# Expression Profiles of the Heat Shock Protein 70 Gene in Response to Heat Stress in Agrotis c-nigrum (Lepidoptera: Noctuidae)

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RESEARCH

# Expression Profiles of the Heat Shock Protein 70 Gene in Response to Heat Stress in *Agrotis c-nigrum* (Lepidoptera: Noctuidae)

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**ABSTRACT.** Heat shock proteins (HSPs) are molecular chaperones, and their overexpression enhances the survivability and stress tolerance of the cell. To understand the characteristics of HSP70 in *Agrotis c-nigrum* Linnaeus larvae, the coding sequence of this protein was cloned, and the effect of heat stress on transcription and protein properties was assessed. The obtained cDNA sequence of HSP70 was 2,213 bp, which contained an ORF of 1,965 bp and encoded 654 amino acid residues. Isolated HSP70 cDNA demonstrated more than 80% identity with the sequences of other known insect HSP70s. Next, HSP70 was expressed in *Escherichia coli* BL21 (DE3) cells and identified using SDS-PAGE and western blotting analyses. In addition, anti-HSP70-specific antisera were prepared using a recombinant HSP70 protein, and the results showed that this antisera was very specific to *Ac*HSP70. Real-time quantitative polymerase chain reaction detected the relative transcription of the HSP70 gene in larvae and the transcription of *A. c-nigrum* in response to high temperatures. Induction of HSP70 was up-regulated to peak expression at 36°C.

**Key Words**: heat shock protein 70, *Agrotis c-nigrum* Linnaeus, expression profile, heat stress

Heat shock proteins (HSPs), which are found in all eukaryotic organisms, play essential roles in protein metabolism, folding, translocation, and the refolding of denatured proteins under both stress and nonstress conditions (Shu et al. 2011, Stephanou and Latchman 2011). According to the approximate relative molecular masses of their encoded proteins, HSPs are categorized as HSP100, HSP90, HSP70, HSP60, and small HSP (Feder and Hofmann 1999). Among the different families of HSPs, the HSP70 family is the most conserved family and has been the most intensely studied in various organisms from bacteria to mammals, including insects (Wang et al. 2012a). In insects, HSP mRNA or protein is induced by a variety of factors, including extreme temperature (Colinet et al. 2010, Bernabò et al. 2011), heavy metal pollution (Warchalowska-Sliwa et al. 2005, Wang et al. 2012a), and parasitic infection (Rinehart et al. 2002, Zhu et al. 2013).

Recent studies have shown that HSP70 genes are involved in protein interactions and as molecular chaperones, and their activities aid in the correct formation of structures under heat shock and protect against damage to cellular structure and function (Ramírez et al. 2011, Wang et al. 2012b). To date, many insect HSP70 genes can be expressed in response to a given stressor and have been identified in model insects, such as *Drosophila melanogaster* (Tissere et al. 1974). Studies have revealed that HSP70 can increase the resistance to stress and survivability in *Chilo suppressalis*, *Liriomyza trifolii*, and *Pteromalus puparum* (Wang et al. 2008, Cui et al. 2010, Zheng et al. 2010). Thus, HSP70 is a key protein that is closely related to the molecular mechanism of insect resistance to the environment. Thus, understanding the differential expression of HSP70 may provide insight into how insects react to the stress environment and provide specific information about the mechanisms of resistance to temperature stress.

*Agrotis c-nigrum* Linnaeus is a serious destructive pest for agriculture. It is distributed worldwide but is predominantly found in China, America, and Japan (Xi et al. 2002). *A. c-nigrum* poses a threat to crops such as tobacco and vegetables (Zhang et al. 1993, 2013). In northeast China, two to three generations of *A. c-nigrum* can seriously damage crops. In recent years, with atmospheric changes, *A. c-nigrum* infestations have become more frequent, and the damage caused has become progressively worse. However, very few reports on *A. c-nigrum* have been made and those that have focus primarily on the ecology and physiology (Zheng and Wang 2010). In this study, we characterized the full-length sequence of HSP70 cDNA from *A. c-nigrum* and investigated the expression of HSP70 in *Escherichia coli* BL21 (DE3). We also produced an antiserum against HSP70, determined the transcriptional pattern of HSP70 using real-time quantitative polymerase chain reaction (RT-qPCR), and provided some information for further studies on the resistance mechanism for environmental stress and ecological adaption of *A. c-nigrum*.

## Materials and Methods

**Insects.** *A. c-nigrum* larvae were collected from Xiang Fang Farm of Harbin, Heilongjiang Province, China. The larvae were reared at 25 ± 1°C and 70% relative humidity (RH) under a photoperiod of 14:10 (L:D) h in an RXZ climatic cabinet. Cabbage leaves were fed to the fourth instar.

**Main Reagents and Strains.** TRIzol reagent was purchased from Invitrogen Corporation (http://www.lifetechnologies.com/home.html). The PMD18-T vector, low melting point agarose, DNA purification kit, *Taq* plus DNA polymerase, 3′-Full RACE kit (TaKaRa Code: D314), and 5′-Full RACE kit (TaKaRa Code: D315) were purchased from TaKaRa Corporation (http://www.takara.com.cn/). Vector pET-21a (+) and CytoBuster Protein Extraction Reagent were purchased from Novagen Corporation (www.novagen.com/). Ponceau S, PVDF membrane, DAB Horseradish Peroxidase Color Development Kit (P0202), mouse His antibody (AH367), and goat antimouse antibody (Code: A0216) were purchased from Beyotime Institute of Biotechnology (http://www.beyotime.com/). Freund's complete adjuvant (F5881) was purchased from Sigma Corporation (http://www.sigmaaldrich.com/). $Ni2^{+}$-NTA agarose was purchased from Qiagen Corporation (http://www.qiagen.com/). Pierce BCA Protein Assay Kit (Code: 23227) was purchased from Thermo Scientific Corporation (http://www.matrix-techcorp.com/). ThunderbirdSybr qPCR Mix (Code: QPS-201) and ReverTra Ace qPCR RT Master Mix with gDNA Remover (Code No. FSQ-301) were purchased from ToYoBo Corporation (http://www.

toyobo-global.com/). The *E. coli* strains were maintained in our laboratory, and other reagents were purchased from TransGene Corporation (http://www.transgen.com.cn/).

**RNA Extraction and Isolation of HSP70.** The fourth instar larvae of *A. c-nigrum* were treated for 2 h at 36°C for heat shock and recovered at room temperature for 1 h. Total RNA was isolated using TRIzol according to the protocol of the TRIzol kit. The RNA quality was confirmed using an ultraviolet spectrophotometer, NanoDrop 8000, and 1% agarose gel electrophoresis. The reverse transcription kit was used to synthesize the first-strand cDNA (ReverTra Ace qPCR RT Mastr Mix with gDNA Remover (Code No. FSQ-301).

According to the conserved sequences from other known HSP70, such as *Mythimna separate* (EU306518), *Helicoverpa zea* (GQ389712), *Trichoplusis ni* (DQ845103), and *Spodoptera litura* (GU433378), all primers were designed in this study using Primer Premier 5.0 software and synthesized by Shanghai Sangon Biotech Co Ltd. These primers are listed in Table 1. The thermal program consisted of 94°C for 3 min, followed by 35 cycles of 94°C for 30 s, 57.8°C for 30 s, 72°C for 90 s, and a final elongation step at 72°C for 10 min. The product was confirmed in a 1% agarose gel and was then purified using a PCR purification kit. The purified PCR products were cloned into the PMD18-T vector and transformed into competent DH5α *E. coli* cells. 3′-RACE and 5′-RACE were performed using the relative kit protocol for the amplification of HSP70, and the primers of P3 and P4 were used to amplify full-length HSP70.

**Sequence Analysis.** The full-length sequence of *Ac*HSP70 was analyzed using DNAStar software. The identity analysis was performed by BLAST, which is available on the NCBI website (http://www.ncbi.nlm.gov/BLAST/). Multiple sequence alignment and phylogenetic analysis were performed using the ClustalW program (http://www.ebiac.uk/clustalw/) and MEGA 4.1 software using the neighbor-joining method. Analyses of protein domains were calculated using ScanProsite (http://prosite.expasy.org/scanprosite/) and Swiss-Model (http://www.swissmodel.expasy.org/SWISS-MODEL.html).

**Prokaryotic Expression of HSP70 and Western Blot Analysis.** The PCR product of HSP70 was digested with *EcoR* I and *Xho* I and ligated into the pET-21a (+) plasmid (digested with the same enzymes), resulting in the recombinant plasmid pET-HSP70. The pET-HSP70 plasmid was transformed into *E. coli* BL21 (DE3), and correct insertion was confirmed by enzyme digestion and sequencing. One clone with the correct insertion was cultured overnight at 37°C. The overnight culture was diluted into LB (1:100) and cultured for an additional 2–3 h to achieve log-phase bacteria ($OD_{600}$: 0.5–0.6). IPTG was added to the log-phase bacterial culture at a final concentration of 1 mmol/liter to induce protein expression. The protein samples were analyzed using 12% SDS-PAGE (Su et al. 2009).

Recombinant proteins extracted from *E. coli* BL21 (DE3) strain were separated on 12% SDS-PAGE. Preparation of the purified protein was performed according to the Qiagen protein purification protocol. The precipitation of positive expression production was collected by centrifugation, and the cell lysate of the target protein was obtained by ultrasonication with 20 mmol/liter imidazole, and the supernatant was collected by centrifugation (15,000 r/min) for 20 min. The supernatant and $Ni^{2+}$-NTA Agarose resin were gently mixed and stored at 4°C for 1 h. The unbound protein was washed with 50 mmol/liter imidazole and 250 mmol/liter imidazole. Next, all of the ingredients were collected for SDS-PAGE.

Purified proteins were electrophoretically transferred onto PVDF membranes at 200 mA for 2 h. The membrane was incubated in TBST (0.02 M Tris-HCl, 0.5 M NaCl, 0.05% Tween-20, 0.01 M Levamisole, pH 7.5) containing mouse His antibody (1:1,000) and blocked in TBST for 10 min. Subsequently, the goat antimouse IgG (H+L) secondary antibody, which was diluted 1:1,000 in TBST containing 5% skimmed milk, was applied. The membrane was developed using DAB for 10 min and terminated by rinsing the strips with distilled water.

**Production of Polyclonal Anti-HSP70 Serum and Immunoblot Analysis.** Five male BALB/c mice (5-wk-old) were immunized intradermally with recombinant HSP70 protein mixed with an equal volume of Freund's complete adjuvant. An additional five mice were not immunized as negative controls. The mice were booster immunized with a mixture of antigen and Freund's incomplete adjuvant (Sigma) three times every 2 wk. Three days after the last immunization, blood was collected, clarified by overnight incubation at 4°C, and centrifuged at $3,000 \times g$ for 15 min. The supernatant was collected and stored at −20°C. At the same time, the negative control group was immunized with PBS as antigen using the same method.

Total proteins extracted using CytoBuster protein extraction reagent from the fourth-instar larvae of *A. c-nigrum*, which were treated at 33°C, 36°C, 39°C, and 42°C for 2 h, and incubated at room temperature for 1 h. The total protein concentrations were determined and diluted by PBS to 2.0 μg/μl using BCA Protein Assay Kit (Thermo). The same volumes (10 μl) of protein were separated for detection of 12% SDS-PAGE, and each treatment included three replicates. The primary antibody was polyclonal anti-HSP70 serum, and the secondary antibody was goat antimouse IgG (H+L). The procedure was performed as previously described.

**Table 1. Sequence of primers**

| Primer type | Sequences of primers (5′–3′) | Used for |
|---|---|---|
| cDNA isolation (RT-PCR) | | |
| P1 | GACATGAAGCACTGGCCCTTC | cDNA cloning |
| P2 | TCGCTCGCTCGCCCTCGTACA | |
| P3 | CACCTTTGCTGAGTTACTCTACGAGTTAAG | |
| P4 | CAATATCAGTAAAAGCTTGCTGTAATGTTG | |
| HSP70*EcoR* I | CCG *GAATTC* ATGGCAGCAACTAAAGCACCC | Vector construction |
| HSP70*Xho* I | CCG *CTCGAG* GTCGACCTCCTCGATGGTAGG | |
| 5′- and 3′-cDNA end isolation (RACE) | | |
| 5′-GSP 1 | ATCAACCCCGACGAGGCCGTAGCTTA | 5′-RACE |
| 5′-GSP 2 | AGGTACAGGATCTGCTGCTGCTCG A | |
| 5′-RACE outer primer | CATGGCTACATGCTGACAGCCTA | |
| 5′-RACE inner primer | CGCGGATCCACAGCCTACTGATGATCAGTCGATG | |
| 3′-GSP 1 | GCAGAAGCTCCTTCAAGACTTCTTTAATGGCAAG | 3′-RACE |
| 3′-GSP 2 | GTCGGTGGTTCTACCCGTATTCC | |
| 3′-RACE outer primer | TACCGTCGTTCCACTAGTGATTT | |
| 3′-RACE inner primer | CGCGGATCCTCCACTAGTGATTTCACTATAGG | |
| RT-qPCR primers | | |
| HSP70-F | GTGACGCGAAGATGGACAAGTC | RT-qPCR |
| HSP70-R | CACCGTAAGCTACAGCCTCGTC | |
| β-actin-F | CGCGACCTCACAGACTACCTG | |
| β-actin-R | CGTAGGACTTCTCCAGGGAGC | |

**RT-qPCR Analysis of AcHSP70.** The fourth-instar larvae of *A. c-nigrum* were treated under the conditions of 28°C, 30°C, 33°C, 36°C, 39°C, and 42°C for 2 h and then incubated at room temperature for 1 h. Next, RNA from the midgut, epidermis, salivary gland, fat body, and Malpighian tubule was isolated and was used to confirm the transcriptional properties of *Ac*HSP70. The reference gene β-actin was used as an endogenous control to quantify the target genes. This gene is an appropriate control for studies on HSP70 in insects during this wide developmental window, as determined in our previous studies (Jiang et al. 2012, Yu et al. 2012). *Ac*HSP70 and β-actin sequences were amplified from each of the species under each treatment condition using specific primers, ThunderbirdSybr qPCR Mix (Code: QPS-201) and Chromo4 RT-PCR instrument (Bio-Red). All amplifications were performed in triplicate. The final volume of each RT-qPCR reaction was 20 µl, which contained 10 µl 2 × SYBR Mix (ToYoBo), 1 µl of diluted cDNA template, 7.8 µl of PCR-grade water, and 0.6 µl of each 10 µM primer. PCR conditions were as follows: 95°C for 60 s, 40 cycles of 95°C for 15 s, 56°C for 30 s, and 72°C for 30 s.

*Ac*HSP70 expression levels were calculated using the $2^{-\Delta\Delta Ct}$ comparative CT method (Livak and Schmittgen 2001). The means and standard deviations were calculated from experiments performed in triplicate and are presented as the *n*-fold differences in expression. Differences in the *Ac*Hsp70 transcriptional features in different tissues were analyzed using SPSS 16.0. Statistical significances were determined using one-way analysis of variance and post-hoc Duncan multiple range tests. Significance was established at $P < 0.05$.

## Results

**Characterization of AcHSP70 cDNA.** The full-length cDNA of *Ac*HSP70 was 2,213 bp in length and contained a predicted open reading frame of 1,965 bp, beginning with a methionine codon at position 147 and ending with a TAA termination codon at position 2109. The 3′-untranslated region is 86 bp in length, from 2,112 to 2,213 bp, and contains a polyadenylation signal (AATAAA) and a poly (A) tail of 16 bp. The complete sequence has been placed in GenBank with the accession number HQ698836. The encoded 654 amino acid polypeptide had a calculated molecular mass of 71.62 kDa and a predicted isoelectric point of 5.39.

The analysis sequence of *Ac*Hsp70, which was performed using analysis software (http://us. expasy.org/tools/dna.html) and DNAStar software (Wang et al. 2006), provided information regarding three conserved domains and four eukaryotic sequences. One cytoplasmic modification site was found at the end of the C-terminus that could affect the activity of ATP kinase and modify the transcriptional level of HSP70 (Carpenter and Hofmann 2000). Similarly, the signal cleavage site was found between the 18th and 19th amino acid site using SignalP3.0. Furthermore, analysis of the three dimensional structure using Swiss-Model indicated that the protein contains two domains, a 44-kDa ATPase domain (7-382 residues) and a 10-kDa C-terminal domain (539-623 residues). The ATPase domain of HSP70 was highly conserved, whereas the C-terminal domain was less conserved compared with the ATPase domain.

**Phylogenetic Analysis of AcHSP70.** Homology analysis revealed that HSP70 was highly conserved in insects. The deduced amino acid sequence shared a high similarity with other known HSP70s, such as an 86% identity with *Mamestra brassicae* (BAF03555) and 88% with *Spodoptera exigua* (ACQ78180). More than an 80% identity was found with other insects. However, compared with the mammalian HSP70 family, only a 75% identity was found between *Homo sapiens* (NM_005345) and *Rattus norvegicus* (NM_024351). Only a 65% identity was found between *Arabidopsis thaliana* (NM_120327) and *Paeonia lactiflora* (JN180465), which belongs to the plant family.

To understand the evolutionary relationship of HSP70 with the other 17 members, a phylogenetic tree of HSP70 is shown in Fig. 1. These results indicated that different branches of insects were found within the tree. Animals and plants were separated from one another. All of the insects were clustered together and formed four branches, which contained the insects belonging to the same order. In addition, all of the plants and mammals were clustered together.

**Expression of AcHSP70 Gene.** The relative mRNA level of *Ac*HSP70 was quantified using RT-qPCR at various temperature gradients. These results revealed that the transcription of *Ac*HSP70 could not be induced for 2 h at 28°C–30°C, as no significant difference was observed compared with 25°C (Fig. 2). However, a statistically significant up-regulation was shown for the other temperatures tested. The maximal induction of *Ac*HSP70 peaked at 36°C, and with further increases in temperature, the expression was inhibited. The highest intensity of temperature response was a 5.64-fold increase at 36°C compared with 25°C.

To further understand the mechanism underlying *Ac*HSP70 resistance to heat stress, RT-qPCR was used to analyze the expression pattern of *Ac*HSP70 in the midgut, epidermis, salivary gland, fat body, and Malpighian tubule. The expression of *Ac*HSP70 in these five tissues and at seven temperatures showed that the transcription of *Ac*HSP70 was significantly different. The expression levels of *Ac*HSP70 could be induced in the midgut and salivary gland and peaked at 36°C, and in the epidermis, fat body, and Malpighian tubule, *Ac*HSP70 levels peaked at 39°C. These results also indicated that the highest level of expression was present in the midgut at each heat shock temperature (Fig. 3).

**Characterization of AcHSP70 Antibodies.** The recombinant protein was isolated from the protein samples, which were induced using IPTG from *E. coli* BL21 (DE3) with pET-21a (+) for different time periods. A band of 71.62 kDa, which was similar to the result predicted by the software, was detected using 12% SDS-PAGE and revealed that the target product was present as a soluble protein. To obtain more soluble protein, the conditions for optimal expression were 1.0 mmol/liter

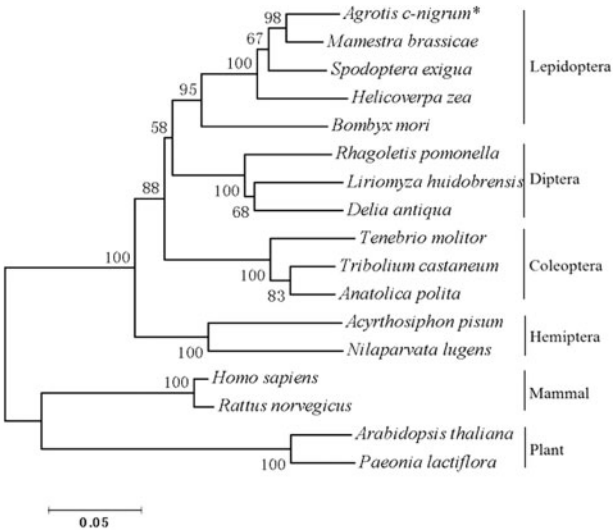


**Fig. 1.** Phylogenetic tree of the HSP70 sequence, constructed using the neighbor-joining method. The values on the branches indicate the percentage frequencies at which the phylogram topology was representative for 1,000 bootstrap replicates. The amino acid sequences of HSP70 were obtained from the following species: Lepidoptera, *A. c-nigrum* (ADZ15147), *Mamestra brassicae* (BAF03555), *S. exigua* (ACQ78180), *H. zea* (ACV32640), and *B. mori* (NP_001037396). Diptera, *Rhagoletis pomonella* (ABL06948), *Liriomyza huidobrensis* (AAW32098), and *Delia antiqua* (AAY28732). Coleoptera, *Tenebrio molitor* (AFE88579), *Tribolium castaneum* (XP_974442), and *Anatolica polita* (ABQ39970). Hemiptera, *Acyrthosiphon pisum* (XP_001949837) and *Nilaparvata lugens* (AFJ20626). Mammal: *Homo sapiens* (NP_005337) and *Rattus norvegicus* (NP_077327). Plant: *Arabidopsis thaliana* (NP_195869) and *Paeonia lactiflora* (AEK70336). For each species, the GenBank accession numbers have been placed in brackets.

IPTG at 36°C with 7 h of incubation (Fig. 4A). In addition, the corresponding bands were recognized using western blotting analyses with the His-antibody (Fig. 4B).

To further determine the expression of HSP70 in *A. c-nigrum*, total crude protein was extracted from *A. c-nigrum*, and polyclonal antiserum against HSP70, which was generated from immunized mice as a primary antibody, was used to confirm the expression of the recombinant and purified proteins. These results showed that bands of 71.62 kDa could be detected in both native and recombinant protein using western blotting analyses (Fig. 4C). In addition, the same experiment was also performed using the serum obtained from PBS-immunized mice and nonimmunized mice as the primary antibody. These results showed that these serums did not react with the protein and did not produce any band. Thus, the polyclonal antibody specifically binds to the HSP70 protein.

To determine the expression difference of *Ac*HSP70 under heat shock treatment, the total proteins of *A. c-nigrum* were extracted after heat shock at 33°C, 36°C, 39°C, and 42°C and detected using the same method. Using BanScan 5.0, the results indicated that the amount of target protein at 36°C was higher than at 33°C, 39°C, and 42°C, which was in agreement with the transcriptional pattern (Fig. 5A and B).

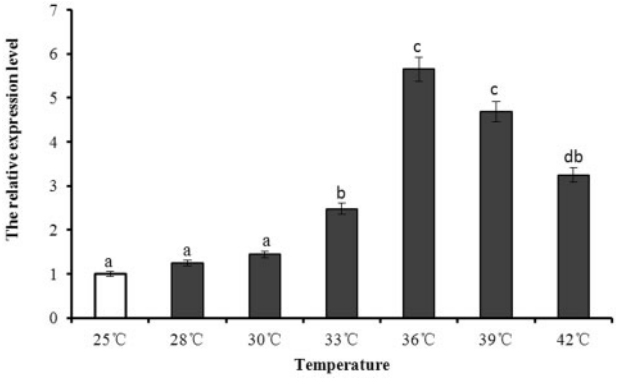


**Fig. 2.** Expression profiles of *Ac*HSP70 mRNA under heat shock temperatures. Control: 25°C; Heat shock temperature: 28°C, 30°C, 33°C, 36°C, 39°C, and 42°C. *Ac*HSP70 mRNA levels were determined using RT-qPCR. Each temperature treatment was repeated three times. The data were represented as the mean ± SD ($n = 3$), and the different letters above the bars (a–d) indicate a significant difference in the means as assessed using multicomparison tests ($P < 0.05$).

## Discussion

In this study, we found that HSP70s are highly conserved proteins, and its amino acid sequence comparisons revealed that there are three signature sequences present, which were consistent with other HSP70 family proteins. In addition, two motifs were identified: an EEVD motif and GGMP tetrapeptide repeats at the C-terminal, which are conserved in most insect HSP70 sequences. The EEVD motif is thought to mediate interactions with chaperone cofactors and is involved in binding with other cochaperones (Boorstein et al. 1994, Freeman et al. 1995, Johnson et al. 1998). There is an EEVD at the C-terminus of *Ac*HSP70. As a conserved repeat, the GGMP motif may be partially responsible for functional differences and was identified in the *Ac*HSP70 protein (Demand et al. 1998, Fuertes et al. 2004). Previous studies have investigated the properties of this domain (i.e., whether it contains several repeats) and whether it would be recognized as a marker domain of the HSP70 and HSC70 family (Wu et al. 2001, Piano et al. 2005). However, many recent studies have shown that GGMP may be found in both HSP70 and HSC70, such as the HSP70 and HSC70 of *Crassostrea gigas*, where both families had a GGMP domain repeating at the C-terminal (Boutet et al. 2003). The induced HSP70 of *C. suppressalis* also has a GGMP domain repeating at the C-terminal (Cui et al. 2010). Thus, it was necessary to further study the structure and function of the HSP70 with GGMP at the C-terminus. In addition, other sites were identified in *Ac*HSP70 for four eukaryotic sequence characteristics and N-glycosylation, which play essential roles in signal transduction and regulation. Glycosylation is important for protein folding, oligomerization, quality control, sorting, and transportation (Demand et al. 1998, Chan et al. 2003).

RT-qPCR data showed that the transcription of *Ac*HSP70 mRNA was present at high levels at 36°C both in the fourth-instar larvae and in different tissues. Compared with the expression of *Ac*HSP70 in an unstressed condition, high expression of *Ac*HSP70 was found in the fourth-instar larvae and in each tissue after heat shock, and in particular, the tissue-specific expression showed different quantities of *Ac*HSP70 in various tissues. The *Ac*HSP70 results showed that the midgut and salivary gland, which comprise the digestive system, were sensitive to heat treatment, and the epidermis, fat body, and Malpighian tubule, which belong to the excretory system, demonstrated relatively lower sensitive. Similar results were obtained by Wang et al. (2012b), who found that the HSP70 mRNA level of the midgut of *Bombyx mori* was higher than other tissues upon thermotolerance after heat shock (Wang et al. 2012b). However, although tissue-specific expression of the HSP70 gene was present in *A. c-nigrum*, the same pattern of *Ac*HSP70 mRNA expression was present in all detected tissues, in which the HSP70 mRNA levels increased initially and then decreased gradually

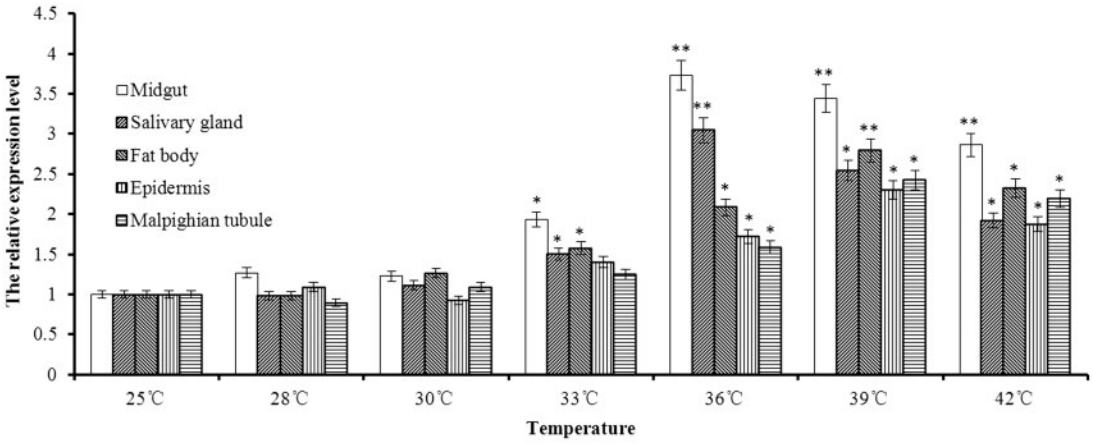


**Fig. 3.** Relative expression levels of HSP70 mRNA in different tissues at 25°C, 28°C, 30°C, 33°C, 36°C, 39°C, and 42°C. Control: 25°C, each temperature treatment was three replicates. Data were represented as the mean ± SD ($n = 3$). The asterisk above the bars indicate a significant difference in the means as assessed using multicomparison tests ($P < 0.05$).

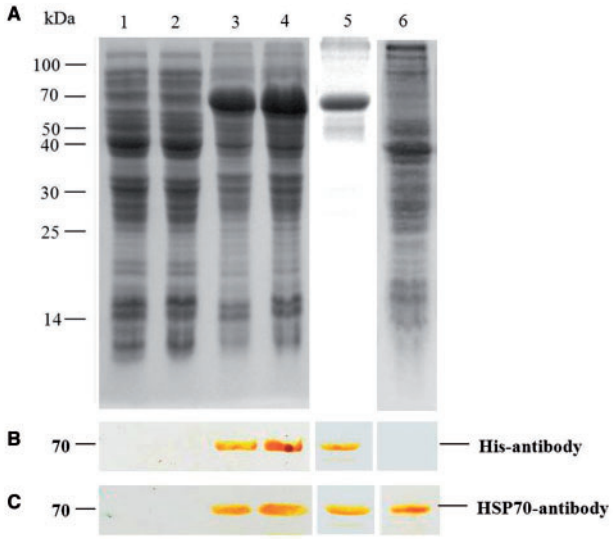


**Fig. 4.** SDS-PAGE and western blotting of *Ac*HSP70 protein. (A) SDS-PAGE of *Ac*HSP70 protein. Lane 1: total protein of noninduced pET-21a (+); lane 2: total protein of pET-21a (+) induced for 7 h; lanes 3 and 4: total protein of pET-21a(+)/*Ac*HSP70 induced for 5 h and 7 h; lane 5: purified protein of pET-21 a(+)/*Ac*HSP70 induced for 7 h; lane 6: total protein of *A. c-nigrum* without treated by heat shock (25°C). (B, C) Western blotting with His-antibody and HSP70-antibody, respectively.

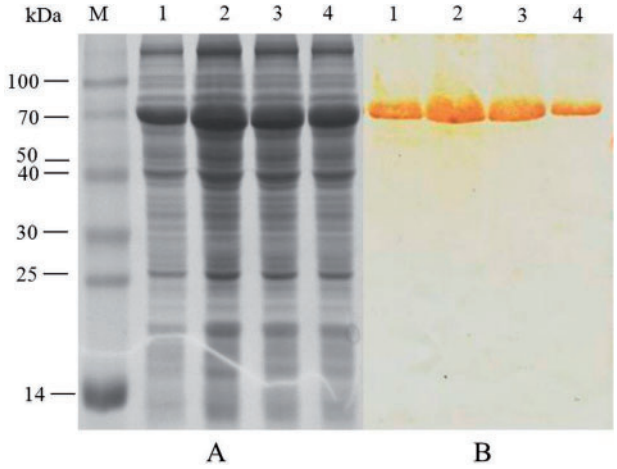


**Fig. 5.** Specificity of HSP70 polyclonal antiserum was determined using western blotting analyses at different temperatures. (A) M, low molecular marker; lanes 1, 2, 3, and 4: total protein of *A. c-nigrum* treated by heat shock at 33°C, 36°C, 39°C, and 42°C for 2 h. (B) Lanes 1, 2, 3, and 4: purified protein of *A. c-nigrum* treated by heat shock at 33°C, 36°C, 39°C, and 42°C for 2 h.

following heat stress. This pattern is due to heat shock causing protein denaturation, as HSP70 proteins function as molecular chaperones that bind denatured proteins to prevent them from aggregating and further assisting in the normal refolding of the denatured proteins or the elimination of permanently denatured proteins (Welch 1992, Kiang and Tsokos 1998). Thus, HSP70 mRNA was abundantly transcribed in different tissues of *A. c-nigrum* when denatured proteins accumulated after heat shock, and the mRNA level gradually decreased when the denatured proteins were normally refolded or eliminated. Furthermore, several published results have shown that the expression pattern is not always consistent in various insects. We obtained the same results in *B. mori* (Wang et al. 2012b) and *H. zea* (Zhang and Denlinger 2010), but we obtained different results in *S. litura* (Shu et al. 2011), in which the level of HSP70 expression in the fat body was higher than in other tissues, although these three insects all belongs to Lepidoptera. In addition, there were some insects with high expression in the brain, similar to *Sarcophaga crassipalpis* (Denlinger et al. 2001), which belongs to Diptera. This phenomenon might be due to differences in the genetic background of the insects and is also consistent with other HSP genes, such as the HSP90 family (Tachibana et al. 2005, Zhang and Denlinger 2010).

SDS-PAGE analysis demonstrated that the amount of protein increased significantly with an increase in induction temperature, and HSP70 proteins were present only in the IPTG-induced *E. coli* BL21(DE3) containing the pET-21a(+)/HSP70 plasmid. Western blotting analyses showed that the polyclonal antiserum generated in mice reacted to purified HSP70 and total protein of *A. c-nigrum*. These results suggested that this antibody was specific to *Ac*HSP70 and could be induced by heat treatment. Compared with the mRNA level, the protein level was also highest at 36°C. This phenomenon also supports prior studies that found that high levels of HSP70 protein may reverse the regulation of the expression of HSP70 (Welch 1992, Kiang and Tsokos 1998).

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