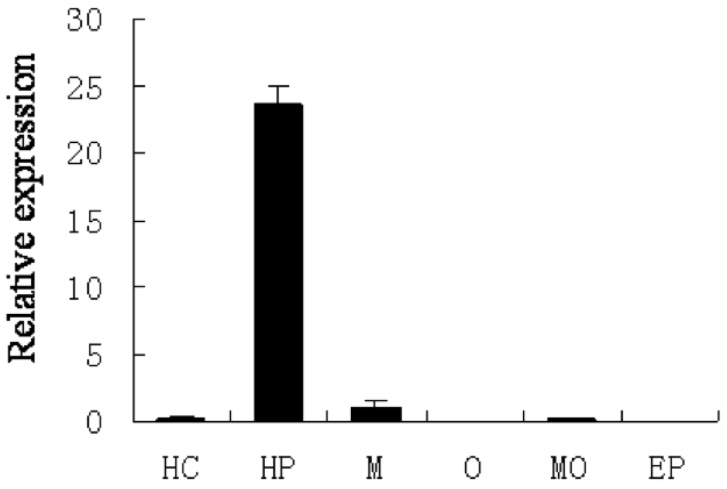

Figure 5. A molecular phylogenic tree of different species hemocyanin based on the neighbor-joining method .The hemocyanin of *M.nipponense* was underlined.

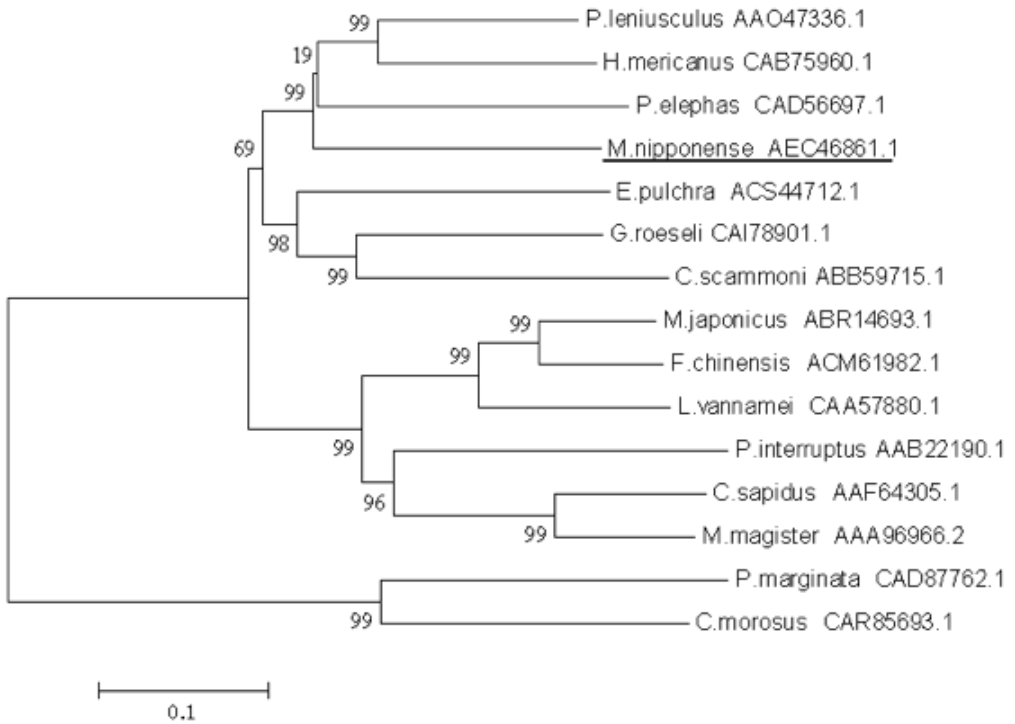

Figure 6. Real-time PCR analysis of MNHc transcripts from different tissues.
HC: haemocytes; HP: hepatopancreas; M: muscles; O:ovarian; MO: mandibular organ; EP: epidermis.

The result indicated that the mRNA transcript of MNHc was expressed highly in the hepatopancreas, weakly in the muscles and haemocytes, and hardly in the mandibular organ, epidermis and ovarian.

Expression of MNHc at different molt stages

MNHc mRNA transcripts were measured in hepatopancreas of prawns from different molt stages using real-time PCR. The MNHc mRNA expression of the prawn was significantly up-regulated in the stage B, and achieved the highest level in the stage C, and was then sharply down-regulated in the stage D0/1 and reached the lowest in the stage D2/D3 till stage A. However, no significant difference was observed in MNHc mRNA transcription between the stages A and D2/D3 of prawns, the same as between the stages B and D0/D1 (Fig. 7).

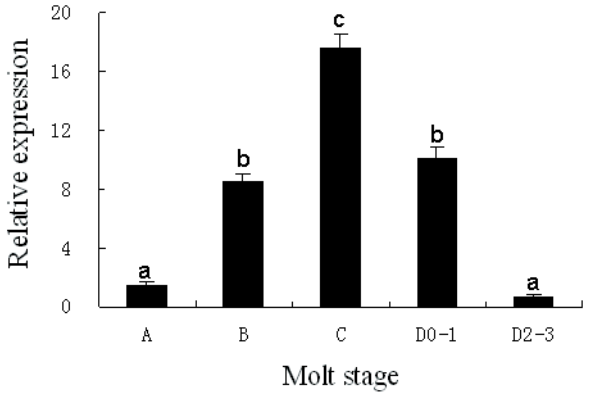

Figure 7. Change of MNHc expression during the molt cycle.

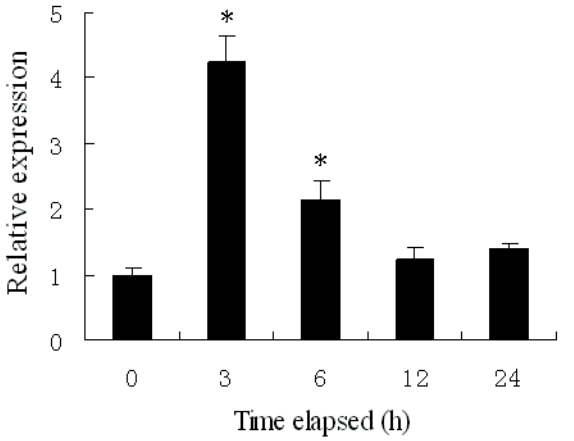

Figure 8. The induction of MNHc expression by *A. hydrophila*. * $P<0.05$, vs 0 h.

MNHc expression of prawns challenged with A. hydrophila

Expression of MNHc mRNA was up-regulated at 3 h after prawns were injected with *A. hydrophila* and down-regulated at 6 h post-injection, and then declined to original level at 12 h post-injection (Fig. 8).

Discussion

Hemocyanin is a multi-gene family that includes hemocyanin, prophenoloxidase, insect hexamer, crustacean cryptocyanin and dipteran hexamer [9]. In this study, we for the first time cloned hemocyanin gene from *M. nipponense* that encoded a peptide with molecular weight of 76.65 kDa, which was similar to that cloned from other crustaceans [2, 5]. Crustaceans hemocyanin is known as a multi-subunits protein complex, which contains different subunits [5]. Each subunit folds into three domains, of which the second domain (hemocyanin-M) contains two copper-binding sites, and the first and third domains are located at the N and C terminus of subunit respectively [11, 12]. SMART analysis indicated that MNHc protein also contained three conserved hemocyanin domains and two copper-binding sites which were coordinated by six histidines and reversibly bound a dioxygen molecule in side-on coordination. The six histidine residues within the two copper-binding motifs are highly conserved in all crustacean hemocyanin, including MNHc in this study (Fig. 4).

In the present study, real-time PCR analysis revealed that MNHc was mainly expressed in hepatopancreas, which is consistent with the results of *Litopenaeus vannamei* [13] . As early as 1970s, Gibson et al. [14] put forward that hepatopancreas is the main organ of crustaceans to synthesize hemocyanin. Whereafter, many scholars confirmed that viewpoint and further proposed that the site of hemocyanin synthesis was hepatopancreas F cell [15, 16]. However, its release approach is still poorly studied.

Real-time PCR results showed that the MNHc mRNA expression levels in hepatopancreas were closely related to the stage of molting and increased from post-molt and reached the highest in inter-molt stage C, which were similar to that of the hemocyanin gene of *L. vannamei* and *Callinectes sapidus* [17, 18]. However, in many crustacean, the MNHc mRNA expression levels in hepatopancreas were significantly increased from post-molt and reached the highest level until pre-molt [18-20]. Changes in hepatopancreas hemocyanin mRNA were somewhat different from that in haemolymph hemocyanin concentrations during the molt cycle, which may be relate to the process of that hemocyanin was synthesized in hepatopancreas and then released into hemolymph. Molting of crustaceans is a complicated physiological process, apart from hormone (mainly ecdysone) regualtion, oxygen-consuming amount and osmotic pressure would significantly increase. Hemocyanin as the sole respiratory protein was also involved in physiological processes such as osmoregulation, protein storage and exoskeleton formation [18, 19]. In addition, *Eurypelma californicum* hemocyanin could bind with ecdysone, participating in hormone transfer and molting regulation [21].

Hemocyanin as a multi-function protein, besides its role as described above, was also found to be involved in defending invaders, including bacteria and fungi [15, 16]. Recently, antibacterial peptides have been isolated and purified from C-terminus of hemocyanin from *L. vannamei*, *Litopenaeus stylirostris* [22], and *P. leniusculus* [23]. After infected by *Vibrio harveyi*, hemocyanin gene of *Penaeus monodon* expression was remarkably enhanced [6] . Our results showed that expression of MNHc mRNA in hepatopancreas was decreased at 3 h after prawns were challenged with pathogenic bacteria *A. Hydrophila* (Fig. 8), these results suggest that MNHc, as an important molecule, involved in immune defence of *A. Hydrophila*.

Acknowledgements

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Conflict of Interests

There is no conflict of interest in respect of this manuscript.

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