



Identification of a novel alternative splicing variant of hemocyanin from shrimp *Litopenaeus vannamei*



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ABSTRACT

Recent evidences suggest that invertebrates express families of immune molecules with high levels of sequence diversity. Hemocyanin is an important non-specific immune molecule present in the hemolymph of both mollusks and arthropods. In the present study, we characterized a novel alternative splicing variant of hemocyanin (cHE1) from *Litopenaeus vannamei* that produced mRNA transcript of 2579 bp in length. The isoform contained two additional sequences of 296 and 267 bp in the 5'- and 3'-terminus respectively, in comparison to that of wild type hemocyanin (cHE). Sequence of cHE1 shows 100% identity to that of hemocyanin genomic DNA (HE, which does not form an open reading frame), suggesting that cHE1 might be an alternative splicing variant due to intron retention. Moreover, cHE1 could be detected by RT-PCR from five tissues (heart, gill, stomach, intestine and brain), and from shrimps at stages from nauplius to mysis larva. Further, cHE1 mRNA transcripts were significantly increased in hearts after 12 h of infection with *Vibrio parahaemolyticus* or poly I: C, while no significant difference in the transcript levels of hepatopancreas cHE was detected in the pathogen-treated shrimps during the period. In summary, these studies suggested a novel splicing variant of hemocyanin in shrimp, which might be involved in shrimp resistance to pathogenic infection.

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1. Introduction

Invertebrates were once believed to have a simple immune system. However, recent data suggest that invertebrates also can express families of immune response proteins with high levels of sequence diversity, which are responsible for resistance to different pathogenic infection [1–5]. For instances, the Down syndrome cell adhesion molecule in the crustacean *Pacifastacus leniusculus* (PIDscam) can generate more than 22,000 different unique isoforms. Bacteria infection or LPS injection will enhance the expression of PIDscam; whereas, infection of white spot syndrome virus (WSSV) or injection with peptidoglycans shows no response [3]. Penaeidins in penaeid shrimp have five classes, which are likely the source of variation in anti-microbial responses [4]. Carcinolectins 5 (CL5) of *Carcinoscorpius rotundicauda* can produce

two isoforms (CL5a and CL5b) capable of recognizing and differentiating bacteria and fungi [5].

Recently, hemocyanin present in the hemolymph of both mollusks and arthropods is accepted as a novel non-specific immune molecule [6–14]. A major immune property of hemocyanin is the phenoloxidase-like enzyme activity, which can be activated by sodium dodecyl sulfate (SDS) and antimicrobial peptides [6,7]. In addition, hemocyanin also displays antiviral, antimicrobial and antitumor activities [8–10]. Zhang et al. reported that hemocyanin isolated from shrimp *Penaeus monodon* could act as an antiviral agent against a variety of viruses including DNA and RNA viruses [8]. Jiang et al. documented that hemocyanin of horseshoe crab *Carcinoscorpius rotundicauda* showed antimicrobial activity [9]. Becker et al. reported that two subunits of hemocyanin from *Concholepas* possessed an antitumor effect on the bladder carcinoma cell line MBT-2 [10]. Moreover, our previous findings showed that hemocyanin from shrimp *Litopenaeus vannamei* or crab *Scylla serrata* possessed antigenic, agglutinative, hemolytic, and immune-enhancing activities [11–16]. Interestingly, our recent evidences demonstrated that *L. vannamei* hemocyanin displayed single nucleotide polymorphisms (SNPs) and glycosylation diversity, which might contribute to the functional diversity of hemocyanin

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Table 1
Nucleotide sequences of primers and probes.

Clone sequence	Primers or probes	Sequences 5'–3'	Purpose
5'-terminus fragment of cHE/cHE1	cHE-5-F	GAGGGTCTTAGTGGTCTCT	RT-PCR
	cHE-5-R	AAATCGGCTTTGGTGTAT	RT-PCR
3'-terminus fragment of cHE/cHE1	cHE-3-F	TCATAAAGACAACAACGGAATC	RT-PCR
	cHE-3-R	ATCAGTCACCGCCACCAC	RT-PCR
cHE/cHE1	cHE-F	ATGAGGGTCTTAGTGGTCTTGGG	RT-PCR
	cHE-R	CATATGAAGAGTTGTAAGCTTGAATC	RT-PCR
beta-actin	beta-actin-F	CCGAGCGAGAAATCGTTCGTGAC	RT-PCR
	beta-actin-R	GGAGTTGTAGGTGGTCTCGTGGAT	RT-PCR
cHE1	cHE-1-F	TCGTCTATCTAGTAAGTCGATAATGT	RT-qPCR
	cHE-1-R	TGGCACGTTCCGTTAGAGTTA	RT-qPCR
	Probe-1	TCAATTGAACGCAAGTCCACGGAATCT	RT-qPCR
cHET (cHE1+cHE)	cHE-T-F	ATACCAGCGAGTTGGATAAGTTC	RT-qPCR
	cHE-T-R	TGGCACGTTCCGTTACAGTTA	RT-qPCR
	Probe-T	TCAATTGAACGCAAGTCCACGGAATCT	RT-qPCR

[17,18]. Thus, it is of great interest to further investigate whether hemocyanin possesses some isoforms.

In the present study, we identified a novel alternative splicing variant of hemocyanin cHE1 in shrimp *L. vannamei*. Its mRNA transcript profile could be modulated by pathogenic infection. We believe that the results will assist in the investigation of shrimp immune defense mechanisms.

2. Materials and methods

2.1. Experimental animals

Live healthy shrimps (*L. vananmei*) including ripe and juvenile shrimps were obtained from Shantou Huaxun Aquatic Product Corporation (Shantou, China) and maintained in 25 L open-circuit filtered seawater tanks at room temperature with aeration. Shrimps were acclimatized to laboratory conditions for 2 d before experiments.

2.2. RNA isolation and cDNA synthesis

Total RNA was extracted from *L. vannamei* tissues using RNAiso Plus (Takara, Dalian, China) according to manufacturer's instructions. The extracted RNA was treated with RNase-Free DNase (Takara, Dalian, China) to remove contaminating DNA. cDNA was synthesized using the M-MLV RTase cDNA Synthesis Kit (Takara, Dalian, China).

2.3. Primer design and cHE/cHE1 cloning

The primers for the complete cHE and cHE1 and their 5'- and 3'-terminus fragments were designed according to NCBI sequence of *L. vannamei* shrimp hemocyanin mRNA (X82502.1) (Table 1). Reverse transcriptase PCR (RT-PCR) was performed in a volume of 50 µl containing 1 unit of Taq-DNA Polymerase (Takara, Dalian, China), 1 µl of cDNA, 1 × PCR buffer, 1.5 mM of MgCl₂, 0.2 mM of each deoxynucleotide and 0.4 µM of each primer. The 5'-terminus fragments of cHE and cHE1 including exons 1 and 2 were amplified by use of primers cHE-5-F and cHE-5-R (Table 1). The thermal cycling conditions were initially 4 cycles of 94 °C for 30 s, 57 °C for 30 s and 72 °C for 90 s, then 6 cycles of 94 °C for 30 s, 55 °C for 30 s and 72 °C for 90 s, and finally 22 cycles of 94 °C for 30 s, 53 °C for 30 s and 72 °C for 90 s. The 3'-terminus fragments of cHE and cHE1 including exons 2 and 3 were amplified by use of primers cHE-3-F and cHE-3-R (Table 1) with thermal cycling conditions of 32 cycles of 94 °C for 30 s, 60 °C for 30 s and 72 °C for 30 s. The complete cHE and cHE1 including exons 1–3 were amplified by use

of primers cHE-F and cHE-R (Table 1). The thermal cycling conditions were 30 cycles of 94 °C for 30 s, 57 °C for 30 s and 72 °C for 150 s. The PCR products were gel purified and inserted into pMD-19T (Takara, Dalian, China) and then transformed into *Escherichia coli* DH5α (Promega, Madison, WI). Clones were selected randomly and sequenced in both directions with primers M13. The amino acids sequences were predicted using the BLAST algorithm (NCBI, <http://www.ncbi.nlm.nih.gov/BLAST/>).

2.4. DNA extraction and HE cloning

DNA was extracted from *L. vannamei* heart tissue using the Universal Genomic DNA Extraction Kit (Takara, Dalian, China) according to manufacturer's instructions. Primers cHE-F and cHE-R (Table 1) were used for amplification of HE (genomic hemocyanin). The PCR amplification, cloning and sequencing of HE were carried out the same as cHE1 described in Section 2.3.

2.5. mRNA transcript levels of cHE1

Total RNA was extracted from seven tissues including the heart, hepatopancreas, lymph, gill, stomach, intestine and brain, and from shrimps of three developmental stages (nauplius larva, protozoa and mysis larva). 2 µg of the total RNA was then used for cDNA synthesis. The 5'- and 3'-terminus fragments of cHE1 cDNA were amplified using primer sets cHE-5-F/cHE-5-R, and cHE-3-F/cHE-3-R, respectively. PCR amplification and agarose gel electrophoresis were practiced as described in Sections 2.2 and 2.3. As an internal loading control, shrimp beta-actin cDNA fragment was amplified with primers beta-actin-F and beta-actin-R (Table 1) under the same PCR conditions.

2.6. Preparation of bacterial suspension and poly I:C

Vibrio parahaemolyticus was routinely inoculated in Luria Bertani broth (LB) at 28 °C with constant aeration for 18 h. The cells were harvested, washed, and then diluted to 10⁷ CFU/ml in filtered seawater for bacterial challenge experiments. The double-stranded synthetic RNA, polyinosinic-polycytidylic acid (poly I:C, SIGMA, Shanghai, China), was diluted to 1 mg/ml for use in challenge tests.

2.7. Immune challenge tests

For immune challenge, two experimental groups and a control group were set up with each group having 30 shrimps. The experimental group was injected individually with 0.1 ml filtered

seawater containing either *V. parahaemolyticus* (10^7 CFU/ml), or poly I:C (1 mg/ml), while the control group was injected with 0.1 ml bacteria-free filtered seawater. Five shrimp hearts or hepatopancreas from each group were collected at 0, 6, 12 and 24 h after injection and pool together for RNA extraction.

Following cDNA synthesis, a quantitative real-time RT-PCR assay (RT-qPCR) was further performed with the ABI PRISM 7300 Sequence Detection System (Applied Biosystems, Foster City, CA) as previously described [19]. Primers and probes using for RT-qPCR were shown in Table 1. The thermal cycling conditions were 95 °C for 30 s, followed by 40 cycles of 95 °C for 5 s and 60 °C for 31 s. Each sample was run in triplicate and the relative changes in gene expression were normalized to beta-actin mRNA levels and presented as mean $\pm$ SE. The statistical significance between multiple groups was analyzed by one way ANOVA followed by an unpaired, one-tailed *t*-test.

3. Results

3.1. Cloning of *cHE1* from shrimp

As shown in Fig. 1A and B, unlike the two predicted bands of about 1200 and 300 bp in shrimp hepatopancreas, two novel bands of about 1500 and 600 bp were observed in shrimp heart from RT-PCR amplification of the 5'- and 3'-terminus of hemocyanin (cHE), respectively. Sequence analysis of the two additional bands suggested that an alternative splicing variant of hemocyanin (cHE1) might exist. To confirm the prediction, RT-PCR amplification, cloning and sequencing analysis of the cHE1 were further performed. It turned out that there is the other transcript in shrimp in addition to the well-known hemocyanin cHE, which was named cHE1 and consisted of 2579 bp. Additional 296 and 267 bp sequences were found inserted in the 5'- and 3'-terminus respectively after compared with sequence of cHE (Fig. 1C and D).

3.2. Intron retention in *cHE1*

To examine the potential protein sequence encoded by cHE1, the BLAST algorithm was applied. Unexpectedly, cHE1 did not produce an open reading frame, suggesting that it might contain one or several introns of genomic hemocyanin. To testify this, hemocyanin (HE) gene were cloned from the extracted genomic DNA and sequenced (Fig. 1C). Sequence alignments revealed that cHE1 possessed the same sequence as that of HE, which reserved two complete introns, suggesting that cHE1 was an alternative splicing variant resulting from the retention of introns (Fig. 2A and B).

3.3. Characteristic mRNA transcript profile of *cHE1*

Characteristic mRNA transcript levels of cHE1 were determined using RT-PCR. Two pairs of primers, cHE5-F and cHE5-R, cHE3-F and cHE3-R, were applied (see method section for details). As shown in Fig. 3A, a predominant band with an expected size of 1561 bp for the 5'-terminus was observed in five examined tissues including the heart, gill, stomach, intestine and brain, in which the mRNA transcript in the intestine was the highest, but it was not detected in the hepatopancreas and lymph. In addition, we found that a faint band of 580 bp for the 3'-terminus appeared in nauplius, protozoa and mysis larva (Fig. 3B), suggesting that cHE1 transcript was expressed at low levels in different developmental stages of shrimp.

3.4. Responses of *cHE1* to pathogen challenge

In order to test whether cHE1 possesses potential immune function, a RT-PCR analysis was carried out to determine the

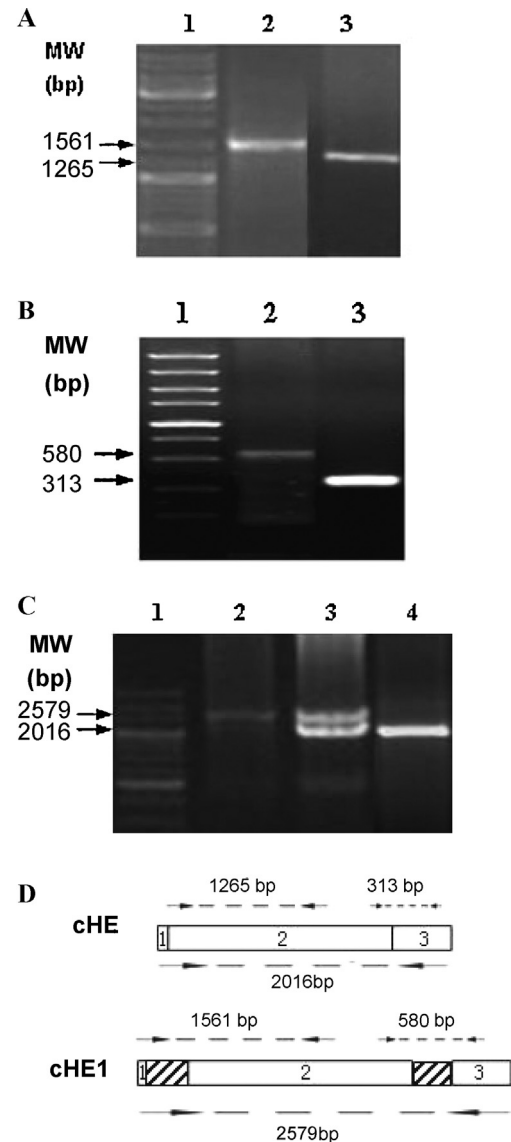


Fig. 1. Cloning of cHE1 transcript from *Litopenaeus vannamei*. (A) RT-PCR amplification of 5'-terminal fragments of cHE1 and cHE in the heart and hepatopancreas. Lane 1, markers; Lane 2, heart; Lane 3, hepatopancreas. (B) RT-PCR amplification of 3'-terminal fragments of cHE1 and cHE in the heart and hepatopancreas. Lane 1, markers; Lane 2, heart; Lane 3, hepatopancreas. (C) PCR or RT-PCR amplification of cHE1 and cHE. Lane 1, markers; Lane 2, heart DNA (PCR); Lane 3, gill cDNA (RT-PCR); Lane 4, hepatopancreas cDNA (RT-PCR). (D) Schematic diagram of hemocyanin from *L. vannamei*. Numbers of 1–3 represent hemocyanin exon 1, exon 2 and exon 3, respectively. Two introns are reserved in cHE1 mRNA while spliced in cHE mRNA, they are 296 (between exon 1 and exon 2) and 267 bp (between exon 2 and exon 3), respectively.

transcription levels of cHE1 in hearts of *L. vannamei* treated with pathogens. As shown in Fig. 4B and C, the transcript of cHE1 increased dramatically after infection with *V. parahaemolyticus* (or poly I:C) for 12 h, and then decreased at 24 h. No obvious variation of cHE1 transcript levels was detected in the mock-treated group at any time point (Fig. 4A). It was noteworthy that there was no significant difference in the transcription levels of hepatopancreas cHE in the pathogen- or mocked-treated shrimps during the entire 24 h period.

To further confirm the above analysis, a quantitative real-time RT-PCR (RT-qPCR) analysis was performed. Significant differences in cHE1 transcription levels were observed between the control group and treatment group with *V. parahaemolyticus* at 12 h (Fig. 5A, left panel), or with poly I:C for

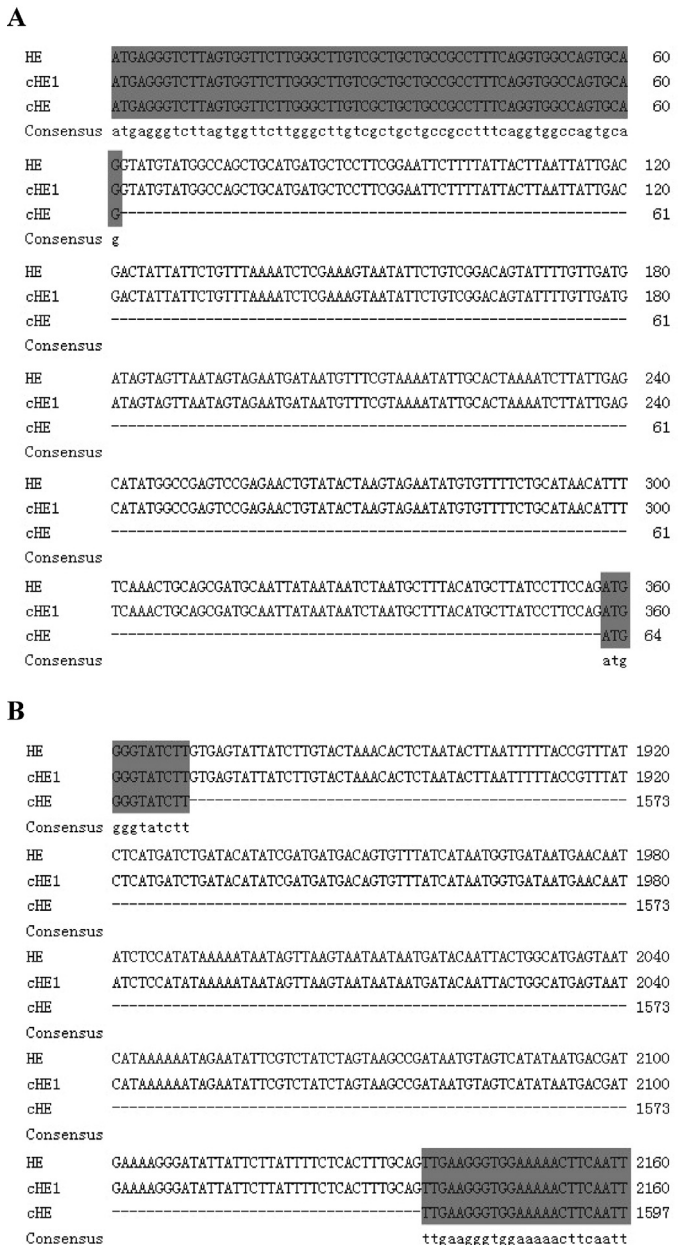


Fig. 2. Multiple alignments of hemocyanin partial nucleotide sequences. Alignment of (A) the 5'- and (B) 3'-terminal fragments of cHE1, cHE and HE. Dashes indicate respective intron in the alignment. Identical nucleotides are highlighted in gray.

12 and 24 h (Fig. 5B, left panel), respectively. All of them were induced at about 1.5-fold. Similar results for the total amount of cHE1 and cHE (Fig. 5A and B, right panel) further confirmed the immune response to the pathogen challenge.

4. Discussion

Recent evidence suggests that alternative splicing plays a significant role in generating the diversities of immune molecules in invertebrates [20,21]. In this study, an alternative splicing variant of hemocyanin (cHE1) in *L. vannamei* was cloned and characterized (Figs. 1–3). It is a very interesting finding to associate alternative splicing with hemocyanin, which may act as one of the molecular basis for hemocyanin multifunction.

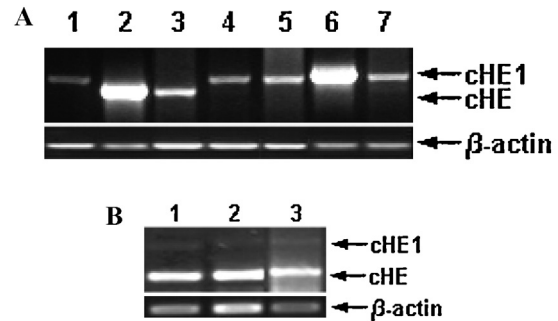


Fig. 3. mRNA transcripts analysis of cHE1. (A) Detection of cHE1 mRNA transcripts in various tissues from healthy shrimps. Total RNA isolated from seven tissues was used for RT-PCR analysis with the 5'-terminal cHE1 gene-specific primers. Lanes 1–7 represent the heart, hepatopancreas, lymph, gill, stomach, intestine and brain. (B) Detection of cHE1 mRNA transcripts in various developmental stages of shrimps. Total RNA isolated from three developmental stages of shrimps was used for RT-PCR analysis with the 3'-terminal cHE1 gene-specific primers. Lanes 1–3 represent nauplius, protozoa and mysis larva. Beta-actin was used as a control.

Interestingly, the cHE1 was found to contain two additional introns of 296 and 267 bp in its 5'- and 3'- terminus, respectively, in comparison to that of the classical form of hemocyanin (cHE) (Fig. 2). Taken into consideration of the findings that alternative splicing variants could be generated by various mechanisms, via exon skipping, alternative 3'-splice site (SS) selection, alternative 5'-SS selection and intron retention [22–24], we would like to offer a hypothesis that cHE1 isoform in shrimp might be derived from retention of introns.

To further characterize the mRNA expression of cHE1 isoform, tissular and developmental stage expression analysis were performed. Results indicated that the transcripts of cHE1 had a stable and low expression level in different developmental stages of shrimp compared to that of cHE. It was also interesting to note that

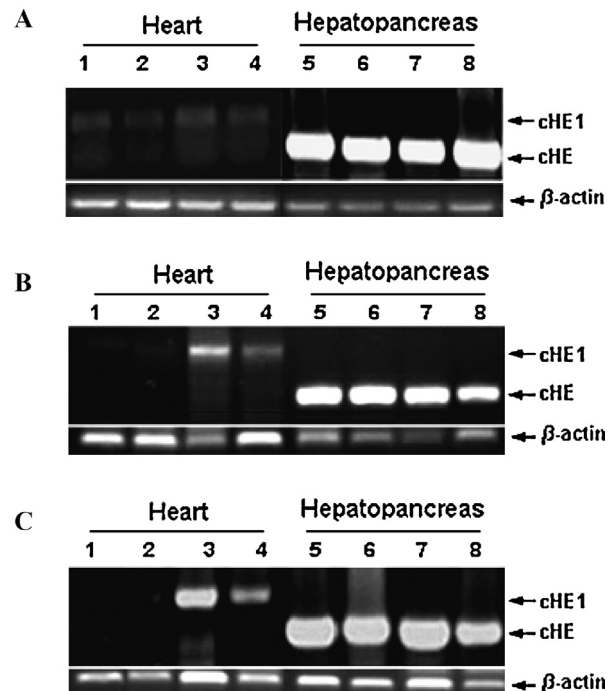


Fig. 4. RT-PCR analysis of cHE1 mRNA transcripts in pathogen-treated shrimp. Shrimps were treated with (A) filtered seawater, (B) *V. parahaemolyticus*, or (C) poly I: C for 0, 6, 12 and 24 h. Lanes 1–4 represent hearts from shrimps treated for 0, 6, 12 and 24 h, respectively; Lanes 5–8 represent hepatopancreases from shrimps treated for 0, 6, 12 and 24 h, respectively.

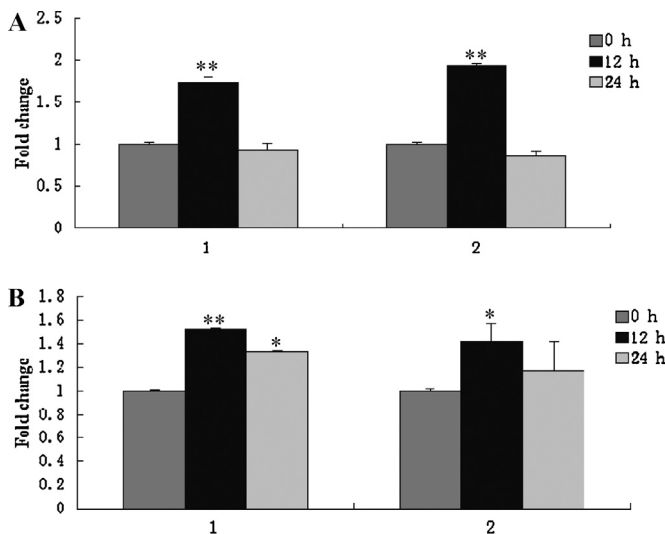


Fig. 5. Quantitative real-time PCR analysis of cHE1 or total of cHE1 and cHE mRNA transcripts. Shrimp hearts were harvested after 0, 12 and 24 h injection with (A) *V. parahemolyticus*, or (B) poly I: C. cHE1 or total of cHE1 and cHE mRNA levels were normalized with beta-actin mRNA levels. 1, cHE1; 2, cHE1 and cHE. Bars represented the mean $\pm$ SD ($n = 3$). * ($p < 0.05$) or ** ($p < 0.01$) indicated significant differences between 0 h and 12 h or 24 h treatment group.

shrimp hemocyanin (cHE) mRNA was mainly expressed in the hepatopancreas [25,26] but not in the intestine, while cHE1 mRNA was abundantly expressed in the intestine but not in the hepatopancreas (Fig. 3). This result is similar to the findings that tissue-specific mRNA expression patterns are different between some classical and alternatively spliced isoforms [27,28]. Therefore, although the exact synthesizing site of cHE1 mRNA is yet to be further investigated, the results shown here strongly suggest that cHE1 is a novel alternative splicing variant of hemocyanin in shrimp.

Furthermore, it is demonstrated that intron retained variants not only produce protein products, but also participate in the regulation of the protein expression of classical forms [29–33]. For instances, Mansilla et al. [31] indicated that a proinsulin mRNA variant from chick embryos generated by intron 1 retention in the 5' untranslated region could regulate proinsulin expression in adaptation to environmental or developmental changes. Seong et al. [32] found that a gonadotropin-releasing hormone (GnRH) pre-mRNA transcript containing intron 1 in rat was involved in the decrease of GnRH translation efficiency. Moran et al. [33] suggested that intron retention might act as a posttranscriptional regulatory mechanism of neurotoxin (N. vectensis toxin 1, Nv1) expression at early life stages of the starlet anemone *Nematostella vectensis*. Based on these, it would be interesting to know whether the cHE1 also has some similar biological properties.

To confirm this hypothesis, challenge tests with pathogens were applied. Results indicated that cHE1 transcription levels were increased significantly after *V. parahemolyticus* or poly I: C treatment for 12 h. In contrast, no significant difference in the transcription levels of hepatopancreas cHE was found in the pathogen-treated shrimps during the period (Figs. 4 and 5). Nonetheless, the other published studies found that PmcHE (Hemocyanin from *Penaeus monodon*) mRNA transcription is strongly up-regulated after 48 h infection with *Vibrio harveyi* [34], and that PjcHE (Hemocyanin from *Penaeus japonicus*) expression shows an up-regulation in the hepatopancreas of WSSV (white spot syndrome virus)-resistant shrimp [35]. This discrepancy probably reflects differences in periods of shrimp treatment with pathogens. Therefore, these findings might suggest that *L. vannamei* cHE1 and cHE contribute to different response (early and late response, respectively) to pathogen stimulation.

In addition, Xu et al. [36,37] reported that a splicing variant of apoE (Neuronal expression of apolipoprotein E4, apoE) with intron-3 retention (apoE-I3) can control apoE expression by processing of apoE-I3 into mature apoE mRNA in response to injury. It will also be interesting to investigate in future study whether cHE1 can act as a cHE precursor or a regulator to activate cHE expression and protein translation.

In conclusion, the present study found for the first time that *L. vannamei* hemocyanin gene could generate a novel alternative splicing variant (cHE1). The isoform might be produced by retention of introns. Furthermore, cHE1 transcripts showed a significant up-regulation in pathogen-treated shrimps. This work will provide useful information for further studying the shrimp immune defense mechanisms.

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