Fish & Shellfish Immunology (2008) **25**, 250–257

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Identification of the small heat shock protein, HSP21, of shrimp *Penaeus monodon* and the gene expression of HSP21 is inactivated after white spot syndrome virus (WSSV) infection

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Received 24 April 2008; revised 9 June 2008; accepted 10 June 2008
Available online 18 June 2008

KEYWORDS
White spot syndrome virus (WSSV);
Small heat shock protein (smHSP);
Penaeus monodon;
Chaperone activity;
Expressed sequence tag (EST)

Abstract The white spot syndrome virus (WSSV) is the causative agent of a severe disease in cultivated shrimp. The virus causes high mortality and leads to heavy stress on shrimps. In response to a variety of stresses, living organisms express particular sets of genes such as *HSPs*. In this study, a *HSP21* gene, categorized into the small heat shock protein (smHSP) family, of shrimp *Penaeus monodon* was identified by annotating the EST databases established from WSSV-infected and WSSV-uninfected shrimp. The shrimp *HSP21* gene was 555 bp in length. The thermal aggregation assay showed that the *HSP21* had chaperone activity. The result of real-time PCR indicated that *HSP21* was constitutive and inducible and was highly expressed in almost all organs such as the epithelium, gill, stomach, midgut, lymphoid organ, hepatopancreas, nervous tissue and heart, but less expressed in haemolymph. However, *HSP21* gene showed down-regulation after WSSV infection. It suggests that gene regulation of *HSP21* was seriously affected by WSSV.

Introduction

Aquaculture industry is an important economic income of many countries. However, it is threatened by many pathogens, especially viruses. Among these viruses, the white

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doi:10.1016/j.fsi.2008.06.002

spot syndrome virus (WSSV) is the most serious one. While the WSSV infection mechanism and the host responses induced by WSSV infection stay unclear, studies on investigating the host immune response after WSSV infection and prophylactic/therapeutic methods become pertinent [1—5]. Moreover, WSSV structural proteins such as VP19, VP28 and VP26 have been adopted to develop a prophylactic vaccine against WSSV [6,7]. In spite of this, to investigate the molecules and mechanisms relevant to the immune response is still relevant fundamental work.

To date, many approaches, such as expressed sequence tags (ESTs), RT-PCR, microarray chips and so on, have attempted to identify virus-host interactions [8]. Among these methods, ESTs have widely been applied as the foundation sequence of some genome-scale analyses. Such reconstructomic analyses use the EST cluster assemblies and singletons as an equivalent to a whole genome's gene collection. EST derived cluster sequences have been widely annotated with tentative functions [9]. Recently, a series of heat shock proteins (HSPs) were identified while widely annotating the EST databases established from many crustaceans. In response to a variety of stresses such as temperature extremes, xenobiotics, heavy metals, UV, oxidizing agents, or high levels of growth hormones, living organisms express particular sets of genes such as *HSPs*. *HSPs* are expressed for the stress tolerance and promote cell survival, especially by refolding proteins and preventing their denaturation [10,11], as well as participating in a variety of normal cellular processes including protein trafficking, signal transduction, DNA replication and protein synthesis [12]. The *HSP* genes are highly conserved in all eukaryotes and prokaryotes, and based on the sequence homology and molecular weight of proteins, the genes have been divided into families such as *hsp110*, *hsp100*, *hsp90*, *hsp70*, *hsp60*, *hsp40*, and *hsp20* [13]. These gene families consist of stress-inducible and constitutively expressed genes. Inducible genes are expressed at low levels under non-stress conditions but are expressed increasingly in response to stress. Nevertheless, basal levels of constitutive genes are high and change relatively little in response to stress [14,15].

In this study, we identified a *HSP21* gene, categorized into the small heat shock protein (smHSP) family, of shrimp *Penaeus monodon*. The results showed that the HSP21, with chaperone activity, was constitutive and heat-inducible. We also analyzed the transcriptional changes after WSSV infection. However, real-time PCR revealed that the *HSP21* gene was down-regulated after WSSV infection suggesting that gene regulation of *HSP21* was seriously affected by WSSV.

Materials and methods

Virus, EST database and EST-based transcription analysis

WSSV, referred to as the WSSV Taiwan-1 strain (WSSV T-1 strain), collected in 1994 in Taiwan from infected *P. monodon* [16] was used to construct WSSV genomic libraries. At the same time, WSSV-infected and WSSV-uninfected ESTs, named as PmTwI and PmTwN, respectively, were constructed using total RNA isolated from the WSSV T-1 strain infected and uninfected *P. monodon* postlarvae (PL20). The details of library-construction and transcription analysis of identified protein genes were described in Wang et al. [8].

Amino acid sequence comparison and phylogenetic construction

The complete coding sequence was compared with the GenBank database using the program BlastX. Amino acid sequences of nine small heat shock proteins (smHSPs) isolated from other invertebrate animals were retrieved from GenBank, and were used for sequence alignment and phylogenetic analysis. Multiple sequence alignment was done using the program ClustalX and edited with Genedoc software. The complete coding sequences of these *smHSPs* were subsequently subjected to phylogenetic analysis and were performed with Mega4.0 software using the Neighbor-Joining algorithm. One thousand bootstrap replicates were generated to test the robustness of the trees. Sequences used in the alignment analysis and phylogenetic tree, followed by their GenBank accession number, were *Nasonia vitripennis* (XP_001607669.1), *Locusta migratoria* (ABC84493.1), *Apis mellifera* (XP_392405.3), *Bombyx mori* (NP_001036985.1), *Heliconius erato* (ABS57447.1), *Tribolium castaneum* (ABS57447.1), *Aedes aegypti* (XP_001657982.1), *Drosophila melanogaster* (NP_608326.1), and *Caenorhabditis elegans* (NP_001024375.1).

Experimental animals, tissue expression of *HSP21* transcription and heat shock experiment

Tiger shrimp *P. monodon* (body length, 20 ± 2 cm; weight, 30 ± 5 g) were collected from the Tungkang Biotechnology Research Center located in southern Taiwan. The organs — epithelium, gill, stomach, midgut, lymphoid organ, hepatopancreas, nervous tissue, haemolymph, and heart — were collected from *P. monodon*.

Shrimps were kept in a plastic tank supplied with seawater with 3% salinity. In the heat shock experiment, three shrimps were transferred from water maintained at room temperature of 24 ± 1 °C to a tank containing seawater preheated to 36 ± 1 °C. After 1 h at 36 ± 1 °C, as the heat shock treatment, the temperature of the water was decreased within 1 min to room temperature by draining the tank's preheated water and adding seawater of room temperature (24 °C) at the same time. After this, the tank was kept at room temperature for 0.5 h for the shrimps' recovery. Three shrimps in the control group were transferred to another tank of water which was kept at room temperature, and after 1 h this tank was also drained of its original water while adding water of room temperature. A pleopod of three shrimps from each group was withdrawn before the heat shock as well as 0, 1, 2, 4 and 6 h after recovery and separately preserved in liquid nitrogen immediately for RNA extraction to analyze the *HSP21* gene expression. Total RNA was isolated using TRIzol Reagent (Invitrogen) according to the instructions provided by the manufacturer and the modified detail was as described previously [17].

Quantification of *HSP21* mRNA expression by real-time PCR

Real-time PCR of shrimp *smHSP* mRNA was performed using gene-specific primers. *β-actin* mRNA was used as the normalization control. PCR primers for gene-specific primer sets were designed using Primer Express V.2.0 software (Applied Biosystems Inc.). The sense and antisense primers for *HSP21* were 5′- CACGAGGAGAAGTCTGACAAC-3′ and 5′-GAGAGCGAGGACTTGATGAG-3′, respectively. The forward and reverse primers for *β-actin* were 5′-CYGAGCGYG GYTACWCCTT-3′ and 5′-TAGCAMAGYTTCTCCTTGATGTCA-3′, respectively. Real-time PCR was performed in a two-step process. In the first step, sample RNA was reverse transcribed with 50 ng random hexamers in a volume of 20 μl using 200 U of Superscript III Reverse Transcriptase (Invitrogen) and 40 U of RNaseOUT Recombinant RNase Inhibitor (Invitrogen). In the second step, real-time PCR was carried out in a MicroAmp Optical 96-well plate using power SYBR Green PCR Master Mix (Applied Biosystems Inc.), with 2 μl cDNA in each well. PCR reactions were monitored in real time using the ABI PRISM 7300 Real-time PCR system (Applied Biosystems Inc.). The thermal cycling conditions for real-time PCR were 50 °C for 2 min, then 95 °C for 10 min, and 40 cycles of denature (95 °C, 15 s) and annealing/extension (60 °C, 1 min). The real time PCR was conducted in triplicate with different shrimp samples. The data obtained from real-time PCR analysis were analyzed using the comparative C_t method according to the user bulletin of ABI PRISM 7300 system and then subjected to paired sample *t*-test. Differences were considered significant at $p < 0.05$.

Recombinant *HSP21* over-expression in *Escherichia coli* and antiserum production

The DNA fragment encoding the full-length *HSP21* was amplified from shrimp cDNA by PCR with the S-F/S-R primer set (5′-TACGGATCCAATGGAGGGCTTCAAGCAC-3′/ 5′-ATCCTCGAGTCAGTTCT GTGCGATGGG-3′; the underlined bases indicate, respectively, the *Bam*HI and *Xol*I restriction sites that were used for subsequent cloning) and ligated to pGEM®-T Easy (Promega) plasmid. After confirming the sequence, the resultant plasmid, pGEM-T-HSP21 was cleaved with *Bam*HI and *Xol*I, and the amplified fragment was then cloned to pER28b+ (Novagen) at *Bam*HI and *Xol*I sites. The resultant recombinant plasmid, pET28-HSP21 was transformed into *E. coli* BL21 (DE3) strain. *E. coli* BL21 (DE3) cells were cultured in LB medium with 25 mg/ml kanamycin and the protein was induced with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG). The recombinant HSP21 proteins tagged with 6 consecutive histidines (6·His-tagged HSP21) were purified by using QIAexpressionist™ nickel-nitrilotriacetic acid (Ni-NTA) metal-affinity chromatography (Qiagen) according to the manufacturer's recommendations. The resins were washed with buffer (pH 8.0) containing 25 mM Tris and 8 M urea and eluted with the buffer containing 500 mM imidazole. The eluted protein was then concentrated using Amicon Ultra-15 centrifugal filters (Millipore) in PBS buffer and stored at −20 °C for further antiserum production and thermal aggregation assay.

New Zealand white rabbits were hyperimmunized by injection with 250 μg proteins emulsified in complete Freund's adjuvant. Subsequent booster injections were carried out with 250 μg protein emulsified in incomplete Freund's adjuvant. The antiserum was collected after the antibody titer had peaked.

Western blot analysis

For Western blotting analysis, proteins that had been separated in SDS-PAGE were transferred onto a polyvinylidene difluoride (PVDF) membrane (Micron Separations) by semi-dry blotting. Membranes were blocked in 5% skim milk (Difco Laboratories) in TBS (0.2 M NaCl and 50 mM Tris—HCl, pH 7.4). Immunodetection was performed by incubation of the blot in a polyclonal rabbit anti-HSP21 serum diluted 1:5000 in TBS with 5% skim milk for 1 h at room temperature. Subsequently, anti-rabbit IgG antibody conjugated with horseradish peroxidase (Sigma) was used at a concentration of 1:5000 and detection was performed with a Western Blot Chemiluminescence Reagent (NEN Life Sciences).

Thermal aggregation assay

Thermal aggregation of citrate synthase (from porcine heart, Sigma) was monitored in HEPES buffer (50 mM HEPES, pH 8.0, 25 mM NaCl, 0.5 mM DTT) at 45 °C. The buffer containing recombinant HSP21 at different concentrations was preincubated for 10 min at 45 °C before the addition of citrate synthase to a final concentration of 20 μg/ml.

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CACGAGGTCTGCCCTCCCATTCCCCATCCAGGCTCCTTCACGCGAGGAAGACACGAATCG          60

GCAGCTAAGTTTAACAGAATAAACAAACTTAATACATAAAAAATGGAGGGCTTCAAGCAC         120
                                          M  E  G  F  K  H            6
ATTCCCGTTAAACTGGGAGACTTCAGCGTGATCGACCAGGAATTCAATTCAATTCGGGAG         180
 I  P  V  K  L  G  D  F  S  V  I  D  Q  E  F  N  S  I  R  E          26
AAATTCGACAGTGAAATGAAGAAAATGGAAGATGAGATGGCTCGCTTCCGCAATGAATTG         240
 K  F  D  S  E  M  K  K  M  E  D  E  M  A  R  F  R  N  E  L          46
ATGAATCGGGAATCGTCTCTCTTCCAGCGCTCAATGCTCACTTCCTCCAGCCAGCAGCAG         300
 M  N  R  E  S  S  L  F  Q  R  S  M  L  T  S  S  S  Q  Q  Q          66
GAATCCAGCGCCAACCAGGGCACCGGCTCGTGGCTGGAGGGCATGAACTCGCCTCTCATC         360
 E  S  S  A  N  Q  G  T  G  S  W  L  E  G  M  N  S  P  L  I          86
CAGCAAGAGGGTGACTGCAAACAACTCAAGCTAAGATTTGACGTCAGCCAGTACAAGCCC         420
 Q  Q  E  G  D  C  K  Q  L  K  L  R  F  D  V  S  Q  Y  K  P         106
GAGGAGATTGTTGTCAAGACAGTTGATAATAAGCTTCTGGTCCACGCCAAGCACGAGGAG         480
 E  E  I  V  V  K  T  V  D  N  K  L  L  V  H  A  K  H  E  E         126
AAGTCTGACAACCGCTCCGTCTACCGCGAGTACAACAGAGAGTTCCTGCTTCCCAAGGGC         540
 K  S  D  N  R  S  V  Y  R  E  Y  N  R  E  F  L  L  P  K  G         146
ACCAACCCCGAGCTCATCAAGTCCTCGCTCTCCAAAGACGGCGTCCTGACGGTGGAAGCT         600
 T  N  P  E  L  I  K  S  S  L  S  K  D  G  V  L  T  V  E  A         166
CCCCTGCCGGCCATAGCTGGGAACGAAGAGAAGGTCATCCCCATCGCACAGAACTGATTG         660
 P  L  P  A  I  A  G  N  E  E  K  V  I  P  I  A  Q  N  *             184
ACTGATTTCATCGCTTCTGTCGTTACGAATTTTCGCCACAGTGTTGAAGGAAGGAAGCCA         720

CGTGTAAGAGGATTGGAGGAATCATCTCGCCAGCATCAGTGTTTTTTTTATATATATATA         780

TCAAACCAAATTATGTCCATGATCATATGTTAAATGTACAACTGCCAGTAATTTAGACAA         840

TAAGGTAATATGTTTGTTTTGATAGGTAATGCACCATTTCCAAGGAT                      887
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Figure 1 The nucleotide sequence and deduced amino acid sequence of the *Penaeus monodon* small heat shock protein gene.

Reactions were continuously monitored at 320 nm in a Ultrospec™ 3100 pro (Amersham Pharmacia, UV/Visible spectrophotometer). The absorbance of citrate synthase alone at 60 min of heating was defined as 100% aggregation.

Immune challenge

In order to reveal whether the transcription of *HSP21* was affected by WSSV infection, shrimps were injected with WSSV and tissues were collected after infection using procedures described in Chen et al. [17]. The control shrimps were injected with 0.9% NaCl solution. A pleopod of three shrimps from each group was withdrawn at 0, 2, 4, 6, 8, 12, 18, 24, 36 and 72 h post-injection and separately preserved in liquid nitrogen immediately for RNA extraction. The expression level of *HSP21* mRNA in the WSSV challenged group and control group were also analyzed by real-time PCR.

Results

Sequence analysis of shrimp small heat shock protein (smHSP)

According to the EST database, an unpublished sequence with an opening reading frame (ORF) was identified (Fig. 1). This ORF encodes a protein of 184 amino acids with a theoretical size of about 21 kDa (and a predicted isoelectric point of 5.38). The nucleotide sequence surrounding the

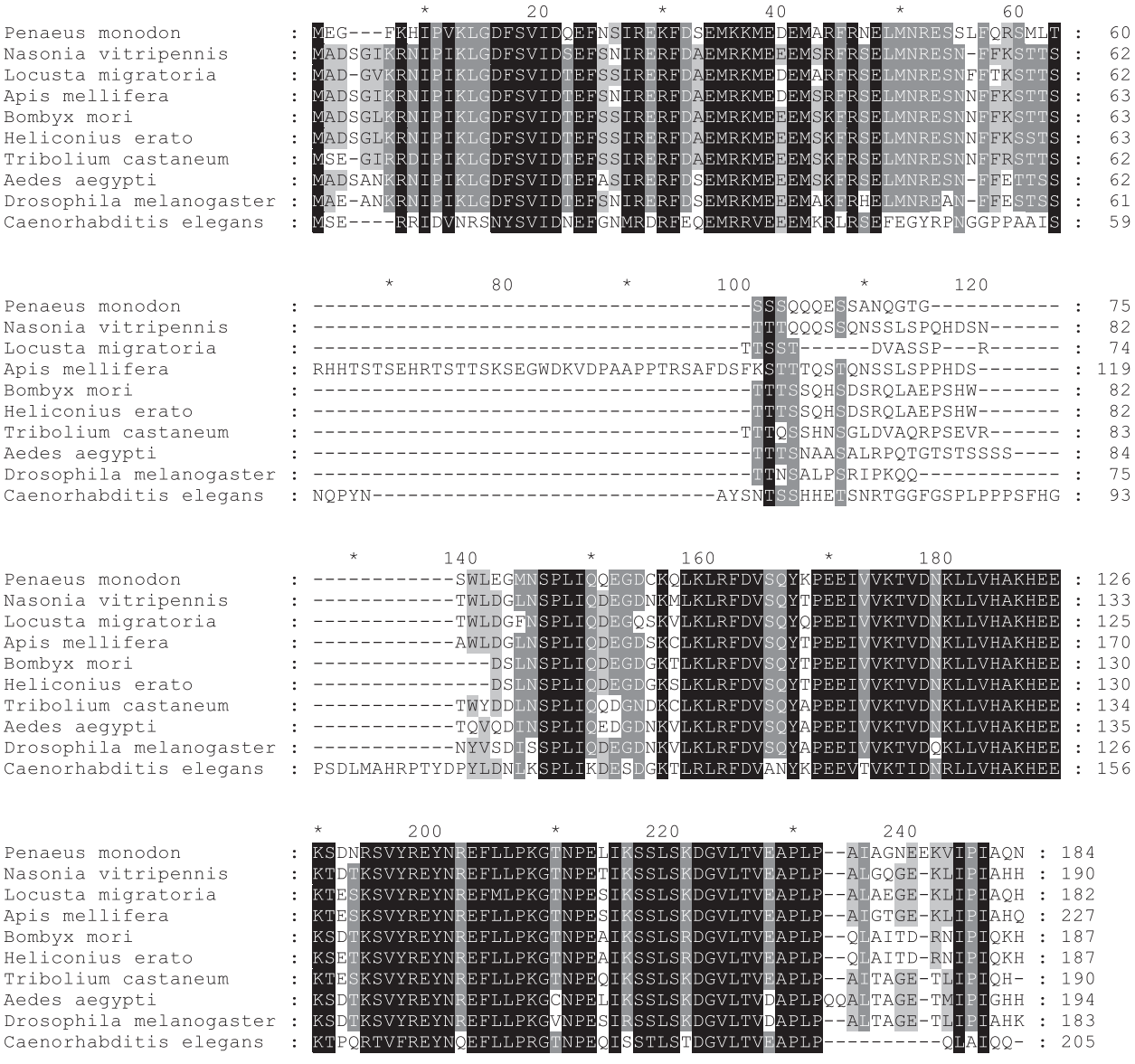

Figure 2 Comparisons between members of the smHSP family from invertebrate organisms including shrimp are shown in multiple sequence alignment and phylogenetic analysis.

methionine start codon (AAA*ATG*G) of the predicted protein conformed to the Kozak rule of an efficient context for eukaryotic translation initiation. A polyadenylation signal (AATAA) was located 181 bp downstream of the translational stop codon.

The deduced amino acid sequence of the ORF was homologous with known small heat shock protein (smHSP) (Fig. 2). Nine smHSPs from diverse genera were selected for analysis. The sequence of shrimp smHSP identified to a high degree with those of similar genes in eight insect species and one roundworm.

A phylogenetic tree was constructed by analyzing the amino acid sequences of *P. monodon* smHSP and the nine similar smHSPs (Fig. 3). The result indicated that *P monodon* smHSP shared higher identity with insects than with roundworm.

Tissue expression pattern of the *HSP21*

The expression of *HSP21* mRNA in different tissues was assessed by real-time PCR. The result showed that *HSP21* was highly expressed in almost all organs such as epithelium, gill, stomach, midgut, lymphoid organ, hepatopancreas, nervous tissue and heart, but poorly in haemolymph (Fig. 4).

To determine whether *P. monodon HSP21* was inducible by heat shock, three shrimps were also subjected to heat shock treatment. Pleopods were used in this analysis. As shown in Fig. 5, heat shock treatment increased the amount of *HSP21* mRNA transcript in *P. monodon*. The transcripts were found before treatment as well as 0, 1, 2, 4, and 6 h after recovery. *HSP21* expression reached its greatest peaks during the first hour after heat shock treatment and recovery. It gradually decreased to the pretreatment level after 6 h. The significant difference ($p < 0.05$) in *HSP21* expression was found between the heat shock treated and the control group at 0, 1, 2 and 4 h after recovery. It indicated that *HSP21* was expressed constitutively and inducible immediately by heat shock.

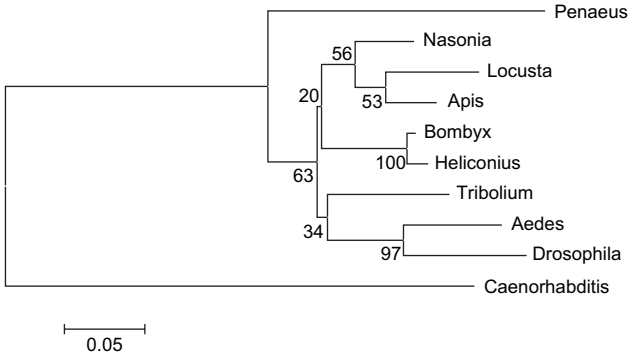

Figure 3 Phylogenetic tree was constructed by the Neighbor-Joining method and shows relatedness of nine smHSPs from invertebrate organisms. Robustness was tested by 1000 bootstrap replications and the indicated distance given the value of 0.1 means the 10% differences in amino acid residues between compared sequences. Bootstrap values are given in percentages.

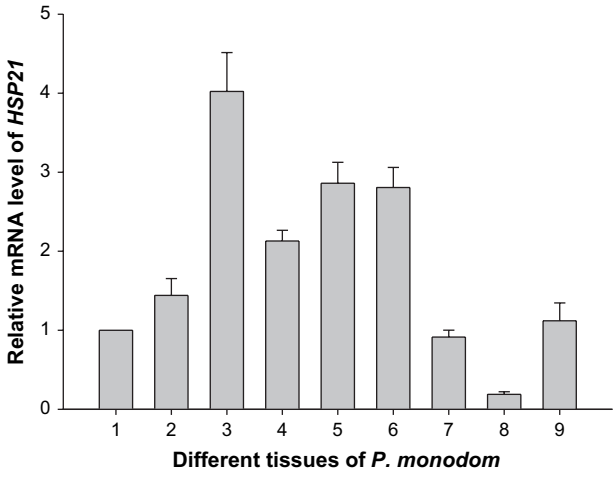

Figure 4 Detection of HSP21 transcripts by real-time PCR. Lanes; (1), epithelium; (2), gills; (3), stomach; (4), midgut; (5), lymphoid organ; (6), hepatopancreas; (7), nervous tissue; (8), haemolymph and (9), heart.

Purification of recombinant *HSP21* and detection *HSP21* in *P. monodon*

The *HSP21* gene was expressed in *E. coli* as a 6·His-tagged fusion protein. After induction with IPTG at 37 °C, the SDS-PAGE result showed that a band (about 23 kDa) corresponding to the HSP21-6·His fusion protein was observed in the induced bacterium (Fig. 6A, lane 2). No protein was found at the same position in the non-induced control (Fig. 6A, lane 1). The data indicated that the *HSP21* gene was expressed and a purified HSP21-6·His fusion protein was obtained (Fig. 6A, lane 3). Western blotting was

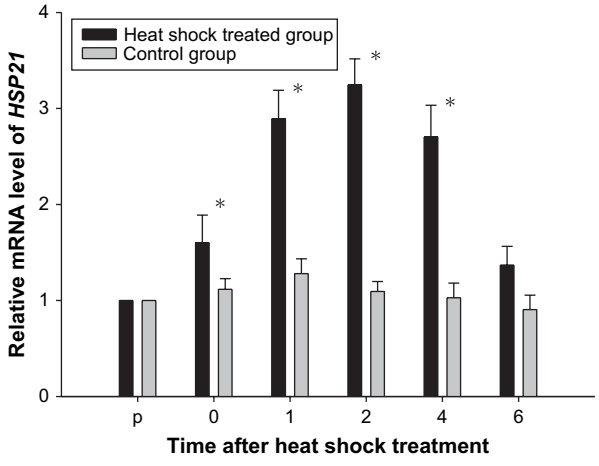

Figure 5 Detection of the *HSP21* transcripts before and after heat shock treatment by real-time PCR. P, pretreated; 0, 1, 2, 4, and 6 show the hours after heat shock treatment. The significant difference ($p < 0.05$) of *HSP21* expression between the heat shock treated and the control group are indicated with asterisks.

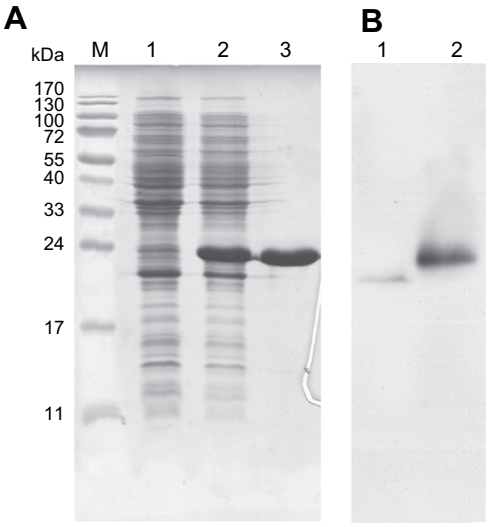

Figure 6 Recombinant expression in *E. coli* purification and western blot of shrimp HSP21. (A), SDS-PAGE of expressed and purified of protein. Lanes M, pre-stained molecular weight marker; (1), non-induced; (2), induced; (3), purified HSP21-6·His fusion protein. (B), Western blotting of HSP21. Lanes 1, epithelium tissue lysate; 2, purified HSP21-6·His fusion protein.

performed to detect the HSP21 in *P. monodon*. Epithelium tissue was selected to be tested owing to the result that HSP21 was highly expressed in the tissue and it was easily extracted in buffer. As shown in Fig. 6B, an obvious band about 21 kDa was detected (lane 1). As compared with purified HSP21-6·His fusion protein (lane 2), the molecular weight of HSP21 detected in tissue is less because of the lack of the His tag (and some amino acids co-expressed by expression vector pET28b+). It indicated that the real molecular weight of HSP21 in shrimp tissue was 21 kDa.

Chaperone function of *HSP21*

HSP21 was active in a standard chaperone assay, which measures the heat-induced aggregation of citrate synthase [18]. At an HSP21/citrate synthase ratio of 1:1 (w/w), HSP offers protection to the extent of only 20%. The extent of protection increases as the concentration of HSP21 increased, and at a ratio 8:1 (chaperone/target) approximately 85% protection is observed (Fig. 7). Thus, recombinant HSP21 is an active chaperone in this assay, comparable in specific activity to other HSP21s that have been studied.

Transcription analysis of *HSP21* in WSSV-infected shrimps

Real-time PCR analysis was used to detect *HSP21*-specific transcripts from shrimp specimens before injection (0 h) and at 2, 4, 6, 8, 12, 18, 24, 36 and 72 h after intramuscular injection of WSSV. The result showed that the *HSP21* mRNA level was significantly decreased after WSSV infection (Fig. 8). The expression level of *HSP21* mRNA was reduced

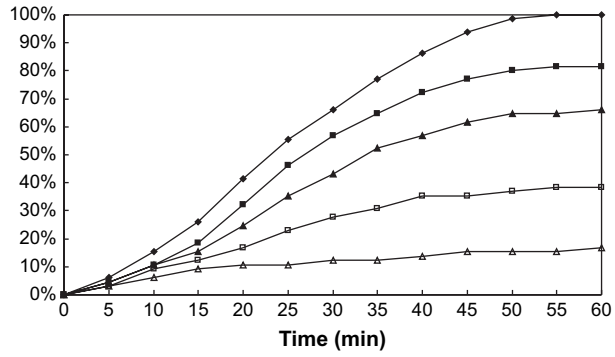

Figure 7 Chaperone activity of HSP21. Thermally induced aggregation of citrate synthase (20 μg/ml) alone (◆); and in the presence of HSP21 at 1:1 (■); 2:1 (▲); 4:1 (□); and 8:1 (△) ratio of HSP21/citrate synthase (w/w). The light scattering of the solution was monitored at 320 nm as described under ''Materials and Methods''.

to about 50% after 2 h post-infection. At 6 h post-infection, the *HSP21* mRNA level even reduced to 10%. The significant difference ($p < 0.05$) of *HSP21* expression between the WSSV challenged and the control group appeared at all sampling times after WSSV injection. It indicated that the expression mechanism of *HSP21* was highly affected by WSSV.

Discussion

Diseases caused by viruses are the greatest challenge to world wide shrimp aquaculture. However, up to this date, there is no effective method to control viruses, especially white spot syndrome virus (WSSV). A better understanding of shrimp immune responses will be very helpful for disease control. For this purpose, a gene named small heat shock protein (smHSP) was firstly identified by annotating the EST

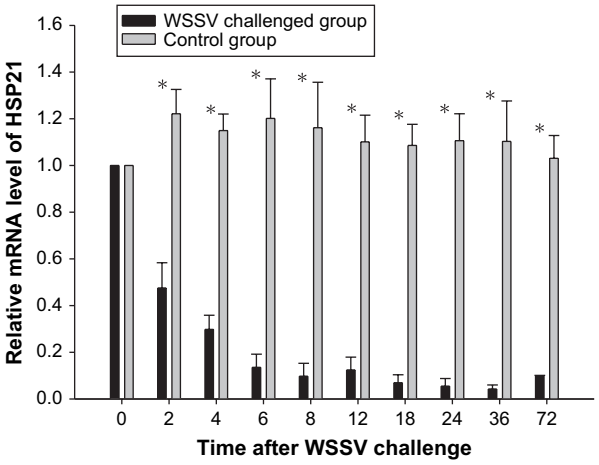

Figure 8 Analysis of *HSP21* expression in the WSSV challenged group and control group by real-time PCR at 0, 2, 4, 6, 8, 12, 18, 24, 36 and 72 h post-injection. The significant difference ($p < 0.05$) of HSP21 expression between the WSSV challenged and the control group are indicated with asterisks.

databases and was compared with the GenBank database. The subunit molecular weights of smHSPs range from approximately 12 to 43 kDa, although most fall within from 17 to 30 kDa, and generally exist as large multimeric assemblies insolution. In this study, we identified a smHSP of shrimp *P. monodon* with a molecular weight of 21 kDa. A thermal aggregation assay indicated that smHSP and HSP21 had chaperone activity.

The real-time PCR result showed that *HSP21* was expressed less in haemolymph. In this tissue, *HSP21* was also expressed less after heat shock (data not shown). This might be because the main function of the haemolymph tissue was not to express the proteins with chaperone activity.

Research on HSPs indicated that the heat shock response is a basic and highly conserved process that is essential for surviving environmental stresses. That is, HSPs are induced in expression against environmental stress [10,11,19]. Therefore, the speculation that the expression of *smHSP* should be induced after heat shock and after WSSV infection was reasonable. Actually, *HSP21* was inducible after heat shock. However, contrary to expectation, the expression of HSP21 was repressed after WSSV infection. We speculate that this might be in relation with the host's apoptosis. Referring to HSP27, it was discovered to have the function in blocking apoptosis [20,21]. That is, reducing the expression of small HSP could finally lead to a cells' apoptosis. Previous studies have indeed found signs of increasing numbers of cells showing DNA fragmentation, an obvious hallmark of apoptosis, by TUNEL assay in *P. monodon* infected with WSSV [22]. Thus, we speculate that the apoptosis induced by WSSV infection might be in connection with the down-regulation of *HSP21*. But, the next question is, what turned off HSP21? WSSV or the host itself?

Besides apoptosis, the studies of smHSP, Mj HSP16.5, of *Methanococcus jannaschii* provided a reasonable construction. Kim et al. observed the crystal structure of Mj HSP16.5 and then proposed the chaperone mechanism of smHSP. Mj HSP16.5 contained a ''windows'' structure in which denatured proteins caused by stress can be attracted to [23]. This can prevent proteins aggregating and miss-folding. As the stress was removed, the proteins renature and might fall off from the ''windows'' because of the renaturation process or disassembly of the smHSP complex [24] thus providing a mechanism for the host to protect proteins from miss-folding. However, during a viral infection, the host's resources are turned to produce the components of viruses. As the result shown in Fig. 8, the quantity of *HSP21* mRNA was reduced to about 10% after 6 h post-infection. We speculate that WSSV occupied host resources so that the host could not express smHSP or WSSV might turn off *HSP21* to stop the chaperone activity in order to increase viral protein expression. However, further research is required to confirm these speculations.

Acknowledgments

This work was supported by the National Council Grant. We are indebted to Mr. Vince J. Genis and Miss Angela Wang for their helpful criticism of the manuscript.

References

[1] Adams A. Response of penaeid shrimp to exposure to *Vibrio* species. Fish Shellfish Immunol 1991;1:59–70.
[2] Itami T, Yan Y, Takahashi Y. Studies on vaccination against vibriosis in cultured kuruma prawns *Penaeus japonicus* – I. Effect of vaccine concentration and duration of vaccination efficacy. J Shimonoseki Univ Fish 1992;40:83–7.
[3] Itami T, Yan Y, Takahashi Y. Studies on vaccination against vibriosis in cultured kuruma prawns *Penaeus japonicus* – II. Effect of different vaccine preparations and oral vaccination efficacy. J Shimonoseki Univ Fish 1992;40:139–44.
[4] Kurtz J, Franz K. Evidence for memory in invertebrate immunity. Nature 2003;425:37–8.
[5] Wu JL, Nishioka T, Mori K, Nishizawa T, Muroga K. A time-course study on the resistance of *Penaeus japonicus* induced by artificial infection with white spot syndrome virus. Fish Shellfish Immunol 2002;13:391–403.
[6] Witteveldt J, Cifuentes CC, Vlak JM, van Hulten MCW. Protection of *Penaeus monodon* against white spot syndrome virus by oral vaccination. J Virol 2004;78:2057–61.
[7] Namikoshi A, Wu JL, Yamashita T, Nishizawa T, Nishioka T, Arimoto M, et al. Vaccination trials with *Penaeus japonicus* to induce resistance to white spot syndrome virus. Aquaculture 2004;229:25–35.
[8] Wang HC, Wang HC, Leu JH, Kou GH, Wang AH, Lo CF. Protein expression profiling of the shrimp cellular response to white spot syndrome virus infection. Dev Comp Immunol 2007;31:672–86.
[9] Rudd S. Expressed sequence tags: alternative or complement to whole genome sequences? Trends Plant Sci 2003;8:321–9.
[10] Feder ME, Hofmann GE. Heat-shock proteins, molecular chaperones, and the stress response: evolutionary and ecological physiology. Annu Rev Physiol 1999;61:243–82.
[11] Picard D. Heat-shock protein 90, a chaperone for folding and regulation. Cell Mol Life Sci 2002;59:1640–8.
[12] Hartl FU. Molecular chaperones in cellular protein folding. Nature 1996;381:571–80.
[13] Nover L, Scharf KD. Heat stress proteins and transcription factors. Cell Mol Life Sci 1997;53:80–103.
[14] Craig EA, Ingolia TD, Manseau LJ. Expression of *Drosophila* heat-shock cognate genes during heat shock and development. Dev Biol 1983;99:418–26.
[15] Rybczynski R, Gilbert LI. cDNA cloning and expression of a hormone-regulated heat shock protein (hsc70) from the prothoracic gland of *Manduca sexta*. Insect Biochem Mol Biol 2000;30:579–89.
[16] Wang CH, Lo CF, Leu JH, Chou CM, Yeh PY, Chou HY, et al. Purification and genomic analysis of baculovirus associated with white spot syndrome (WSBV) of *Penaeus monodon*. Dis Aquat Org 1995;23:239–42.
[17] Chen LL, Lu LC, Wu WJ, Lo CF, Huang WP. White spot syndrome virus envelope protein VP53A interacts with *Penaeus monodon* chitin-binding protein (PmCBP). Dis Aquat Org 2007;74:171–8.
[18] Leroux MR, Melki R, Gordon B, Batelier G, Candido EP. Structure-function studies on small heat shock protein oligomeric assembly and interaction with unfolded polypeptides. J Biol Chem 1997;272:24646–56.
[19] Hasday JD, Singh IS. Fever and the heat shock response: distinct, partially overlapping processes. Cell Stress Chaperones 2000;5:471–80.
[20] Mehlen P, Schulze-Osthoff K, Arrigo AP. Small stress proteins as novel regulators of apoptosis. Heat shock protein 27 blocks Fas/APO-1- and staurosporine-induced cell death. J Biol Chem 1996;271:16510–4.
[21] Mehlen P, Kretz-Remy C, Preville X, Arrigo AP. Human hsp27, *Drosophila* hsp27 and human αB-crystallin expression-mediated increase in glutathione is essential for the protective

activity of these proteins against TNFα-induced cell death. EMBO J 1996;15:2695—706.

[22] Sahtout A, Hassan M, Shariff M. DNA fragmentation, an indicator of apoptosis, in cultured black tiger shrimp *Penaeus monodon* infected with white spot syndrome virus (WSSV). Dis Aquat Org 2001;44:155—9.

[23] Kim KK, Kim R, Kim SH. Crystal structure of a small heat-shock protein. Nature 1998;394:595—9.

[24] Kim R, Lai L, Lee HH, Cheong GW, Kim KK, Wu Z, et al. On the mechanism of chaperone activity of the small heat-shock protein of *Methanococcus jannaschii*. Proc Natl Acad Sci U S A 2003;100:8151—5.