



Short communication

Alternation of *Venerupis philippinarum* Hsp40 gene expression in response to pathogen challenge and heavy metal exposure

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ABSTRACT

HSP40 was an understudied protein family with co-chaperone activity. In the present study, a HSP40 homology was cloned from *Venerupis philippinarum* haemocytes (designated as VpHSP40) by EST analysis and RACE approaches. The expression profiles of VpHSP40 under *Vibrio anguillarum* challenge and heavy metal exposure were investigated by quantitative real-time RT-PCR. The bacterial challenge could significantly up-regulate the mRNA expression, and the highest expression level was detected at 24 h post-infection with 6.0-fold increase compared with that in the control group. During heavy metal exposure experiment, the expression of VpHSP40 could also be induced by Cu²⁺ and Cd²⁺ at different concentration, where Cu²⁺ displayed more toxic effect on clam than that of Cd²⁺. Concerning the same heavy metal, varied effect on VpHSP40 expression was detected at different concentration of heavy metal. Taking together, these results suggested that VpHSP40 was perhaps involved in mediating the immune responses and environmental stresses in *V. philippinarum*.

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1. Introduction

It is widely accepted that HSP40/DnaJ family function as co-chaperones with the well-studied molecular chaperones HSP70 and promote protein folding, assembly and transport of proteins within the cell [1–3]. Recent study indicates that they bind to HSP70 through a well conserved “J domain” normally located in the N-terminal ends [4]. The J domain consists of four helices and a loop region between helices II and III that contains the highly conserved HPD motif [5]. In addition to J domain, two other comparatively conserved domains, the glycine-rich region (G domain) located between the J domain and other regions of HSP40 proteins and the central domain containing four repeats of a CXXCXGXG motif (CRR domain) involved in polypeptide binding [6], also exist in some HSP40s. Depending on the presence of these regions, HSP40 could be categorized into three subgroups. Type I HSP40 proteins possess all three domains similar to that of DnaJ. Type II HSP40 proteins lack

the CRR domain, and Type III proteins possess only the J domain [3,4,6].

Since the first discovery of HSP40/DnaJ in bacteria, the number of identified HSP40/DnaJ homology expanded rapidly. More than 7000 sequences, ubiquitously existed from bacteria to mammalian, have been deposited in Genbank up to data. However, little knowledge is available to the important chaperones molecule in mollusk and its expression profiles under pathogen challenge and heavy metal exposure. In order to fill in the gap, the present study aims to: (1) clone the full-length cDNA of HSP40 from *Venerupis philippinarum*; (2) investigate temporal expression profile of VpHSP40 after being infected by *Vibrio anguillarum* pathogen or heavy metal exposure.

2. Materials and methods

2.1. Clams and challenge

The clams *V. philippinarum* (7.5–11 g in weight) were purchased from a local farm, and acclimated for a week before commencement of the experiment. After the acclimation period, the clams

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were randomly divided into ten flat-bottomed rectangular tanks with 50 L capacity, each containing 50 clams. The temperature was held at 20–22 °C throughout the whole experiment. The salinity for the supplied seawater was kept at 30‰.

For the *V. anguillarum* challenge experiment, one tank served as the control. Another five tanks were immersed with high density of *V. anguillarum* with final concentration of 10^7 CFU mL⁻¹. The infected clams were randomly sampled at 6 h, 12 h, 24 h, 48 h, 72 h and 96 h respectively. For heavy metal exposure experiment, clams cultured in seawater were used as the control group. The other four tanks were treated with cadmium chloride (10 and 40 µg L⁻¹ CdCl₂) or copper chloride (10 and 20 µg L⁻¹ CuCl₂) under two different final concentration levels, respectively. Stock solutions containing the corresponding heavy metal ions were prepared. After 24 h, 48 h and 96 h exposure, the haemolymphs were collected from the control and the treated groups using a syringe individually and centrifuged at 2000×g, 4 °C for 10 min to harvest the haemocytes. There were five replicates for each time point.

2.2. Cloning the full-length cDNA of VpHSP40

A cDNA library was constructed with the haemocytes of a clam as previously described [7]. BLAST analysis of all the 3226 EST sequences revealed that one EST of 527 bp was highly similar to the previously identified HSP40. Two primers, P1 (GTGACCTC-TACCTTCCCTT) and P2 (CTTTCTGCCACGCCAACTA), were designed to amplify the full-length cDNA of VpHSP40. The amplified PCR products were gel-purified and cloned into pMD18-T vector (Takara). After transformed into the competent cells of *Escherichia coli* JM109, the positive recombinants were identified through ampicillin selection and PCR screening with M13-47 and RV-M primers. Three of the positive clones were sequenced on an ABI3730 Automated Sequencer, and the resulting sequences were subjected to cluster analysis. The VpHSP40 gene sequence was analyzed using the BLAST algorithm at NCBI web site (<http://www.ncbi.nlm.nih.gov/blast>) and the deduced amino acid sequence was analyzed with the Expert Protein Analysis System (<http://www.expasy.org/>). Specific domain prediction was detected by SMART service (<http://smart.embl-heidelberg.de/>).

2.3. Temporal expression profile of VpHSP40 mRNA in haemocytes post *V. anguillarum* challenge or heavy metal exposure

Comparative qualification of VpHSP40 transcript in haemocytes after *Vibrio* or heavy metal challenge was measured by quantitative real-time RT-PCR in Applied Biosystem 7500 fast Real-time PCR System. Two VpHSP40 gene-specific primers, P3 (TCAGGTAGAAA-CAGGAGCAT) and P4 (TCAAGGGAAAGGTAGAGG), were used to amplify a product of 210 bp. The product was purified and sequenced to verify the PCR specificity. A set of actin primers, P5 (CTCCCTTGAGAAGAGCTACGA) and P6 (GATACCAGCAGATTCCA-TACCC), were used to amplify a 121 bp fragment as internal control. The reaction component, thermal profile, and the data analysis were conducted as previously described [7]. All data were given in terms of relative mRNA expression as means ± S.E. The results were subjected to analysis of *t*-test, and the *P* values less than 0.05 were considered statistically significant.

3. Results and discussion

3.1. cDNA cloning and analysis of VpHSP40

Compared with HSP70 and HSP90, no mollusk HSP40 sequence information has been deposited in Genbank. In this study, a 1352 bp product representing the full-length cDNA of VpHSP40

were obtained by overlapping EST and the fragments from RACE. The sequence was deposited in GenBank under accession no. GQ384397. The deduced amino acid sequence of VpHSP40 was shown below the corresponding nucleotide acid sequence in Fig. 1.

The complete sequence of VpHSP40 cDNA contained a 5' untranslated region (UTR) of 64 bp, a 3' UTR of 334 bp with a polyA tail. An open reading frame (ORF) of 954 bp encoded a polypeptide of 317 amino acids with the predicted molecular weight of 35.74 kDa. No signal peptide was identified from the deduced amino acid of VpHSP40, which further demonstrated VpHSP40 was a non-secreted protein as other HSP40s. Blast analysis indicated the deduced amino acid of VpHSP40 shared significant homology to HSP40 protein family, such as 69% identity with HSP40 from *Ciona intestinalis* (NP_001027731.1), 63% identity with *Rattus norvegicus* (AAQ17187.1), 48% identity with DnaJB1 from *Homo sapiens* (CAG38724.1), 45% identity with *Danio rerio* (CAN88790.1).

The characteristic conserved DnaJ domain for HSP40 was also existed in the N-terminus of VpHSP40 by SMART analysis. The domain was located from Val^{3rd} to Lys^{61st} in the deduced amino acid of VpHSP40, where conserved HPD tripeptide was replaced by HPE (Fig. 1). This substitution might not affect the function of the tripeptide, for aspartic acid had similar physiological characteristics

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1  GACCCATATTTGTGTACAGTCAATAATAACCAAAAAGAAACACACTTTATCACAA
61  CAAAATGGGTGTCGATTATTACAATTTGTTAAATTTAACCGGTCTGCAACTGATCGGGA
1  M G V D Y Y N L L N L T R S A T D A D
121 TATCAAAAAGCATTACGAAAATATCTTTAAATTTCCATCCAGAGAAGTCCCTGGTGA
20  I K K H Y R K L S L K F H P E K S P G D
181 CCAAGCAGCTGCTGACAAATTCACAAAGTTGAGAGGCATATGATGTTTTATCAGATCC
40  Q A A A D K F K Q V A E A Y D V L S D P
241 AAGAAAGCGGGCTGTTTATGATCAGTTTGGTGAAGGGTTTGAAGAATGGAGTCTCTAG
60  R K R A V Y D Q F G E L K N G V P S
301 TGGTCAAGTAGAAAACAGGAGCATGGACTCAAGGATATACTTTCCACGGAAATGCTGACAA
80  G Q V E T G A W T Q G Y T F H G N A D K
361 AGTTTTCCGGGATTTCTTTGGAGGAGATAATCCATCCAGAATTTTATGACAGAGTTGA
100 V F R D F F G G D N P F Q E F Y D R V D
421 TGGAGATATGAGTATGTCATTTGGAGGCTAGTTGGCGGTGGCAGAAAGAACGAGATCC
120 G D M S M S F G G L V G R G R K K Q D P
481 ACCTATTGAACGTGACCTTACCTTTCCCTTGAAGAAGTTTCCATGGCTGTACAAGAA
140 P I E R D L Y L S L E E V F H G C T K K
541 AATGAAATATCAAGAAGAGTAATGAATGAAGATGGTCATACATCAAGCATTAGAGATA
160 M K I S R R V M N E D G H T S S I R D K
601 GATTTTAAACACTACTGTGAAGAAAGGTTGGAACCAATAACAAGAATCACATTTCCAGA
180 I L T I T V K K G W K P N T R I T F P E
661 GGAAGCGACCAAGGTCCAATAATGTACCAGTCGACATTTGTTTATTTGTGAAGACAA
200 E G D Q G P N N V P A D I V F I V K D K
721 ACAACATCAGAGATTTAGACGCGAGGTTGCAACCTTATACACAGCAGCAAAAGTACCACT
220 Q H Q R F R R E G V N L I H T A K V P L
781 TGGTAAAGCCCTGACAGGTTGTACAGTAGATATCTAACATTTGGATGAAAGAATGCTTCA
240 G K A L T G C T V D I L T L D E R M L H
841 TATACCAATCAATGATATCATCAACCTGGTTACAGAAAATGTGTACCAAAAAGAAAGAA
260 I P I N D I I K P G Y R K C V P K E G M
901 GCCTCTTCTGCTGATCCTACACAGAAAGGAGATTTGATCATTTGAATTTGACATTGAATT
280 P L S A D P T Q K G D L I L E F D I E F
961 CCCTGCCACTTAAACACAGAAAAGAAATTTGGTCAAGGCACCCCTTCTTACCTAGAG
300 P A A T L T P E K K D L V K A A L L H *
1021 ACAATATTTGTTATAAAGAATATCTGTGATAGAGTGAATGATTTATGTTTTATGAGTA
1081 TCTGACTATTTTATATGCTTAAATGATGTCGAATGATATATGTTGTTGTAGAAAAAATG
1141 AAATACAATGATTTAAACAAATTTCAAAATTTCAATAACAAATACTGAGAAATATATTTT
1201 AATAGAACTTATATGGGAAAAGAAATATCTTTAAATTTGGCATTGAATTTGTTAACAGC
1261 TTTTGTGCTACTGATCATCATAGTTTACTGATTTGTCAGATGCCAAATGTATAAA
1321 TCTAAAAGAATTTTAAAAAATAAAAAAAAAA

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Fig. 1. The nucleotide sequence (above) and deduced amino acid sequence (below) of VpHSP40. Nucleotides were numbered from the first base at the 5' end. The polyadenylation signal was in bold and italics. The asterisk indicated the stop codon. Conserved DnaJ domain was underlined. DnaJ_C motif was dash-dot lined. HPD tripeptide was boxed.

with glutamic acid. Another specific motif DnaJ_C with 120–170 residues size was also identified in the N-terminus of VpHSP40 (Fig. 1). Conserved DIF (Asp-Ile-Phe) sequence for G/F domain and CXXCXGXG sequence for CRR domain was not found in the deduced amino acid of VpHSP40. All these results indicated that VpHSP40 belonged to type III DnaJ family.

3.2. The expression profile of VpHSP40 after *Vibrio* challenge

It is well known that HSPs could respond to high temperature, pathogen microorganism and other environmental stressors [8–14]. These intensive studies were mainly concentrated on HSP70, HSP90 and some small HSPs. The expression profile of HSP40 after pathogenic bacterial challenge was only investigated in *Paralichthys olivaceus* [6]. In the present study, we investigated the temporal mRNA expression of VpHSP40 in the haemocytes of clams post-*Vibrio* challenge (Fig. 2). During the first 24 h after pathogen challenge, the expression level of VpHSP40 mRNA was up-regulated gradually and increased 6.0-fold compared with that in the control group at 24 h ($P < 0.05$). After that, the expression level was sharply decreased at 48 h, which was perhaps due to the increase in degranulation of VpHSP40-producing haemocytes responding to pathogen challenge. As time progressed, the second peak of VpHSP40 expression was detected at 72 h post-infection with 5.9-fold of the control group ($P < 0.01$). The recruitment of VpHSP40-producing haemocytes into circulating system probably contributed to the drastic increase of VpHSP40 transcript at this time point. At 96 h, VpHSP40 mRNA level dropped greatly and was only 1.9-fold higher than the control. An unpaired, two-tailed *t*-test with control and challenged groups showed statistically significant difference in VpHSP40 gene expression at 12 h ($P < 0.05$), 24 h ($P < 0.05$), 72 h ($P < 0.01$) and 96 h ($P < 0.05$) post-infection. However, no significant difference was observed in other time points of the challenge group. These results indicated that VpHSP40 was perhaps involved in the clam immunity responses to pathogen infection.

Although the increased expression patterns were also observed for type I and II HSP40 in UV-inactivated SMRV infected FEC cell, different types of HSP40s displayed distinct time courses and expression level. Type I HSP40 was not only responded more quickly than that of type II, but also had the higher expression increment [6]. The mRNA expression of type III HSP40 was rarely investigated to our knowledge. As a co-chaperone with HSP70, the expression profile of type III HSP40 was perhaps accordance with that of HSP70. Further study was needed to define the time-course expression profile of clam HSP70 proteins after pathogenic challenge.

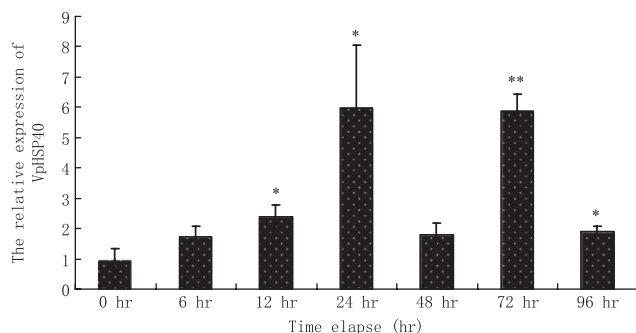


Fig. 2. Time-course expression level of VpHSP40 transcript in haemocytes after *Vibrio anguillarum* infection measured by quantitative real-time PCR at 0 h, 6 h, 12 h, 24 h, 48 h, 72 h and 96 h. Each symbol and vertical bar represented the mean \pm S.D. ($n = 5$). Significant differences across control were indicated with an asterisk at $P < 0.05$ and two asterisks at $P < 0.01$.

3.3. The expression profile of VpHSP40 in response to heavy metal exposure

Evidence continues to accumulate on the modulating effects of environmental contaminants on host immunity. It has been well acknowledged that HSPs are not only expressed in response to pathogen infection, but also in response to chemical stressor like heavy metal or organic pollutants. In *Chironomus riparius*, the HSP40 and HSP90 mRNA expressions were both significantly up-regulated in response to short and long-term exposure to DEHP at concentrations of 1, 10, and 30 $\mu\text{g L}^{-1}$ [15]. In scallop, HSPs responded to various chemical stresses (Cd^{2+} , Cu^{2+} , Pb^{2+} or PAH) with a dose-dependent expression pattern were also observed by previous studies [11,16–18]. How HSP40 responded to heavy metal in mollusk was not clear up to data. In this study, the heavy metals Cu^{2+} and Cd^{2+} were found to induce the expression of VpHSP40 at different concentration levels.

In Cu^{2+} treatment group (Fig. 3), the expression level of VpHSP40 was increased and reached around 3.0-fold compared with that in the control group from 24 h to 96 h when Cu^{2+} concentration was of 10 $\mu\text{g L}^{-1}$. The statistically significant difference was observed at 24 h ($P < 0.05$) and 48 h ($P < 0.05$). After increasing Cu^{2+} concentration to 20 $\mu\text{g L}^{-1}$, no significant change was found during the first 24 h. As time progressed, the expression of VpHSP40 mRNA increased significantly, and the expression level were 2.9-fold at 48 h ($P < 0.01$) and 2.2-fold at 96 h ($P < 0.05$) compared with that in the control group. VpHSP40 mRNA in Cd^{2+} treatment group showed discrepant expression profile under different concentration of heavy metal (Fig. 4). In the lower concentration group (10 $\mu\text{g L}^{-1}$), the expression of VpHSP40 was up-regulated with the 1.6-fold increase compared to the control during the first 24 h post-exposure. Then the expression of VpHsp40 was sharply down-regulated to the minimum point at 48 h. After that, the second increase trend of VpHSP40 expression was detected at 96 h post-exposure. When Cd^{2+} concentration increased to 40 $\mu\text{g L}^{-1}$, VpHSP40 mRNA expression decreased firstly at 24 h. Then, the maximum expression of VpHSP40 was detected with 2.8-fold increase compared to the control at 48 h. At 96 h post-exposure, the expression level decreased sharply and reached 1/5 of the control group.

The distinct effect of heavy metals on VpHSP40 expression indicated the existence of difference in the toxicity of Cu^{2+} or Cd^{2+} . At the concentration of 10 $\mu\text{g L}^{-1}$ Cu^{2+} or Cd^{2+} , the VpHSP40 gene expression level induced by Cu^{2+} was about two times higher than

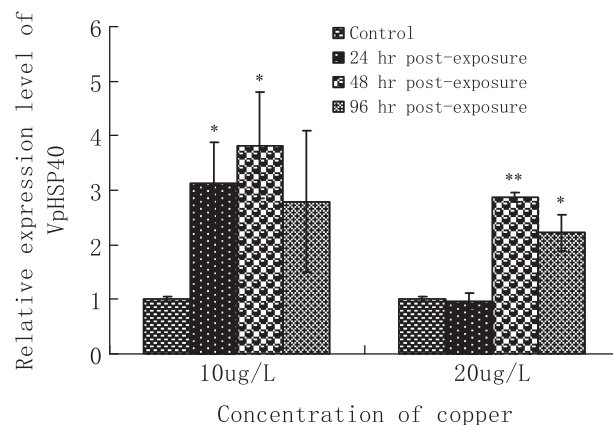


Fig. 3. The VpHSP40 mRNA expression levels analyzed by real-time PCR in clams exposed to different concentrations of Cu^{2+} after 24 h, 48 h and 96 h exposure. Each symbol and vertical bar represented the mean \pm S.D. ($n = 5$). Significant differences across control were indicated with an asterisk at $P < 0.05$ and two asterisks at $P < 0.01$.

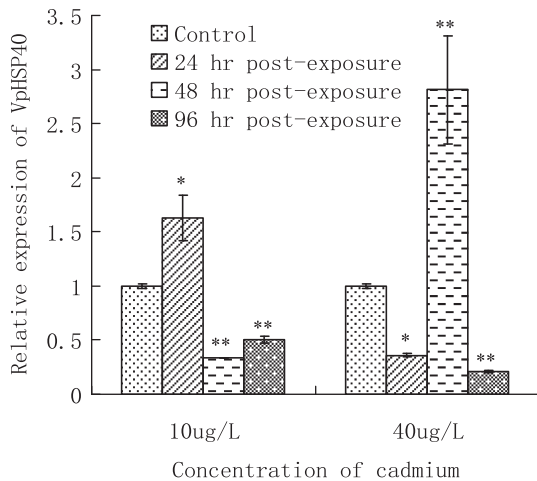


Fig. 4. The VpHSP40 mRNA expression levels analyzed by real-time PCR in clams exposed to different concentrations of Cd^{2+} after 24 h, 48 h and 96 h exposure. Each symbol and vertical bar represented the mean \pm S.D ($n = 5$). Significant differences across control were indicated with an asterisk at $P < 0.05$ and two asterisks at $P < 0.01$.

that of for Cd^{2+} . In another words, the same induction intensity of the gene expression corresponded to higher concentration of Cd^{2+} or longer time exposure than that of Cu^{2+} , which suggested Cu^{2+} could be more toxic than Cd^{2+} for clams. Change in haemocyte count might account partially for the discrepant expression profile for different heavy metal challenge. In shrimp, heavy metal exposure caused a decrease in the haemocyte count during the first 8 h, and the greatest decrease in haemocyte numbers was induced by Pb followed, in descending order, by Zn, Hg, Cr, Cu and Cd [19]. Moreover, it has been shown that transcriptional regulation of heat shock protein genes is mediated by the interaction of heat shock factor (HSF) transcription factors with heat shock elements in the heat shock protein gene promoter regions [20,21]. Whether the discrepant expression patterns of VpHSP40 to the heavy metal exposure are mediated by transcription factors HSF as well deserved further investigation.

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