Original Research Articles

Expression of Hsp70 in the red swamp crayfish, *Procambarus clarkii* in response to immune challenge

Chao Peng[1,2], Zhongyuan Chen[1], Qing Han[1], Liye Shao[1], Ping Mo[1], Shuiqing Wu[2a]

[1] Hunan University of Arts and Science, Hunan Changde 415000, China, [2] Key Laboratory of Cultivation and High-value Utilization of Marine Organisms in Fujian Province Fisheries Research Institute of Fujian, Xiamen 361000, China

Israeli Journal of Aquaculture - Bamidgeh

Vol. 75, Issue 2, 2023

A heat shock protein 70 (Hsp70) was identified from *Procambarus clarkii* (named as PcHsp70). The open reading frame of the PcHsp70 cDNA was 1917 bp, encoding a 638 aa protein with signature sequences of the Hsp70 family. Tissue distribution results showed that PcHsp70 has relatively high expression in immune-related tissues such as hepatopancreas, gill, and hemocytes. The expressions of PcHsp70 in hepatopancreas and gill were significantly induced by Gram-positive bacterium, Gram-negative bacterium, and white spot syndrome virus stimulation, indicating that PcHsp70 may be involved in the immune response to pathogen invasion. The research will provide a foundation for understanding the resistance and stress mechanism of Hsp70 in *P. clarkii*.

INTRODUCTION

The heat shock proteins (Hsps) are a highly homologous protein family which act as molecular chaperones to regulate the balance of cell life and death.[1] They were produced in response to thermal stress, bacterial and viral infections, UV irradiation exposure, oxygen radicals, heavy metal pollution, or other toxic exposure.[2] By assisting in folding nonfunctional proteins to reach functional conformation, Hsps have multiple functions.[3]

Under adverse conditions such as pathogen invasion, Hsp70 can improve cell resistance by regulating the protein folding modification.[4] The human Hsp70 can activate innate immunity by regulating the activity of other proteins.[5] Hsp70 could also regulate the adaptive immune response and enhance several cytokine secretions by activating macrophages.[6] Recently, it was demonstrated that Hsp70 participates in pathogen infections in shrimp. For example, Hsp70 from *Litopenaeus vannamei* could be upregulated by white spot syndrome virus (WSSV), and Hsp70 helps shrimp survive WSSV infection by modulating the proPO system and apoptosis.[7] Upon infection, hypodermal and hematopoietic necrosis virus (IHHNV) infection, the expression of Hsp70 protein was induced.[8] Induction of Hsp70 could enhance tolerance to *Vibrio parahaemolyticus* infection by activating the proPO system when shrimp was facing non-lethal heat shock.[9] Transcription of *L. vannamei* Hsp70 could be upregulated by the Gram-positive bacterium *Staphylococcus aureus* and the Gram-negative bacterium *Vibrio alginolyticus* infection.[10] The expression of

Fenneropenaeus chinensis Hsp70 in hepatopancreas and gills was significantly induced by heat shock and *Vibrio anguillavium* challenge.[11] Further studies showed that recombinant protein Hsp70 could improve the survival rate of *L.vannamei* under *V. parahaemolyticus* infection.[9,12]

The red swamp crayfish, *Procambarus clarkii*, is a freshwater crustacean invertebrate native to northern Mexico and the southern United States. *P. clarkii* is the sixth-largest freshwater aquaculture species in China. In 2021, the output of *P. Clarkii* in China reached 2,633,600 tons. However, due to increased breeding density and the frequent outbreak of shrimp disease caused by pathogenic bacteria, the crayfish breeding industry has suffered severe losses.[13] *P. clarkii* lacks an acquired immunity and can only rely on innate immune responses to resist the invasion of pathogenic microorganisms.[14] Thoroughly analyzing the antiviral defense mechanism and exploring the vital immune factors in *P. clarkii* is essential in formulating prevention and control strategies of shrimp disease. Hsp70 acts as a "guardian," which can improve resistance and protect stress when organisms are subjected to environmental stress. Hence, understanding the function and mechanism of Hsp70 in crayfish benefits healthy shrimp culture. In this study, the Hsp70 was identified in *P. clarkii*, and the spatial distribution and temporal expression of Hsp70 were analyzed after Gram-positive bacteria, Gram-negative bacteria, and virus stimulation.

a * Corresponding author. Shuiqing Wu e-mail: 15602258587@163.com

Table 1 Primer sequences.

Primers	Sequence（5'→3'）	Usage
Hsp70-F	ATGCCTAAAGCTGTTGGCATTG	ORF cloning
Hsp70-R	GTCAACTTCTTCAATGGTAGGTCCA	ORF cloning
qHsp70-F	TATTGAGATAGACTCCCTG	qRT-PCR
qHsp70-R	TTCTTGTAGAACCACCC	qRT-PCR
β-actin-F	AGGGCGTGATGGTTGGT	qRT-PCR
β-actin-R	CATAGCGGGAGTGTTGAAAG	qRT-PCR

MATERIALS AND METHODS

EXPERIMENTAL ANIMALS

Healthy red swamp crayfish (*P. Clarkii*, 10±2 g per crayfish) were collected from an aquaculture base in Changde, Hunan Province, China. Shrimp were cultured in tanks for 5 days at approximately 25 °C.

CDNA CLONING AND ANALYSIS

The total RNA of crayfish was extracted using a TRIZOL reagent. The cDNA was synthesized by the first strand synthesis kit (Simgen, Wuhan, China). The transcript of PcHsp70 ORF obtained from the transcriptome database of *P. Clarkii* was amplified using the PcHsp70-F/R primers (Table 1). The PCR product was further verified by electrophoresis, and the desired sequence was then purified and ligated into the pUCm-T Vector (Sangon Biotech, China). The product was transformed into DH5α cells, and the positive clones were picked for sequencing (Sangon Biotech, China). Sequence features, multiple sequence alignment, and phylogenic analysis were analyzed as described in a previous study.[15] The relevant GenBank registration numbers of aligned Hsp70 are as follows: *Cherax quadricarinatus* ADM94257.1; *Metapenaeus ensis* AHY94555.1; *Eriocheir sinensis* ACF98297.1; *Penaeus vannamei* XP_027225206.1; *Scylla serrata* AFI61333.1; *Euphausia crystallorophias* AIR72267.1; *Apis cerana* UYC36093.1; *Oncorhynchus kisutch* XP_020358882.1; *Danio rerio* NP_001186941.1; *Mus musculus* AAA37869.1; *Salmo trutta* XP_029629136.1; *Paralichthys olivaceus* XP_019941072.1; *Homo sapiens* BAD96505.1; *Bos indicus* QGW08891.1; *Penaeus monodon* AIE88312.1; *Penaeus japonicas* XP 042878451.1; *Pachygrapsus marmoratus* CAL68994.1; *Portunus trituberculatus* ACL37319.1; *Cotesia vestalis* AGF34718.1; *Polyrhachis vicina* AGF33487.1; *Pteria penguin* ABJ97377.1;

SPATIAL EXPRESSION ANALYSIS

Total RNA from muscle, gill, intestine, hemocytes, lymphoid organ, eye, heart, hepatopancreas, stomach, and brain of three individuals was isolated. Quantitative real-time PCR (qRT-PCR) was executed to detect the expression profile of PcHsp70 mRNA in different tissues. The primers of PcHsp70 used for qRT-PCR are shown in Table 1. The qRT-PCR was performed with a total volume of 20 μL, including 10 μL of 2×MonAmp™ ChemoHS Specificity Plus qPCR Mix (Monad, Suzhou, China), 0.4 μL of each primer, 1 μL of the template, and 8.2 μL of Nuclease-Free water. The qRT-PCR program was performed as described in a previous study.[15] The relative expression level of PcHsp70 was calculated according to the $2^{-\Delta\Delta CT}$ method.[16]

IMMUNE CHALLENGE ASSAYS

The mRNA expression profile of PcHsp70 was analyzed after different immune challenges. *P. Clarkii* (10±2 g per crayfish) was divided into four groups. For the bacterial challenge, 100 μl of *V. parahaemolyticus* (1×10^7 cells per shrimp) and 100 μl of *Staphylococcus aureus* (1×10^7 cells per shrimp) were used to introduce a challenge of Gram-positive or Gram-negative bacteria, respectively.[17] For the viral challenge, 100 μl of WSSV (1×10^6 copies/mL) was used to introduce the virus challenge as previously described methods.[18] For the control group, 100 μl of PBS was injected into shrimp.[19] The Hepatopancreas and gills of three individuals were randomly collected to detect the expression of PcHsp70 mRNA at different times after injection.

RESULTS

BIOINFORMATICS ANALYSIS OF PCHSP70

The ORF of PcHsp70 was 1917, encoding a 638 aa protein. The putative MW and pI of PcHsp70 were calculated as 69.88 kDa and 5.23, respectively. The sequence is predicted to contain phosphorylation sites of 40 serine (Ser), 46 threonine (Thr), and 15 tyrosine (Tyr). 4-598 aa belonged to the Hsp70 domain, and no signal peptide was found (**Figure 1**). A Dnak characteristic motif and two signature sequences of Hsp70 family proteins were found in the PcHsp70. The Dnak characteristic motif was found in the N-terminal section (IDLGTT-F-S), and the two signature sequences were located in the central part of PcHsp70: IFDLGGGTFDVSIL and IVMVGGSTRIPKQK. Moreover, the PcHsp70 protein also contained an ATP-binding site motif (AEAYLGST), a non-organellar motif (RARFEEM), and a conserved C-terminal sequence (GPTIEEVD) (**Figure 1**).

MULTIPLE SEQUENCE ALIGNMENT OF PCHSP70

Multiple sequence alignment results showed that PcHsp70 showed 95.57%, 91.09%, 90.10%, 88.20%, 87.50%, 87.50%, and 87.26% similarity with *C. quadricarinatus* Hsp70, *Penaeus japonicas* Hsp70, *Scylla serrata* Hsp70, *Danio rerio*

```
  1 atgcctaaagctgttggcattgaccttggcaccaccttctcatgtgtgggtgtattccagcatgggaaggtagaaatcattgcaaatgac 90
  1 M  P  K  A  V  G  I  D  L  G  T  T  F  S  C  V  G  V  F  Q  H  G  K  V  E  I  I  A  N  D  30

 91 caggggtaaccgtactacaccatcgtatgtggctttcactgatgcagagaggctcattggtgatgctgccaagaatcaggtagcaatgaac 180
 31 Q  G  N  R  T  T  P  S  Y  V  A  F  T  D  A  E  R  L  I  G  D  A  A  K  N  Q  V  A  M  N  60

181 ccaaacaatacagttttgatgctaaaaggctaataggcagaaaattcaatgatgctagtgttcaagctgatatgaaacactggccattt 270
 61 P  N  N  T  V  F  D  A  K  R  L  I  G  R  K  F  N  D  A  S  V  Q  A  D  M  K  H  W  P  F  90

271 actgtaattagtgatggtggcaaacccaaaattcagattgaatacaagggagaaacgaagtcttttacccagaagaaatttcatcaatg 360
 91 T  V  I  S  D  G  G  K  P  K  I  Q  I  E  Y  K  G  E  T  K  S  F  Y  P  E  E  I  S  S  M  120

361 gtacttatcaagatgaaagaaacggctgaagcttaccttggctccactgtaaaggatgcagttataacagtgcctgcatacttcaatgat 450
121 V  L  I  K  M  K  E  T  A  E  A  Y  L  G  S  T  V  K  D  A  V  I  T  V  P  A  Y  F  N  D  150

451 tcacagcggcaagccacaaaggatgctggaactatctctggtatgaatgtgcttcgtattattaatgaacccactgctgcagccattgca 540
151 S  Q  R  Q  A  T  K  D  A  G  T  I  S  G  M  N  V  L  R  I  I  N  E  P  T  A  A  A  I  A  180

541 tatggtctagataaaaaggttggtggtgaacgtaatgttctcatctttgatcttggtggtggtacatttgatgtttcaatcttgacaata 630
181 Y  G  L  D  K  K  V  G  G  E  R  N  V  L  I  F  D  L  G  G  G  T  F  D  V  S  I  L  T  I  210

631 gaagatggtatttttgaagtaaagtctactgcaggtgatactcaccttggtggagaagactttgataatcgtatggttacccacttcatt 720
211 E  D  G  I  F  E  V  K  S  T  A  G  D  T  H  L  G  G  E  D  F  D  N  R  M  V  T  H  F  I  240

721 caagagttcaaacgcaagtataaaaaggatatgagtgacaacaagcgtgcagtaagacggctacgtacagcatgcgaacgtgctaaaaga 810
241 Q  E  F  K  R  K  Y  K  K  D  M  S  D  N  K  R  A  V  R  R  L  R  T  A  C  E  R  A  K  R  270

811 actttgtcttcatctacacaagcaagtattgagatagactccctgtatgaaggtgtagacttctacacttcaattaccagagctagattt 900
271 T  L  S  S  S  T  Q  A  S  I  E  I  D  S  L  Y  E  G  V  D  F  Y  T  S  I  T  R  A  R  F  300

901 gaagaaatgtgtgctgacctcttcagaggcacacttgatcctgtagaaaagtctctccgtgatgcaaaaatggacaagagtcaaattaat 990
301 E  E  M  C  A  D  L  F  R  G  T  L  D  P  V  E  K  S  L  R  D  A  K  M  D  K  S  Q  I  N  330

991 gaaattgttatggtgggtggtctacaagaattccaaagattcaaaagttgctgcaagatttcttttaatggtaaagagcttaataaatct 1080
331 E  I  V  M  V  G  G  S  T  R  I  P  K  I  Q  K  L  L  Q  D  F  F  N  G  K  E  L  N  K  S  360

1081 attaatcccgatgaagctgttgcttatggtgctgcagttcaagcagcaatcctgtgtgtggagacaagtctgaagctgttcaagacttgttg 1170
361 I  N  P  D  E  A  V  A  Y  G  A  A  V  Q  A  A  I  L  C  G  D  K  S  E  A  V  Q  D  L  L  390

1171 ctgctggatgttgctccactttctcttggtattgaaactgctggtggtgtaatgactgctctcattaaacgcaacactactattcccaca 1260
391 L  L  D  V  A  P  L  S  L  G  I  E  T  A  G  G  V  M  T  A  L  I  K  R  N  T  T  I  P  T  420

1261 aagcagactcagacatttacaacatattcagataatcaaccaggtgttctcattcaggtatatgaaggtaacgtgccatgaccaaagac 1350
421 K  Q  T  Q  T  F  T  T  Y  S  D  N  Q  P  G  V  L  I  Q  V  Y  E  G  E  R  A  M  T  K  D  450

1351 aacaacttcttggcaaatttgaattgactggtattccaccagccccacgtggggttcctcagattgaggttacctttgacattgatgct 1440
451 N  N  L  L  G  K  F  E  L  T  G  I  P  P  A  P  R  G  V  P  Q  I  E  V  T  F  D  I  D  A  480

1441 aatggaattttaaatgtatctgctgcagacaaatccactggcaaagaaaataaaatcaccattacaaatgacaagggacggctcagcaaa 1530
481 N  G  I  L  N  V  S  A  A  D  K  S  T  G  K  E  N  K  I  T  I  T  N  D  K  G  R  L  S  K  510

1531 gaagaaattgagaaatggttcaagatgcagagaaatataaagctgatgatgaaaaccagcgtgaacgaattgcagccaagaatagtctc 1620
511 E  E  I  E  K  M  V  Q  D  A  E  K  Y  K  A  D  D  E  N  Q  R  E  R  I  A  A  K  N  S  L  540

1621 gaatcttattgctttaatatgaaatctactgtggaagacgaaaagttcaaagataaggtttctagtactgatcgcagcaaaatactagat 1710
541 E  S  Y  C  F  N  M  K  S  T  V  E  D  K  F  K  D  K  V  S  S  T  D  R  S  K  I  L  D  570

1711 gcatgcaatgaagcaattaaatggttggattctaatcagtctgctgaaaaggatgaatttgagcataaacagaaggaagtagagcagatt 1800
571 A  C  N  E  A  I  K  W  L  D  S  N  Q  S  A  E  K  D  E  F  E  H  K  Q  K  E  V  E  Q  I  600

1801 tgcaatcctatcataacaaagatgtatgcttcttcaggaggggcacctggtgcacctggtggggcacctgaaggtgcctccactggcggt 1890
601 C  N  P  I  I  T  K  M  Y  A  S  S  G  G  A  P  G  A  P  G  G  A  P  E  G  A  S  T  G  G  630

1891 ggacctaccattgaagaagttgactaa 1917
631 G  P  T  I  E  E  V  D  *  638
```

Figure 1. Full-length nucleotide sequence (above) and deduced amino acid sequence (below) of PcHsp70.

The PcHsp70 domain (4-598 aa) was bold in italics; The predicted 40 serine phosphorylation sites were labeled with ellipses, 46 threonine phosphorylation sites were labeled with squares, and 15 tyrosine phosphorylation sites were labeled with hexagons; The three signature sequences were indicated with a straight underline; The ATP-binding site motif was labeled with a wavy underline; The non-organellar motif was labeled double underline; The conserved C-terminal sequence was a label with shadow.

Hsp70, *Mus musculus* Hsp70, *Homo sapiens* Hsp70, *Apis cerana* Hsp70, respectively (**Figure 2**). The results indicated that the evolution of Hsp70 in *P. Clarkii* was conserved.

PHYLOGENETIC ANALYSIS OF PCHSP70

The phylogenetic tree results showed that PcHsp70 belonged to the invertebrate branch and was most closely related to *C. quadricarinatus* Hsp70, with which it formed a clade (**Figure 3**).

TISSUE EXPRESSION OF PCHSP70

qRT-PCR analysis revealed that PcHsp70 was ubiquitously expressed in the ten tissues examined herein. PcHsp70 had the highest relative expression in hepatopancreas tissues, followed by the gill, hemocytes, lymphoid organ, brain, intestine, eye, heart, and stomach. The relative expression of PcHSP70 in muscle was the lowest (**Figure 4**).
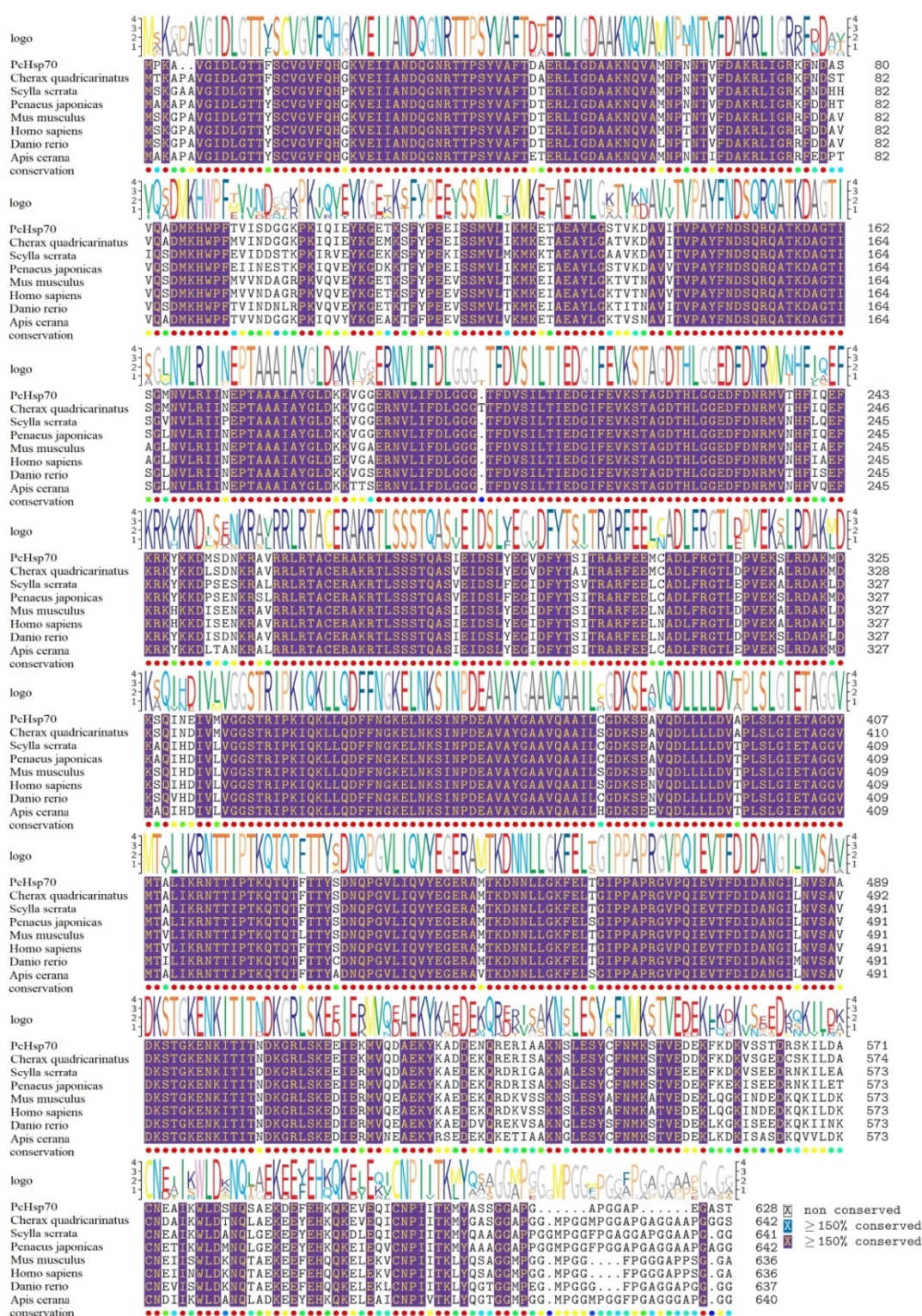
Figure 2. Multiple alignment of PcHsp70 with other species.

TEMPORAL EXPRESSION OF PCHSP70 POST-IMMUNE CHALLENGE

To further study the immune role of crayfish PcHsp70 during pathogen infection, Expression of PcHsp70 were detected after different immune challenge. When challenged with Gram-positive bacteria (*S. aureus*), the expression of PcHsp70 in hepatopancreas increased at 6 h post-injection (hpi), peaked at 24 hpi, and then returned to the average level at 48 hpi (**Figure 5A**). In gill, the expression of PcHsp70 increased at 24 hpi, peaked at 48 hpi, and then returned to the average level at 72 hpi (**Figure 5B**). Following injection with *V. parahaemolyticus*, the expression trend of PcHsp70 in hepatopancreas and gill was similar, significantly increasing during 6-48 hpi (**Figure 5C** and **5D**, *P <0.01*). And the expression of PcHsp70 in the hepatopancreas and gill peaked at 12 hpi (**Figure 4C** and **4D**). Af-
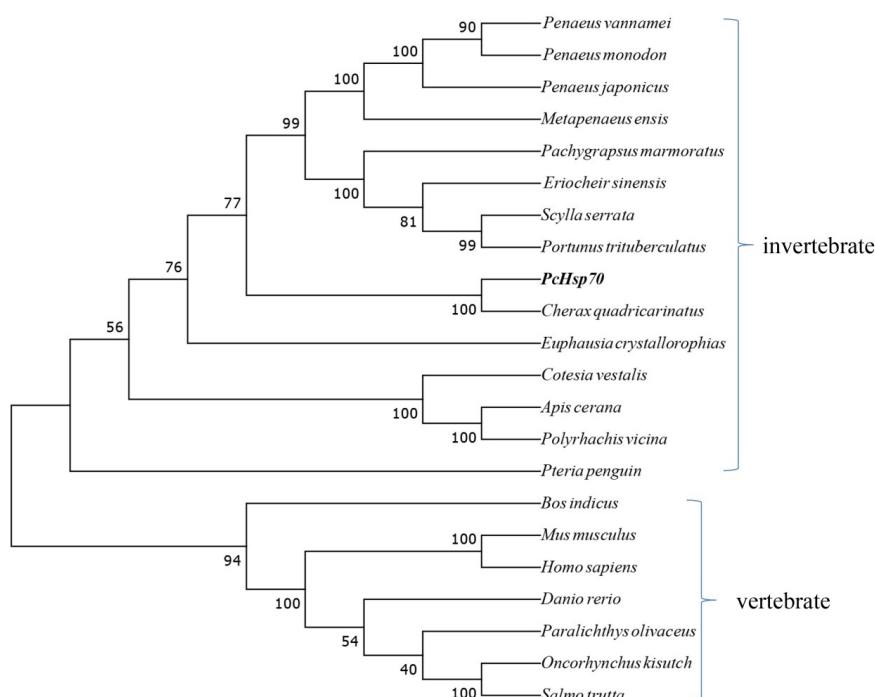
Figure 3. Phylogenetic tree of the PcHsp70 compared with Hsp70 from other species.
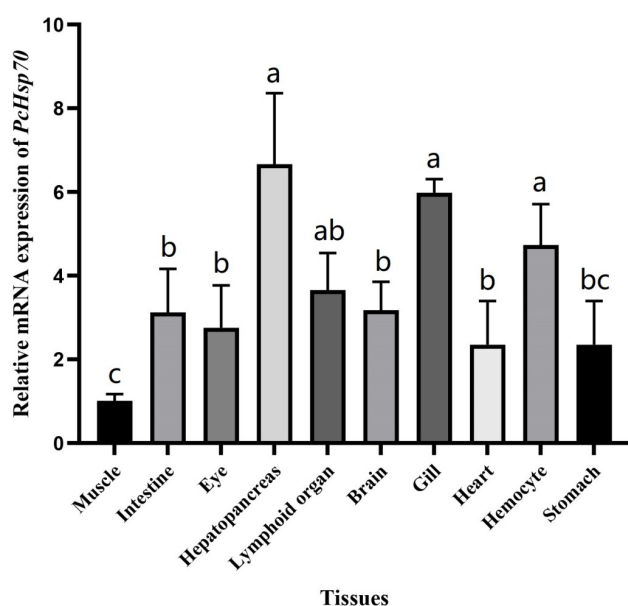
Figure 4. Tissue distribution of PcHsp70 in *P. clarkii*.

Each Bar represents the mean ± SD (n=3). Different lowercase letters indicate statistically significant differences (*P<0.05*).

ter the WSSV challenge, the expression of PcHsp70 in hepatopancreas was increased significantly during 12-48 hpi, while the expression in gill was significantly increased during 6- 48 hpi ([Figure 5C and 5D], *P <0.05*). However, the peak expression in hepatopancreas and gill was 12 phis and 6 hpi, respectively (**[Figure 5C]** and **[5D]**).

DISCUSSION

Hsp70 was one of the most well-studied Hsps, which played an essential biological role in improving cell tolerance to stress and maintaining cell self-stability.[20] We cloned an Hsp70 from *P. Clarkii* (PcHsp70) in this study. The PcHsp70 protein contained a Dnak characteristic motif, an ATP-binding site motif, a non-organellar motif, and two signature sequences of the Hsp70 family, indicating that the PcHsp70 had ATPase activity and belonged to cytoplasmic Hsp70 of Dnak type.[21] There is a highly conserved cytoplasmic specific regulatory motif at the C-terminal of PcHsp70, which existed in most eukaryotes of Hsp70. This motif affected the activity of the ATPase domain and the binding activity to the substrate and regulated the amount of Hsp70 mRNA transcription in the heat shock state.[22,23] Multiple sequence alignment results showed that PcHsp70 had high similarity with other Hsp species in the N terminal ATPase and substrate peptide binding domains. In contrast, its C-terminal domain was less conserved, consistent with the conserved and structural characteristics of the Hsp70 family (date was not shown).[24]

To preliminarily investigate the role of PcHsp70 in *P. Clarkii*, the spatial distribution and temporal expression of PcHsp70 were analyzed after different immune stimulation. Tissue distribution results showed that PcHsp70 was widely distributed in different tissues. The highest expression was found in the hepatopancreas, followed by gill and hemocyte. In crustaceans, hepatopancreas was essential for detoxification and immune protection.[25] Gills were in direct contact with the external environment and were important immune barriers.[26] Hemocytes are essential in immune responses against pathogens, cell encapsulation, and phagocytosis.[25] The high expression of PcHsp70 in vital im-
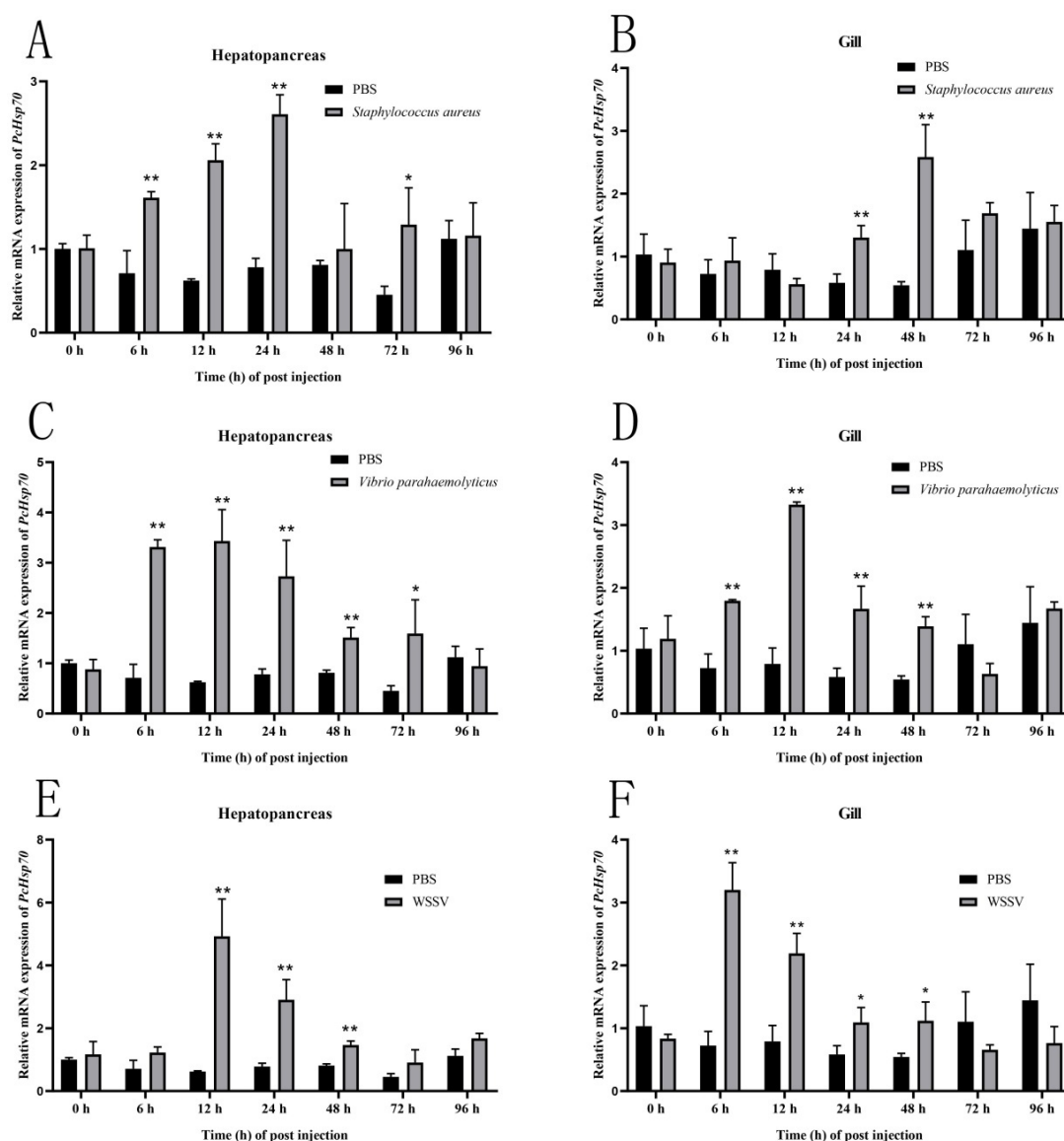
Figure 5. Temporal expression patterns of PcHsp70 post immune challenge.

Figures 5A and 5B show the expression of PcHsp70 in hepatopancreas and gill post Gram-positive bacteria (*S. aureus*) stimulation. Figures 5C and 5D show the expression of PcHsp70 in hepatopancreas and gill post Gram-negative bacteria (*V. parahaemolyticus*) stimulation. Figures 5E and 5F show the expression of PcHsp70 in hepatopancreas and gill post-WSSV stimulation. The error bars indicate significant group differences (*$P < 0.05$, **$P < 0.01$). Data are shown as means ± SDs (n = 3).

mune organs of hepatopancreas, gill, and hemocyte showed that these tissues were essential sites for PcHsp70 to play the role of immunomodulatory, anti-stress or antioxidant effects. Following *S. aureus, V. parahaemolyticus,* and WSSV virus stimulation, the expression of PcHsp70 in hepatopancreas and gill was significantly upregulated, suggesting that PcHsp70 might participate in the immune response during Gram-positive bacteria, Gram-negative bacteria, and virus infection in *P. Clarkii*. There may be several reasons for the increased expression of PcHsp70 in crayfish after being infected with pathogens. In humans, Hsp70 can activate innate immunity by regulating the activity of other proteins.[27] In addition, pathogen invasion could induce reactive oxygen species (ROS) or toxic substances, damaging the DNA and proteins.[28] As a result of ROS or toxic substances production, the accumulation of denatured proteins might trigger the expression of Hsp70 to solubilize

denatured protein, restore protein function, and clear damaged proteins.[17] In the present study, a common characteristic of the expression of PcHsp70 in the hepatopancreas and gills after stimulation with *S. aureus, V. parahaemolyticus,* or WSSV stimulation was that the expression of PcHsp70 was mainly induced between 6-48 hpi, and then tended to be average compared with the control group. This may be due to the production of many ROS or toxic substances in the early stage of pathogen invasion. Hence, PcHsp70 is induced to eliminate the damage of ROS or toxic substances to the host DNA and protein. In the later stages of infection, pathogens may occupy host resources to promote their replication and hence result from inhibiting the expression of PcHsp70. Compared with *S. aureus* or *V. parahaemolyticus* stimulation, the expression of PcHsp70 induced by WSSV stimulation was more acute, which increased by up to 4.92-fold at 12 hpi after WSSV stimulation

in hepatopancreas. This may be due to the more significant toxicity of WSSV. The regulatory patterns between hepatopancreas and gills are generally similar, but some differences remain. The reasons for the different regulatory patterns between different tissues and between different pathogens need to be further studied. Studies have shown that the up-regulated expression of Hsp70 was beneficial in resisting the invasion of pathogens. In the giant tiger prawn *Penaeus monodon*, increasing Hsp70 expression could inhibit virus replication and increase shrimp Field's antiviral ability.[29] When the expression of Hsp70 was knocked down by RNAi technology in *Artemia franciscana*, it reduced tolerance to bacterial infection.[30] Therefore, induction of Hsp70 expression after pathogen invasion may be a protective mechanism for crayfish facing environmental stress.

In conclusion, the PcHsp70 was identified in *P. Clarkii*, and the expression patterns of PcHsp70 were analyzed after different immune challenges for the first time in *P. Clarkii*. Our results indicated that PcHsp70 was potentially involved in immune responses to Gram-positive, Gram-negative, and virus infections. This will help to understand the significance of PcHsp70 in the immune defense of crayfish. The molecular mechanism and pathway of PcHsp70 involved in immune response need to be further elucidated.

AUTHOR CONTRIBUTION

Conceptualization, Writing - original draft preparation: Chao Peng; *Resources*: Zhongyuan Chen; *Writing - review and editing*: Qing Han; *Software, Methodology*: Liye Shao; *Formal analysis and investigation*: Ping Mo; *Supervision, Funding acquisition*: Shuiqing Wu.

REFERENCES

1. Huang WJ, Leu JH, Tsau MT, Chen JC, Chen LL. Differential expression of LvHSP60 in shrimp in response to environmental stress. *Fish Shellfish Immunol*. 2011;30(2):576-582. doi:10.1016/j.fsi.2010.12.001

2. Bolhassani A, Agi E. Heat shock proteins in infection. *Clin Chim Acta*. 2019;498:90-100. doi:10.1016/j.cca.2019.08.015

3. Multhoff G. Heat shock protein 70 (Hsp70): membrane location, export and immunological relevance. *Methods*. 2007;43(3):229-237. doi:10.1016/j.ymeth.2007.06.006

4. Gołaś E, Maisuradze GG, Senet P, et al. Simulation of the opening and closing of Hsp70 chaperones by coarse-grained molecular dynamics. *J Chem Theory Comput*. 2012;8(5):1750-1764. doi:10.1021/ct200680g

5. Moseley P. Stress proteins and the immune response. *Immunopharmacology*. 2000;48(3):299-302. doi:10.1016/s0162-3109(00)00227-7

6. Srivastava P. Roles of heat-shock proteins in innate and adaptive immunity. *Nat Rev Immunol*. 2002;2(3):185-194. doi:10.1038/nri749

7. Janewanthanakul S, Supungul P, Tang S, Tassanakajon A. Heat shock protein 70 from Litopenaeus vannamei (LvHSP70) is involved in the innate immune response against white spot syndrome virus (WSSV) infection. *Dev Comp Immunol*. 2020;102:103476. doi:10.1016/j.dci.2019.103476

8. Valentim-Neto PA, Moser JR, Fraga APM, Marques MRF. Hsp70 expression in shrimp Litopenaeus vannamei in response to IHHNV and WSSV infection. *Virus Dis*. 2014;25(4):437-440. doi:10.1007/s13337-014-0236-6

9. Junprung W, Supungul P, Tassanakajon A. Litopenaeus vannamei heat shock protein 70 (LvHSP70) enhances resistance to a strain of Vibrio parahaemolyticus, which can cause acute hepatopancreatic necrosis disease (AHPND), by activating shrimp immunity. *Dev Comp Immunol*. 2019;90:138-146. doi:10.1016/j.dci.2018.09.011

10. Zhou J, Wang WN, He WY, et al. Expression of HSP60 and HSP70 in white shrimp, Litopenaeus vannamei in response to bacterial challenge. *J Invertebr Pathol*. 2010;103(3):170-178. doi:10.1016/j.jip.2009.12.006

11. Zhenyu G, Chuanzhen J, Jianhai X. Heat-shock protein 70 expression in shrimpFenneropenaeus chinensis during thermal and immune-challenged stress. *Chin J Ocean Limnol*. 2004;22(4):386-391. doi:10.1007/bf02843633

12. Junprung W, Supungul P, Tassanakajon A. HSP70 and HSP90 are involved in shrimp Penaeus vannamei tolerance to AHPND-causing strain of Vibrio parahaemolyticus after non-lethal heat shock. *Fish Shellfish Immunol*. 2017;60:237-246. doi:10.1016/j.fsi.2016.11.049

13. Zhu JJ, Ye ZZ, Li CS, et al. Identification and molecular characterization of a novel anti-lipopolysaccharide factor (ALF) from red swamp crayfish, Procambarus clarkii. *Int J Biol Macromol*. 2019;132:43-50. doi:10.1016/j.ijbiomac.2019.03.167

14. Marmaras VJ, Lampropoulou M. Regulators and signalling in insect haemocyte immunity. *Cell Signal*. 2009;21(2):186-195. doi:10.1016/j.cellsig.2008.08.014

15. Peng C, Zhao C, Wang PF, Yan LL, Fan SG, Qiu LH. Identification of a TRIM32 from Penaeus monodon is involved in autophagy and innate immunity during white spot syndrome virus infection. *Developmental & Comparative Immunology*. 2021;123:104169. doi:10.1016/j.dci.2021.104169

16. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)). *Methods*. 2001;25(4):402-408. doi:10.1006/meth.2001.1262

17. Zhang Z, Zhang Q. Molecular cloning, characterization and expression of heat shock protein 70 gene from the oyster Crassostrea hongkongensis responding to thermal stress and exposure of Cu2+ and malachite green. *Gene*. 2012;497(2):172-180. doi:10.1016/j.gene.2012.01.058

18. Wang S, Zhao XF, Wang JX. Molecular cloning and characterization of the translationally controlled tumor protein from Fenneropenaeus chinensis. *Mol Biol Rep*. 2009;36(7):1683-1693. doi:10.1007/s11033-008-9369-2

19. Zhao C, Peng C, Wang P, Yan L, Fan S, Qiu L. Identification of a Shrimp E3 Ubiquitin Ligase TRIM50-Like Involved in Restricting White Spot Syndrome Virus Proliferation by Its Mediated Autophagy and Ubiquitination. *Front Immunol*. 2021;12:682562. doi:10.3389/fimmu.2021.682562

20. Iryani MTM, Lv A, Sun J, et al. Effects of heat shock protein 70 knockdown on the tolerance of the brine shrimp Artemia franciscana to aquaculture-related stressors: Implications for aquatic animal health and production. *Aquaculture*. 2022;550:737872. doi:10.1016/j.aquaculture.2021.737 872

21. Ravaux J, Toullec JY, Léger N, Lopez P, Gaill F, Shillito B. First hsp70 from two hydrothermal vent shrimps, Mirocaris fortunata and Rimicaris exoculata: characterization and sequence analysis. *Gene*. 2007;386(1-2):162-172. doi:10.1016/j.gene.2006.09.0 01

22. Wu R, Sun Y, Lei LM, Xie ST. Molecular identification and expression of heat shock cognate 70 (HSC70) in the Pacific white shrimp Litopenaeus vannamei. *Mol Biol*. 2008;42(2):234-242. doi:10.1134/ s002689330802009x

23. Kawamoto Y, Akiguchi I, Shirakashi Y, et al. Accumulation of Hsc70 and Hsp70 in glial cytoplasmic inclusions in patients with multiple system atrophy. *Brain Research*. 2007;1136:219-227. doi:10.1016/j.brainres.2006.12.049

24. Kiang J, Tsokos GC. Heat Shock Protein 70 kDa Molecular Biology, Biochemistry, and Physiology. *Pharmacology & Therapeutics*. 1998;80(2):183-201. doi:10.1016/s0163-7258(98)00028-x

25. Söderhäll K, Cerenius L. Role of the prophenoloxidase-activating system in invertebrate immunity. *Current Opinion in Immunology*. 1998;10(1):23-28. doi:10.1016/s0952-7915(98)8002 6-5

26. Clavero-Salas A, Sotelo-Mundo RR, Gollas-Galván T, et al. Transcriptome analysis of gills from the white shrimp Litopenaeus vannamei infected with White Spot Syndrome Virus. *Fish Shellfish Immun*. 2007;23(2):459-472. doi:10.1016/j.fsi.2007.01.010

27. Asea A. Heat Shock Proteins and Toll-Like Receptors. *Toll-Like Receptors (TLRs) and Innate Immunity*. 2008;183(183):111-127. doi:10.1007/97 8-3-540-72167-3_6

28. Ramaglia V, Harapa GM, White N, Buck LT. Bacterial infection and tissue-specific Hsp72, -73 and -90 expression in western painted turtles. *Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology*. 2004;138(2):139-148. doi:10.1016/j.cca.2004.06.007

29. de la Vega E, Hall MR, Degnan BM, Wilson KJ. Short-term hyperthermic treatment of Penaeus monodon increases expression of heat shock protein 70 (HSP70) and reduces replication of gill associated virus (GAV). *Aquaculture*. 2006;253(1-4):82-90. doi:1 0.1016/j.aquaculture.2005.07.041

30. Iryani MTM, MacRae TH, Panchakshari S, et al. Knockdown of heat shock protein 70 (Hsp70) by RNAi reduces the tolerance of Artemia franciscana nauplii to heat and bacterial infection. *Journal of Experimental Marine Biology and Ecology*. 2017;487:106-112. doi:10.1016/j.jembe.2016.12.004