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Cloning, Sequencing and Expression of a Heat Shock Protein 70 Gene from *Tenebrio molitor*

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Abstract: Heat Shock/Stress Proteins (HSPs) are a group of stress proteins which are closely associated with organisms' adaptability to environment and the heat shock protein 70 is the most conserved and important member. Researchers cloned an *hsp70* gene from *Tenebrio molitor* larvae by PCR and RACE Method and determined the mRNA abundance in the beetle developmental stages by real-time qPCR. The cDNA cloned was 2,282 bp in full length containing a 115 bp 5'untranslated region rich in adenine, a 1,935 bp open reading frame and a 232 bp 3'untranslated regionrich in adenine and thymine. It also had seven repeats of the Heat Shock Element (HSE) nGAAn in its 5'UTR and a 22 bp Poly (A) tail in the 3'UTR. The deduced heat shock protein had a highly conserved N-terminal ATPase domain and a conserved C-terminal peptide-binding domain. The tertiary structure of ATPase domain was made of two large globular subdomains which were separated by a deep cleft and the peptide-binding domain was a sandwich of 2 four-stranded β-sheets with four loops protruding upwards and two α-helices. Real-time qPCR analysis revealed that the hsp70 mRNA expression in *T. molitor* was characterized by heat-inducible and developmental-regulation features. The results form a basis for further research on structure, function and expression regulation of HSPs from *T. molitor* as well as to decipher the relationship between HSPs and stress-resistance in the beetle.

Key words: HSPs, stress proteins, PCR, shock protein, HSE, China

INTRODUCTION

All living organisms respond to a variety of stresses such as extremes in temperature, exposure to heavy metals or xenobiotics, UV, oxidizing agents or even high levels of growth hormones by expressing a specific set of genes with resultant stress responsive proteins. The Heat Shock Proteins (HSPs) belong to this particular category and are mainly divided into HSP100, HSP90, HSP70, HSP60, HSP40 and small molecule HSPs based on their molecular size (Schlesinger, 1990). A number of studies have shown that the sequence and biological function of HSPs are highly conserved during evolution (Borchiellini *et al*., 1998; Chen *et al*., 2005; Huang *et al*., 2008). HSPs primarily function as molecular chaperones to regulate biological functions of many kinds of proteins and a variety of vital processes. In particular, they are involved in the folding and transporting of nascent peptides and assembly, aggregation and degradation of certain proteins thereby playing a critical role in the maintenance of normal cell signaling and regulation of cell life (Sorger, 1991).

Secondly, as important cell damage repair factors, HSPs repair denatured and inactivated proteins, protect the cytoskeleton (Brown *et al*., 1996) improve protective functions and antioxidant capacity of protective enzymes. They also avoid cell impairments by inhibiting denaturation and aggregation of intracellular proteins (Mestril and Dillmann, 1995). In addition, HSPs are involved in a variety of physiological and pathological processes such as apoptosis, heat tolerance, immunoregulation, oncogenesis and viral infection (Schlesinger, 1990). These prominent biological functions and their highly conserved structure triggered a great interest in the applied aspects of research on HSPs. For example, HSPs are used as molecular trackers to study apolexis of organisms, origin of organelles and evolution of species (Catherine *et al*., 2000). They are also used for the development of novel anti-tumor vaccines and anti-cancer molecular targets (Workman, 2004) and serve as biomarkers for environmental monitoring, genetic breeding and medicolegal investigations (Dunlap and Matsumura, 1997; Gordon and Clark, 2004). Since, their

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discovery in the salivary glands of *Drosophila melanogaster* in 1962 the structure, function, expression and application of various kinds of HSPs have become research hotspots in life sciences. *HSP* genes have been cloned and sequenced from several insect species representing different insect orders (Konstantopoulou and Scouras, 1998; Landais *et al*., 2001; Karouna-Renier *et al*., 2003; Sonoda *et al*., 2006; Jiang *et al*., 2012).

Tenebrio molitor Linnaeus (Coleoptera: Tenebrionidae, Tenebrio) is native to South America and is one of the grain pests in cold regions (Liu *et al*., 2006). It is very nutritious, easy to culture has low dispersal ability and no hidden ecological danger. Because of these features it is utilized as a resource insect and has wide applications in agriculture (Huang *et al*., 2005), animal husbandry, food, medical care (Zhang *et al*., 2009) research, etc. At present the mealworm beetle is bred in many countries and new industries that raise and utilize *T. molitor* have emerged in some regions. The resistance of *T. molitor* to unfavorable environments and their productive and economic characters are critical for the maintenance of a healthy and sustainable mealworm beetle industry. The HSP genes *hsp70* and *hsp90* have been reported to be up-regulated in response to cold and heat shock in several insects (Schlesinger, 1990; Goto and Kimura, 1998; Yocum, 2001; Sinclair *et al*., 2007). In order to study the mechanism of stress-resistance that could be applied towards the breeding of *T. molitor* an *hsp70* gene which is the most conserved in the *hsp* gene family was cloned and analyzed from mealworm beetle larvae and the mRNA levels in *T. molitor* developmental stages were determined by real-time qPCR.

MATERIALS AND METHODS

Insects: *Tenebrio molitor* was from the Forest Protection Laboratory of Sichuan Agriculture University in China. They were fed on bran and supplement feed such as seasonal vegetables including carrot, lettuce leaves and Chinese cabbage and small dried fish. All insects were cultured at 25°C, 60% RH and 12:12 h (L:D) photoperiod for approximately 2 years..

Genomic DNA isolation: Six, 60 days old larvae of *T. molitor* that were starved for 3 days were frozen in liquid nitrogen and ground to powder. Genomic DNA was isolated from the powders using 2×CTAB. DNA concentration and purity were determined by 0.8% agarose gel electrophoresis and UV-Vis spectrophotometer (DU®800 Spectrophometer, Beckman Coulter) and the DNA sample was stored at -20°C until further use.

Total RNA isolation: Six, 60 days old larvae of *T. molitor* were frozen in liquid nitrogen and ground to powder after exposure to heat stress at 42°C for 1 h. Total RNA was isolated from the powder according to the instructions in the RNAiso plus kit (Takara). RNA concentration and purity were determined by 1.0% agarose gel electrophoresis and UV spectrophotometer and the sample was stored at -70°C until further use.

Cloning and sequencing of HSP70 cDNA from *T. molitor*: A pair of primers (HSPF1/HSPR1) (Table 1) for the amplification of hsp70 from *T. molitor* were designed based on the conserved regions in *Tribolium castaneum* HSP70 mRNA (GenBank accession: XM_968428) and Anatolica polita borealis HSP70 mRNA (GenBank accession: EF569673). Several PCR reactions were performed using *T. molitor* genomic DNA as template and various primer combinations (HSPF1/HSPR1). The reaction conditions included 94°C for 4 min, 30 cycles of 94°C for 30 sec, 55°C for 30 sec, 72°C for 1 min and a final extension at 72°C for 10 min. Amplified products were detected on a 1.0% agarose gel and the target fragment was cloned into pMD20-T vector and transformed into *Escherichia coli* DH5α. Positive clones were selected by colony PCR and sequenced by the Shenzhen Huada Gene Research Institute and analyzed using BLAST program in the NCBI database.

5'RACE: For 5'RACE PCR, specific primers including GSP1 and GSP2 (Table 1) were designed from the beginning of the isolated Tmhsp70 core sequence and 5'RACE adaptor primer, outer and inner primers (Table 1) provided by the 5'-Full RACE kit (Takara). A series of reactions including dephosphorylation, decapping, ligation with 5'RACE adaptor and reverse transcription were performed according to the instruction manual. After reverse transcription, nested PCR was carried out under two different PCR conditions. Cycling condition for the

Table 1: Primers used for amplification of hsp70 from *Tenebrio molitor*

Primers	Sequence[a] (5'→3')
HSP F1	GAGATCATCGCCAACGACCAAG
HSPR1	TGCGTTCGATGATCTTCGTCAT
5'RACE adaptor	AAGCAGTGGTATCAACGCAGAGTACG CGGG
GSP1	GCCGTTGAAGTTGTTCTGGATGAGT
GSP2	TAGCGTTGGTTCTCAAGTCCT
5'RACE outer primer	CATGGCTACATGCTGACAGCCTA
5'RACE inner primer	CGCGGATCCACAGCCTACTGAT GATCAGTCGATG
3'RACE adaptor	GGAGATTAAGGGTAGGAGCTTTT TTTTTTTTTTTTT
3'RACE downstream primer	GGAGATTAAGGGTAGGAGC
3'RACE outer primer	GAGATCATCGCCAACGACCAAG
3'RACE inner primer	TCCACTCGCATACCCAA

[a]Primers were obtained from kits or designed from ncbi database sequences with primer 5.0

first or outer PCR consisted of pre-denaturation at 94°C for 3 min, 30 cycles at 94°C for 30 sec, 56°C for 30 sec, 72°C for 3 min and a final extension at 72°C for 10 min. The second or inner PCR conditions were: 94°C for 3 min, 32 cycles at 94°C for 30 sec, 55°C for 35 sec, 72°C for 90 sec and a final extension at 72°C for 10 min.

Amplified DNA fragments were detected on a 1.0% agarose gel and fragments of the predicted size were subcloned into pMD20-T vector according to the manufacturer's protocol and transformed into *Escherichia coli* DH5α. Positive clones were selected by colony PCR and sequenced by the Shenzhen Huada Gene Research Institute.

3'RACE: The isolated Tmhsp70 core sequence was used to design a set of 3'RACE primers including 3'RACE adaptor primer, downstream primer, 3'RACE outer and inner primers (Table 1). First strand cDNA was synthesized using 1 μg total RNA as template according to RT reagent kit instructions and the first or outer PCR was carried out in a 15.0 μL reaction volume that included 2.0 μL cDNA template, 0.5 μL 10 μmol L^{-1} 3'RACE outer primer, 7.5 μL PCR Master mix and 5.0 μL ddH_2O. Reaction parameters were as follows: 4 min at 94°C, 25 cycles of 30 sec at 94°C, 30 sec at 55°C and 150 sec at 72°C. The second PCR was the adaptor reaction performed in a 15.0 μL volume consisting of 1.0 μL outer PCR mixture, 0.6 μL 5 μmol L^{-1} adaptor primer, 7.5 μL PCR Master Mix and 5.9 μL ddH_2O with reaction conditions consisting of 4 min at 94°C, 5 cycles of 30 sec at 94°C, 30 sec at 40°C, 90 sec at 72°C. The last or inner PCR was performed in a 25.0 μL volume including 2.0 μL adaptor reaction mixture, 2.0 μL 5 μmol L^{-1} 3'RACE inner primer, 2.0 μL 5 μmol L^{-1} downstream primer, 12.5 μL PCR Master Mix and 6.5 μL ddH_2O with the following conditions: 4 min at 94°C, 38 cycles of 30 sec at 94°C, 30 sec at 52°C, 90 sec at 72°C and a final extension for 10 min at 72°C. Amplified products from the inner PCR were detected on a 1.0% agarose gel and fragments of the predicted size were cloned into pMD20-T vector and transformed into *Escherichia coli* DH5α. Positive clones were selected by colony PCR and sequenced by the Shenzhen Huada Gene Research Institute.

Sequence analysis of Tmhsp70: The core sequence, 5'terminal and 3'terminal sequences of Tmhsp70 cloned into the pMD20-T vector were spliced together to obtain the complete Tmhsp70 sequence. Nucleotide sequence homology was analyzed using BLASTN in the NCBI database and the ORF, coding region and deduced amino acid sequence were analyzed using the ORF Finder in the database.

Predicting the physico-chemical property and structure of the deduced HSP70 from *T. molitor*: The amino acid composition, isoelectric point and molecular weight of the deduced HSP70 (named as TmHSP70) encoded by Tmhsp70 were predicted using the expasy/protparam and expasy/compute pI/MW program on SwissProt. Signal peptide and transmembrane domains in TmHSP70 were predicted using cbs/signalP, cbs/TMHMM-2.0 and ch.emb/TMpred, respectively. The secondary structure, structural domain and tertiary structure were predicted using npsa/gor4, ebi/interproscan and expasy/swissmodel, respectively.

Real-time qPCR: Different days old larvae, pupae and adults exposed to 42°C for 1 h and corresponding controls at 25°C were comparatively analyzed by real-time qPCR. The larvae tested were respectively 30 and 90 days old from eggs eclosion. The pupae used in the test were respectively 1 and 5 days old after pupation from larvae. The adults tested were res pectively 1 and 30 days old after emergence from pupae. Insect stages were immediately frozen in liquid nitrogen after exposure to 42°C and 60% RH for 1 h in environmental growth chambers. A control treatment was prepared and handled similarly, using insect stages exposed to 25°C and 60% RH for 1 h. Total RNA was extracted from whole insect bodies using RNAiso plus kit (Takara) and was further cleaned using an RNeasy Minielute Cleanup kit (Qiagen). The quality and concentration were determined by an UV/visible spectrophotometer (DU®800, Beckman Coulter). The integrity of the RNA was confirmed by formaldehyde agarose gel electrophoresis. Total RNA from each stage was checked for genomic DNA contamination by PCR amplification of 1 μL RNA sample by using gene-specific primers (Table 2) for hsp70. The amplified products and the DNA ladder were analyzed on 2% agarose gel containing ethidium bromide.

First-strand cDNAs were synthesized for qPCR by using total RNA. Then, 2.5 μL of 50 μmol L^{-1} Oligo d (T) primer (Table 2), 2.5 μL of 100 μmol L^{-1} random hexamers, 2.5 μL PrimeScript® RT Enzyme Mix I (Takara) and 10 μL of 5×PrimeScript® Buffer (Takara) were added to 2.5 μg total RNA. The final volume of the first-strand cDNA reaction mixture was adjusted to 50 μL by adding nuclease-free

Table 2: Primers used for real-time qPCR

Primers	Sequence[a] (5'→3')
Oligo d (T)	5'-TTT TTT TTT TTT TTT TTT-3'
18S rRNA (forward)	5'-GAC TAG GGT TCG ATT CC-3'
18S rRNA (reverse)	5'-TTC CTT GGA TGT GGT AGC-3'
hsp70 (forward)	5'-CAA GAT GGA CAA GGG GAT GAT-3'
hsp70 (reverse)	5'-CCG TTG AAG TAG TTC TGG AGG-3'

[a]Primers were obtained from kits or designed from ncbi database sequences with primer 5.0

water based on the volume of the total RNA. The mixtures were heated at 37°C for 15 min and subsequently heated at 85°C for 5 sec to inactivate the reverse transcriptase. All RNA samples from different developmental stages were reverse transcribed simultaneously. The first-strand cDNA reaction was aliquoted and stored at 20°C for later use.

Real-time qPCR was carried out with SYBR® Premix Ex Taq™ II (Perfect Real Time) (Takara) in a final volume of 25 μL reaction mixtures by using *hsp70* gene-specific primers and house-keeping gene 18S ribosomal RNA (*rRNA*) primers (Table 2) in an optical module connected thermal cycler (iQ5 Multicolor Real-Time Detection System, Bio-Rad). First-strand cDNA obtained from the 1 day old pupae (25°C) were used as templates to establish standard curves for each PCR run. The 1 μL of each forward and reverse primer with a concentration of 10 μmol L^{-1} Taq™ II, 2 μL of the first-strand cDNA mixture and 8.5 μL of nuclease-free water for amplification of 18S rRNA. A serial five fold dilution was prepared from cDNA samples. These five different dilutions and three technical replications were used to establish the standard curve. Based on the standard curve, the optimal Cycle threshold (Ct) that was established for the 18S rRNA was selected for further analysis. The templates concentrations among developmental stages of treated and control *T. moltor* were tested against the standard *18S rRNA* house-keeping gene.

The efficiency of PCR amplification for gene-specific primers was analyzed by one cDNA sample with five serial dilutions. A melting curve analysis was carried out to verify primer-dimer formation of gene-specific primers. Once primer efficiency was determined, PCR amplification was carried out in a 25 μL reaction mixture containing 1 μL of each forward primer and reverse primer (concentration of 10 μmol L^{-1}), 12.5 μL SYBR® Premix Ex Taq™ II, 8.5 μL nuclease-free water and 2 μL cDNA template. An identical thermal-cycle program was used for targets of the Tmhsp70 and the 18S rRNA. PCR amplifications were performed in triplicate wells. The touch-down PCR program consisted of a denaturing step at 95°C for 30 sec, PCR amplification step at 95°C for 5 sec and 60°C for 30 sec, repeated for 40 temperature cycles and dissociation step at 95°C for 15 sec, 60°C for 1 min and 95°C for 15 sec to obtain the melting curve. The experiment was repeated three times each with three technical replications by using total RNA isolated from two independent groups of insects. The Ct values obtained by real-time qPCR for different life stages in the treated and control samples were quantified by a comparative Ct threshold method ($2^{-\Delta\Delta Ct}$) for relative quantification (Giulietti *et al*., 2001). The relative expression quantities from real-time qPCR analysis were subjected to two-way Analysis of Variance (ANOVA) of SPSS17.0 to determine significant differences in *Tmhsp70* gene expression levels among *T. molitor* developmental stages and two temperatures. Significant differences among developmental stages within a temperature treatment recognized by the two-way ANOVA were determined by Tukey's multiple range test ($p<0.05$) whenever appropriate.

RESULTS

Cloning and sequencing of hsp70 from *Tenebrio molitor*: A PCR fragment 1,097 bp in length was obtained from the genomic DNA of *T. molitor*. Sequence alignments using BLAST showed that the isolated cDNA shared 83% nucleotide sequence identity with Microdera dzhungarica punctipennis hsp70 (GenBank accession: JF421286) and 82% with Tenebrio castaneum hsp70 (GenBank accession: XM_969349) indicating that the obtained sequence was the core sequence of *hsp70* gene from *Tenebrio molitor*. The amplified sequence was therefore named Tmhsp70. The 5' and 3'ends of Tmhsp70 core sequence were then used to design specific primers for 5' and 3'RACE, respectively. A 5'sequence of 803 bp and a 3'sequence of 1,093 bp were acquired by 5' and 3'RACE, respectively and a full length cDNA sequence of 2,282 bp in length was assembled by sequence splicing. Nucleotide sequence identity between the full cDNA sequence of Tmhsp70 and *M. dzhungarica* punctipennis hsp70 was 81% and between Tmhsp70 and *T. castaneum* hsp70 was 82% similar to the comparison of core sequence. Tmhsp70 sequence was submitted to GenBank with an accession number JQ219848.

The sequence of Tmhsp70 contained a 115 bp 5'Untranslated Region (5'UTR) rich in adenine residues, a 232 bp 3'untranslated region (3'UTR) rich in adenine and thymine residues and a 1,935 bp open reading frame (116-2050 bp) which encoded 644 aa (Fig. 1). As shown in Fig. 1 the deduced HSP70 encoded by *Tmhsp70* contained three signature motifs of HSP70 family, i.e., IDLGTTYS (9-16 residues), IFDLGGGTFDVSIL (197-210 residues) and IVLVGGSTRIPKIQQ (335-349 residues) as well as a EEVD (641-644 residues) motif characteristic of cytoplasmic HSP70s. In addition, Tmhsp70 had seven repeats of the Heat Shock Element (HSE) motif nGAAn in its 5'UTR, a 22 bp Poly (A) tail at the 3'UTR and a Polyadenylation Signal (PAS) at 2,242-2,247 bp.

Amino acid sequence homology between HSP70s from *T. molitor* and other insects was very high as shown in Fig. 2. The deduced sequence of HSP70 (named as

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1 CGAGCCAACGAACAGTAAAACGAAAAGAGGTGAACCAAGCAAAGTTAAAATCAGTTTGAGTAAAGTGAATTCAAGT

77 GAATTTTTTCTCAAATAAAAGTTGAAAAAGAGAAGAGCA

116 ATG GTG AAG TCT CCA GCA ATC GGT ATC GAC CTG GGC ACG ACC TAC TCC TGC GTC GGG GTC TGG CAG CAC
1 M V K S P A I G I D L G T T Y S C V G V W Q H
185 GGC AAG GTC GAG ATC ATC GCC AAC GAC CAA GGT AAC AGA ACC ACC CCC AGC TAT GTC GCC TTC ACC GAC
24 G K V E I I A N D Q G N R T T P S Y V A F T D
254 ACG GAG CGC CTC CTC GGA GAC GCC GCC AAG AAC CAG GTC GCC ATG AAT CCC AGC AAC ACA GTC TTC GAC
47 T E R L L G D A A K N Q V A M N P S N T V F D
323 GCC AAA CGT CTA ATC GGC CGC AAG TAC GAC GAT CCC AAG ATC CAA CAA GAC TTG AAA CAT TGG CCT TTC
70 A K R L I G R K Y D D P K I Q Q D L K H W P F
392 AAA GTC ATC AGC GAC GGT GGA AAA CCG AAG ATT CAA GTC GAC TAC AAA GGC GAG ATC AAG AAG TTT GCA
93 K V I S D G G K P K I Q V D Y K G E I K K F A
461 CCC GAA GAA ATC AGC TCG ATG GTG TTG ACG AAG ATG AAA GAA ACC GCC GAA GCG TAC TTG GGA ACT TCG
116 P E E I S S M V L T K M K E T A E A Y L G T S
530 GTC AGA GAT GCG GTC ATC ACC GTT CCG GCA TAC TTC AAC GAC TCT CAA AGA CAA GCT ACG AAG GAC GCC
139 V R D A V I T V P A Y F N D S Q R Q A T K D A
599 GGC GTC ATC GCC GGT TTG AAC GTG ATG AGG ATC ATC AAC GAA CCG ACG GCG GCA GCT CTA GCC TAC GGT
162 G V I A G L N V M R I I N E P T A A A L A Y G
668 CTG GAC AAG AAT CTG AAG GGC GAG AGA AAC GTG TTG ATC TTC GAT CTA GGC GGA GGC ACT TTC GAC GTC
185 L D K N L K G E R N V L I F D L G G G T F D V
737 TCC ATT TTG ACC ATC GAT GAA GGG TCG CTC TTC GAA GTG AGA GCC ACG GCG GGC GAC ACG CAC TTG GGC
208 S I L T I D E G S L F E V R A T A G D T H L G
806 GGT GAA GAC TTC GAC AAC CGA CTG GTC GAC CAC TTG GCG GAC GAG TTT AAA CGC AAA TAC AAG AAG GAC
231 G E D F D N R L V D H L A D E F K R K Y K K D
875 TTG AGA ACC AAC GCT AGA GCG CTC CGT CGC TTG AGG ACC GCG GCC GAA AGG GCC AAG CGC ACG TTG TCT
254 L R T N A R A L R R L R T A A E R A K R T L S
944 TCC AGC ACC GAA GCT TCC TTC GAG ATC GAC GCC CTC TTC GAC GGT ATC GAT TTC TAT ACT AAA ATC AGC
277 S S T E A S F E I D A L F D G I D F Y T K I S
1013 AGA GCG AGG TTC GAA GAA CTC AAC GCC GAC CTC TTC AGA AGC ACC CTG CAA CCC GTC GAG AAA GCA TTG
300 R A R F E E L N A D L F R S T L Q P V E K A L
1082 ACG GAC GCC AAG ATG GAC AAG GGG ATG ATC CAC GAC ATC GTC TTG GTC GGC GGC TCC ACT CGC ATA CCC
323 T D A K M D K G M I H D I V L V G G S T R I P
1151 AAG ATT CAG CAA CTC CTC CAG AAC TAC TTC AAC GGC AAA TCG CTC AAT CTC TCC ATC AAY CCG GAC GAA
346 K I Q Q L L Q N Y F N G K S L N L S I N P D E
1220 GCC GTC GCC TAC GGT GCC GCC GTC CAA GCG GCC GTC TTG AAC GGA GAG TCC GAC TCG AAG ATC CAA GAC
369 A V A Y G A A V Q A A V L N G E S D S K I Q D
1289 GTC CTC CTG GTC GAC GTC GCT CCT CTG TCT CTG GGC ATC GAG ACG GCT GGA GGT GTT ATG ACG AAG ATC
392 V L L V D V A P L S L G I E T A G G V M T K I
1358 ATC GAG CGC AAC GCG CGA ATC CCG TGC AAA CAA ACG CAA ATC TTC ACC ACT TAC TCT GAC AAC CAA CCC
415 I E R N A R I P C K Q T Q I F T T Y S D N Q P
1427 GCC GTC ACC ATC CGA GTC TTT GAA GGC GAA AGG GCA ATG ACC AAA GAC AAC AAT TTA CTG GGA ACT TTC
438 A V T I R V F E G E R A M T K D N N L L G T F
1496 GAT CTG ACC GGA ATC CCA CCG GCG CCT CGC GGA ATC CCG AAG ATC GAG GTT ACT TTC GAC ATG GAC GCG
461 D L T G I P P A P R G I P K I E V T F D M D A
1565 AAC GGT ATA CTC AAC GTT TCC GCA AAA GAC ACG AGT TCC GGT AAT TCG AAG AAC ATC ACC ATC AAG AAC
484 N G I L N V S A K D T S S G N S K N I T I K N
1634 GAT AAA GGG AGA TTA TCT CAG AAA GAT ATC GAC AGG ATG GTR TCC GAG GCG GAG CAG TAT AAA GAA GAG
507 D K G R L S Q K D I D R M V S E A E Q Y K E E
1703 GAT GAG AAG CAG AGG CAA AAA ATT GCT GCG AGG AAT CAG CTG GAG GCT TAC GTC TTC CAG TTG AAA CAG
530 D E K Q R Q K I A A R N Q L E A Y V F Q L K Q
1772 ACC GTT TCG GAG CAA GGA AGC AAA CTG TCG CCT TCC GAT AAA GAA ACC CTG ACG AGC GAA TGT GAC GGT
553 T V S E Q G S K L S P S D K E T L T S E C D G
1841 TGC TTG CAG TGG TTG GAT GCC AAT ACT CTG GCG GAG AAA GAA GAA TAC GAA GAT AAA CAG AAG CAG CTG
576 C L Q W L D A N T L A E K E E Y E D K Q K Q L
1910 ACT TCG ATT TGT GGT CCT ATA GTG GCT AAA TTG TTT CAA ACA GGA GGA CAA AGT GCG GGA ATG CCC GGA
599 T S I C G P I V A K L F Q T G G Q S A G M P G
1979 AGT TGC GGA CAA CAG GCT GGC GGT TTC GGA GGA TCG AAT AAC GCC GGA CCG ACG ATC GAA GAA GTC GAT
622 S C G Q Q A G G F G G S N N A G P T I E E V D
2048 TAA
     *
2051 GTAAGAATATTGGACAGAAATGTGATTAAAATTGATGTGTTTAGTCTTAGATCTGTGGTCAGTGCCCGGTAGCCGTC
2128 GAGAGTTGTAAATAAAATATACTGCATATACCTAGTATGTATTTATGTAATGTGATATAAATGCATTTCTGTATAGA
2205 GACTGATTGTTTTGTATAGGTACTTAATTGTAAAGAAAATAAATAACTTTTTGAATAAAAAAAAAAAAAAAAAAAAA
2282 A
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Fig. 1: An *hsp70* gene from *Tenebrio molitor* (Tmhsp70) and the deduced amino acid sequence. Numbers on the left represent numbers of bases or amino acid residues, heat shock elements are underlined, signature motifs of HSP70 family are shadowed, cytoplasmic HSP70 signature motif is boxed, termination codon is indicated by asterisk, polyadenylation signal is indicated by double underline

TmHSP70) encoded by Tmhsp70 shared the highest homology with HSP70s from three other beetles including *T. castaneum*, *M. dzhungarica punctipennis* and Anatolica polita borealis.

Physico-chemical properties of the deduced HSP70 from *T. molitor*: The main physico-chemical properties of the deduced TmHSP70 from *T. molitor* were analyzed using expasy/protparam and expasy/compute pI/MW programs at SwissProt and the results are shown in Table 3. Relative molecular mass of TmHSP70 was 70.5 kDa with 644 total amino acids among which ninety were negatively charged and eighty were positively charged. Among the total amino acids, contents of Ala, Gly and Leu were relatively higher and made 8.9, 8.1 and 7.9% of the protein, respectively meanwhile contents of Cys, His and Trp were relatively lower and only comprised 0.9, 0.8 and 0.5%, respectively. The isoelectric point of TmHSP70 was about 5.4 and grand average of hydropathicity was -0.423 indicating the hydrophilic and weak acidic nature of the protein. In addition, the predicted TmHSP70 had no signal peptide or transmembrane domains.

Secondary structure and structural domain of the deduced HSP70 from *T. molitor*: Secondary structure of the deduced TmHSP70 consisted of multiple alpha helices (39.91%), beta sheets (16.93%) and random coils (43.17%) (Fig. 3).

The deduced TmHSP70 had a highly conserved N-terminal ATPase domain of ca. 42 kDa (3-383 residues) and a conserved C-terminal domain of ca. 26 kDa (389-619

Table 3: Physico-chemical properties of the deduced HSP70 from *Tenebrio molitor*

Protein	Relative molecular weight (kDa)	Molecular formula	Amino acid composition: No. of negatively charged amino acids	No. of positively charged amino acids	Total No. of amino acid	Content of representative amino acids (%): Amino acids of higher content: Ala	Gly	Leu	Amino acids of lower content: Cys	His	Trp	pI	Grand average of hydropathicity
TmHSP70	70.5	$C_{3085}H_{4962}N_{862}O_{986}S_{18}$	80	90	644	8.9	8.1	7.9	0.9	0.8	0.5	5.4	-0.423

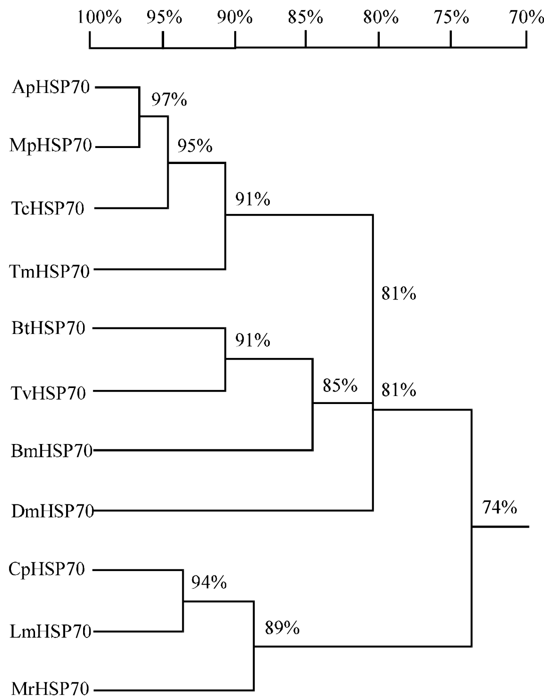

Fig. 2: Homology tree based on the amino acid sequences of HSP70 from *Tenebrio molitor* and other insects. ApHSP70: *Anatolica polita borealis* HSP70 (ABQ39970); MpHSP70: *Microdera dzhungarica punctipennis* HSP70 (AEB52075); TcHSP70: *Tribolium castaneum* HSP70 (XP_974442); TmHSP70: *Tenebrio molitor* HSP70 (AFE88579); BtHSP70: *Bemisia tabaci* HSP70 (ACZ52196); TvHSP70: *Trialeurodes vaporariorum* HSP70 (ACH85201); BmHSP70: *Bombyx mori* HSP70 (BAF69068); DmHSP70: *Drosophila melanogaster* HSP70 (NP_731716); CpHSP70: *Cryptocercus punctulatus* HSP70 (AFK49798); LmHSP70: *Locusta migratoria* HSP70 (AAO21473); MrHSP70: *Megachile rotundata* HSP70 (AAS57864)

residues) which was further subdivided into a conserved peptide-binding subdomain of ca. 18 kDa (389-544 residues) and a non-conserved indeterminate C-terminal subdomain of ca. 9 kDa (538-619 residues) (Fig. 4).

Tertiary structure of the deduced HSP70 from *T. molitor*: Tertiary structure prediction using expasy/swissmodel in SwissProt indicated that the ATPase domain (3-383 residues) of TmHSP70 consisted of two large globular subdomains (I and II; Fig. 5) each further divided into two small subdomains (A and B; Fig. 5). The subdomains are separated by a deep cleft at the bottom of which nucleotide binds in complex with one kalium ion contacting all four subdomains (IA, IB, IIA, IIB; Fig. 5).

The peptide binding moiety (residues 389-544) formed a sandwich of 2 four-stranded β-sheets with four loops protruding upwards (two inner and two outer loops) and two α-helices (A and B; Fig. 6) which were packed against the inner loops ($L_{1,2}$ and $L_{4,5}$; Fig. 6). The substrate binding cavity was formed by the β-sheets 1 and 2 and the loops $L_{1,2}$ and $L_{3,4}$. Helix B constituted a lid which closed the cavity to prevent the escape of peptide substrates that bind.

Exprssion levels determined using real-time qPCR: The parameter Ct (threshold cycle) is defined as the cycle number at which the fluorescence passes the fixed threshold. When Ct values were used to generate a log-linear regression plot, the standard curve for the house-keeping gene 18S rRNA showed a strong relationship (r^2 = 0.995; PCR efficiency = 100.9%). A correlation coefficient of >0.99 shows good primer efficiency and indicates a successful real-time PCR experiment. The efficiencies for PCR amplification for Tmhsp70 and 18S rRNA, tested in separate five serial dilutions of 1 day old pupae cDNA template were approximately equal with the regression slopes of -3.286 for Tmhp70 and -3.301 for 18S rRNA. The efficiency of PCR amplification for target gene *Tmhsp70* showed that the amplification efficiencies were 98.6% for Tmhsp70 primers. A melting curve analysis showed no indication of primer-dimerization for Tmhsp70.

The relative expression of Tmhsp70 mRNA in different developmental stages exposed to two temperatures (25 and 42°C) is shown in Fig. 7. The ANOVA results of the comparative Ct values ($2^{-\Delta\Delta Ct}$) for real-time qPCR revealed that the Tmhsp70 expression was significantly different between the two temperatures

AA MVKSPAIGIDLGTTYSCVGVWQHGKVEIIANDQGNRTTPSYVAFTDTERLLGDAAKNQVAMNPSNTVFDA
Pre-sec ccccceeeeeeceeeeeeeeeeccccceeeeeecccccccccccceeeeeccchhhhhhhhhhhhhcccccchhhh
AA KRLIGRKYDDPKIQQDLKHWPFKVISDGGKPKIQVDYKGEIKKFAPEEISSMVLTKMKETAEAYLGTSVR
Pre-sec hhhhccccccchhhhhccccceeeeecccceeeeccccccccchhhhhhhhhhhhhhhhhhhhhhccccc
AA DAVITVPAYFNDSQRQATKDAGVIAGLNVMRIINEPTAAALAYGLDKNLKGERNVLIFDLGGGTFDVSIL
Pre-sec ceeeeeccccchhhhhhhhhhcccccchhhhhhhcchhhhhhhhhhhhhccccccceeeeeecccccccceeeee
AA TIDEGSLFEVRATAGDTHLGGEDFDNRLVDHLADEFKRKYKKDLRTNARALRRLRTAAERAKRTLSSSTE
Pre-sec eccccchhhhhhcccccccccccchhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhcccccch
AA ASFEIDALFDGIDFYTKISRARFEELNADLFRSTLQPVEKALTDAKMDKGMIHDIVLVGGSTRIPKIQQL
Pre-sec hhhhhhhcccccchhhhhhhhhhhhhhhhhhhhhhcccchhhhhhhhhhhhhcccccceeeeeccccccchhhhhhh
AA LQNYFNGKSLNLSINPDEAVAYGAAVQAAVLNGESDSKIQDVLLVDVAPLSLGIETAGGVMTKIIERNAR
Pre-sec hhhcccccceeccccccchhhhhhhhhhhhhhcccccccceeeeeeeeccccccccceecccccchhhhhhhccc
AA IPCKQTQIFTTYSDNQPAVTIRVFEGERAMTKDNNLLGTFDLTGIPPAPRGIPKIEVTFDMDANGILNVS
Pre-sec cccccccceeeeecccccccceeeeeeccchhhhhcccccceeccccccccccccccceeeeeecccccceeeeee
AA AKDTSSGNSKNITIKNDKGRLSQKDIDRMVSEAEQYKEEDEKQRQKIAARNQLEAYVFQLKQTVSEQGSK
Pre-sec ccccccccccceeeeeccccccchhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhcccc
AA LSPSDKETLTSECDGCLQWLDANTLAEKEEYEDKQKQLTSIOGPIVAKLFQTGGQSAGMPGSOGQQAGGF
Pre-sec ccccceeeccccccchhhhhhcchhhhhhhhhhhhhhceeeccccceeeeeccccccccccccccccccccc
AA GGSNNAGPTIEEVD
Pre-sec cccccccceeeeec

Fig. 3: Secondary structure prediction of the deduced HSP70 from *Tenebrio molitor*. AA: the Amino Acid sequence; Pre-sec: Predicted secondary structure; h: Alpha helix; e: beta sheet and c: random coil

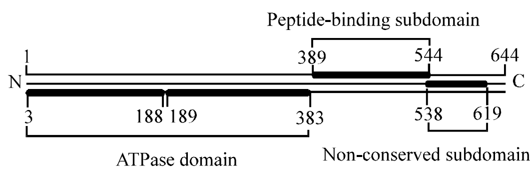

Fig. 4: Domains of deduced HSP70 from *Tenebrio molitor*

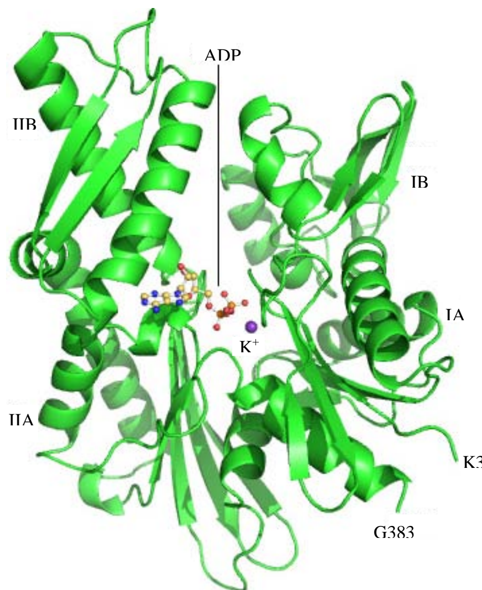

Fig. 5: Tertiary structure of ATPase domain of the deduced HSP70 from *Tenebrio molitor* secondary structure representation of the ATPase domain of TmHSP70 in complex with ADP and one kalium ion

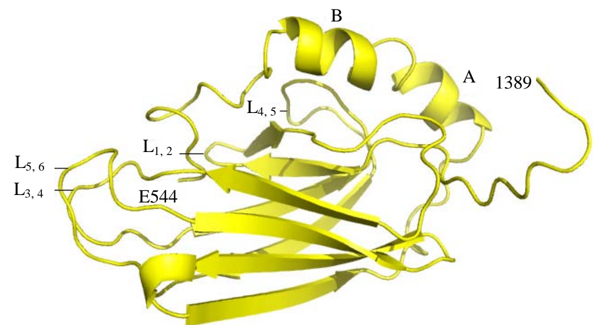

Fig. 6: Tertiary structure of peptide-binding domain of the deduced HSP70 from *Tenebrio molitor*. $L_{1,2}$, $L_{3,4}$, $L_{4,5}$ and $L_{5,6}$ are four loops, A and B are two α-helices

(F = 10,805.16; df = 1, 24; p = 0.000; n = 3) and among the developmental stages (F = 736.85; df = 5, 24; p = 0.000). The interaction between temperature and stage was significant, too (F = 356.59; df = 5, 24; p = 0.000). The expression of Tmhsp70 was up-regulated approximately by 1.3~34.9 fold in *T. molitor* developmental stages exposure to 42°C for 1 h, compared with the control insects (25°C) (Fig. 7). Thus, it seems clear that Tmhsp70 is induced by heat shock. In addition, expression of Tmhsp70 mRNA in *T. molitor* was found to be developmentally dependent because the higher abundance of mRNA was present in the newly-born pupae (P-1 day) within the control insects (25°C) and after heat shock treatment the mRNA came up to the highest level in the old larvae (L-90 days) and pupae (Fig. 7).

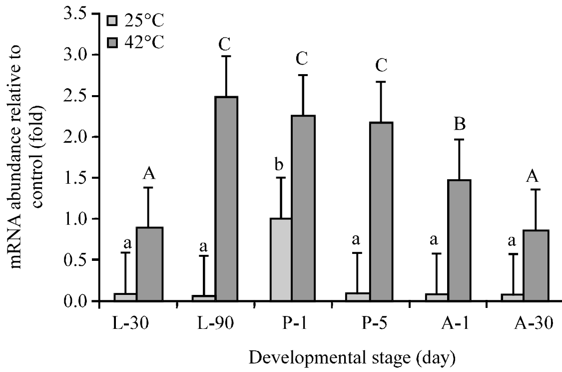

Fig. 7: Relative abundance of Tmhsp70 mRNA in *Tenebrio molitor* developmental stages. Total RNA extracted from the whole organism of different day old larvae (L-30 and L-90 day), pupae (P-1 and P-5 day) and adults (A-1 and A-30 day) were analyzed by real-time qPCR and quantified by a comparative Ct Threshold Method. The mRNA abundance relative to control is given by $2^{-\Delta\Delta Ct}$ where AACt = ACt (sample)-ACt (calibrator) and ACt is the Ct of the target gene (*Tmhsp70*) subtracted from the Ct of the housekeeping gene (*18S rRNA*). The mRNA in 1 day old pupae of *T. molitor* at 25°C was used as a calibrator. Mean±SEM presented, n = 3 in all cases. Means within a temperature treatment followed by different letters are significantly different ($p<0.05$; Tukey's test)

Furthermore, the amount of Tmhsp70 mRNA accumulated constitutively in larvae, 5 days old pupae and adults was fewer than half of that expressed in 1 day old pupae and after exposure to 42°C for 1 h, the amount of Tmhsp70 mRNA expressed in 90 days old larvae was higher than accumulated in 1 and 30 days old adults (Fig. 7).

DISCUSSION

Heat Shock Proteins (HSPs) are important stress proteins that are closely associated with the adaptation of an organism to environmental changes. Traditionally, genes encoding HSPs are divided into inducible and constitutive groups. Genes in the inducible group are induced under stressful conditions but return to a normal expression evel when stress is removed (Lang *et al*., 2000). Genes in the constitutive group are not stress-inducible but are expressed at all times and are generally referred to as Heat Shock Cognates (HSC) (Qin *et al*., 2003; Karouna-Renier *et al*., 2003). A variety of environmental stresses including cold (Goto and Kimura, 1998), heat (Schlesinger, 1990), electric radiation (Ennamany *et al*., 2008), ultraviolet irradiation (Trautinger, 2001), starvation (Yengkokpam *et al*., 2008), anoxia (Prentice *et al*., 2004), ischemia, organic pollutants, trace-metal exposure (Ait-Aissa *et al*., 2000), antibiotics, tissue wound, microbial infection (Jindal and Young, 1992) and tumor (Wei *et al*., 1995) have been reported to induce HSPs in various organisms.

Insects are the most widely distributed biological groups on earth and show very strong adaptability to various environmental conditions. Research on HSPs from insects has great value in understanding insects' stress-resistance. In insects different HSPs can be induced by different environmental factors and the rapidly synthesized HSPs elevate insects' tolerance to unfavorable environments (De Jong *et al*., 2006; Rinehart *et al*., 2006).

Researhers cloned an *hsp70* gene from *T. molitor* larvae (named as Tmhsp70) and determined the mRNA expression by real-time qPCR. The results revealed that the Tmhsp70 mRNA was expressed constitutively very low in *T. molitor* (25°C) and its expression was sharply and dramatically up-regulated when insects were exposed to an elevated temperature (42°C). The overall increase in the levels of Tmhsp70 mRNA in different life stages ranged from 1.3-34.9 fold on the basis of real-time qPCR analysis. It is clear that Tmhsp70 is a heat-inducible *hsp* gene. Furthermore, expression of Tmhsp70 mRNA has the characteristics of developmental regulation. At 25°C, mRNA of Tmhsp70 was more abundant in newly born Pupae (P-1 day) than accumulated in larvae, old Pupae (P-5 day) and adults. After a heat shock, the amount of Tmhsp70 mRNA expressed in 90 days old larvae was a little higher than accumulated in 1 and 30 days old adults. The up-regulated Tmhsp70 mRNA may be contributing to the increased thermotolerance of the old larvae.

Earlier researchers showed that expression of different *hsp* genes even the same *hsp* gene in different tissues or in different developmental stages of an organism is quite different (Lang *et al*., 2000; Yocum, 2001; Karouna-Renier *et al*., 2003; Mahroof *et al*., 2005; Rinehart *et al*., 2006; Jiang *et al*., 2012). The regulation of inducible *hsp* gene expression occurs primarily at the transcriptional level and the mRNA coding for the protein can be induced about 1-1000 fold (Lindquist, 1986). The DNA sequences responsible for regulating heat shock gene expression in eukaryotes are known as Heat Shock Elements (HSE) (Schlesinger, 1990). For maximum heat shock induction, a functional HSE should contain a minimum of three contiguous nGAAn units and two HSEs positioned close to the transcriptional start site (Papadimitriou *et al*., 1998).

In addition, regulation of mRNA stability and translation has also been studied. Under normal temperatures, hsp mRNA are unstable while their stability is increased under heat stress (Petersen and Lindquist, 1988) which is due to their distinctive 3'-Untranslated region (3'UTRs) rich in adenine and uracil residues (Lee, 1998). Furthermore, under heat stress while translation of mRNA from other genes are inhibited hsp mRNA are translated because of the presence of a long and unique 5'-Untranslated region (5'UTRs) rich in adenine residues. This feature also makes it more difficult for hsp mRNAs to form secondary structures unlike other mRNAs under the same heat stress (McGarry and Lindquist, 1985). It is clear that the unique 5' and 3'untranslated regions have critical effect on the regulation of stability and translation of hsp mRNAs. If regulation of the *hsp70* gene occurs at the translational level, the gene will be transcribed but translation will be repressed. Increased translation will occur only in the event of exposure to heat (Lindquist, 1986). Transcription of heat shock genes can be negatively regulated when HSPs have accumulated to a specific concentration that is proportional to the severity of heat treatment (Lindquist, 1986). In this instance, the inherently unstable, inducible hsp70 transcripts are degraded. In contrast to the heat-inducible genes, the basal amounts of constitutive hsp70 mRNA exhibit much greater stability at normal temperatures while contributing and re-establis hing base levels of these genes (Karouna-Renier *et al*., 2003).

The *Tmhsp70* gene we cloned from *T. molitor* larvae contains seven HSEs. Moreover, it has a 5'-Untranslated region (5'UTR) rich in adenine residues and a 3'-Untranslated region (3'UTR) rich in adenine and thymine residues like *hsp* genes from other insects. But regulation of Tmhsp70 expression including transcriptional and post-transcriptional regulation is yet to be determined.

Tmhsp70 contains a 1,935 bp ORF encoding 644 amino acids which are highly identical to HSP70s from other insects. In addition, the deduced heat shock protein (TmHSP70) has three signature motifs of the HSP70 family, i.e., IDLGTTYS, IFDLGGGTFDVSIL and IVLVGGS TRIPKIQQ as well as an EEVD motif that is signature to the carboxyl terminal of cytoplasmic HSP70. It is evident from the presence of these features that the deduced TmHSP70 is a cytoplasmic heat shock protein 70.

Structural domain analysis indicates that TmHSP70 shares some common structural features with other HSP70s studied so far. All of them contain a highly conserved amino terminal region that corresponds to an ATP binding domain and a highly conserved carboxyl terminal region that corresponds to the substrate binding domain. Together the two domains form the molecular foundation for HSP70 chaperone activity.

HSP70s function as chaperones by binding and/or releasing peptide substrates which is often associated with ATP hydrolysis (Feder and Hofmann, 1999). Amino terminal ATPase domain of HSP70 is an ATPase active centre responsible for binding and hydrolysis of ATP while the carboxy terminal peptide-binding domain is responsible for association with unfolded peptide substrates by specific binding to exposed hydrophobic regions of peptides. The binding and/or hydrolysis of ATP may regulate the affinity between HSP70s and peptide substrates while peptides and unfolded protein substrates may promote the activity of ATPase (McKay, 1993; Barthel *et al*., 2001). Peptides or denatured proteins bind to HSP70s during their association with ADP and are released when ADP transform into ATP (Hartl, 1996; Bukau and Horwich, 1998; Blond-Elguindi *et al*., 1993). Depletion of the ATP-binding site results in loss of ATP binding capability, ATPase activity and molecular chaperone function *in vitro* (Miernyk and Hayman, 1996). However, the mechanism regulating ATP-hydrolysis-associated conformation changes in the substrate-binding domain is still unclear.

HSP70 is the most highly conserved member of heat shock proteins which is not only expressed in cells exposed to heat stress but also constitutively expressed in all living cells including nuclei, cytoplasts, endoplasmic reticula, mitochondria, chloroplasts, etc. (Wu *et al*., 2004). As molecular chaperones, HSP70s not only participate in the folding and transportation of newly translated peptides but can also repair misfolded proteins or accelerate degradation of some proteins that are unable to restore their native conformation. This function protects the normal physiological processes of cells (Feder and Hofmann, 1999).

In cells without any stress, many protein precursors bind to cytoplasmic HSP70s soon after synthesis so as to maintain their appropriate structures. These peptides are then transferred to HSP70s on the surface of organelles including mitochondria, chloroplasts and endoplasmic reticula where they play an important role in guiding and translocating nascent peptides into the organelles (Simon *et al*., 1992; Matouschek *et al*., 1997).

In cells under heat or other stresses, protein chains may be unfolded and not be in their native states with change in their molecular spatial configuration. At this time, HSP70s that are rapidly synthesized bind to the denatured or non-native proteins and assist in their dissociation. This decreases the formation of insoluble aggregates which poses danger to cells. Moreover,

HSP70s maintain cell stability, avoid cellular damages, promote repair to damaged cells and elevate tolerance to heat or other environmental stresses by exerting effect on folding, unfolding and transposition of peptides or aggregation of protein complexes and assisting in new protein synthesis (Mayer and Bukau, 2005). Consequently, HSPs including HSP70s are induced in Sliving cells or organisms under a large variety of stresses and are important to an organisms' stress-resistance physiology.

CONCLUSION

In this study, researchers have cloned and sequenced an *hsp70* gene from *T. molitor* larvae for the first time and determined the mRNA relative abundance in the beetle life stages exposed to two different temperatures. The results have laid a foundation for further studying the relationship between HSPs and stress-resistance in the mealworm beetle. But more expression profiles of Tmhsp70 are still to be further determined such as the specific expression in male and female insects or in different tissues exposure to heat and other stresses including cold, heavy metals, insecticides, hormone analogues, pathogenic microorganisms, parasites, etc. Furthermore, earlier reports suggest a correlation between the different body-colors observed on *T. molitor* individuals and stress-resistance (Huang *et al.*, 2011, 2012). Therefore, to decipher the mechanism of stress-resistance and improve variety breeding further research on cloning, expression, structure and function of various *hsp* genes from different color varieties of *T. molitor* is necessary.

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