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Full Length Research Paper

Identification of HSP90 gene from the Chinese oak silkworm, *Antheraea pernyi*

Li Chen[#], Baojian Zhu[#], Guoqing Wei, Lei Yu, Yingying Xu, Chaoliang Liu* and Jia Cao

College of Life Science, Anhui Agricultural University, 130 Changjiang West Road 230036, Peoples Republic of China.

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The heat shock proteins (Hsp) play an important role in protein folding and protection of cells from stress. To investigate the role of Hsp90 in silk-producing insect *Antheraea pernyi* (Lepidoptera: Saturniidae), a full-length cDNA encoding Hsp90 from *A. pernyi* was cloned, sequenced and characterized. The complete cDNA (2,482 bp) contained a 2,154 bp open reading frame encoding 717 amino acid residues and had 94.5% identity with *Antheraea yamamai* Hsp90. The relative expression levels of Hsp90 in five different tissues at normal and high temperatures were evaluated with real-time fluorescence quantitative RT-PCR. The expression of Hsp90 was obviously changed in the examined tissues except for fat bodies after induced by high temperature. SDS-PAGE of purified protein demonstrated that an 86 KD recombinant protein was successfully expressed in transformed *Escherichia coli* cells. These results shed light on studying the mechanism of tolerance in *A. pernyi.*

Key words: *Antheraea pernyi*, HSP90, sequence analysis, expression.

INTRODUCTION

Heat shock proteins (HSPs) are evolutionarily highly-conserved proteins synthesized in cells when they are exposed to stress (Moseley, 1997; Scheibel et al., 1998; Feder et al., 1999; Huang and Ma, 2004). Heat shock proteins were discovered in heat-shocked *Drosophila* salivary glands (Ritossa, 1962) and subsequent research proved that similar proteins were induced by other types of stress, such as hypoxia (Guttmans et al., 1980), ischemia (Marber et al., 1995), acidosis (Weitzel et al. 1985), lack of energy (Sciandra and Subjeck, 1983), heavy metals (Levison et al., 1980), amino acid analogs (Kelley and Schlesinger, 1978), glucose analogs (Pouyssegur et al., 1977) and ultraviolet light (Barbe et al., 1988). HSPs protect stressed cells by maintaining the conformations of vital protein (Beckmann et al., 1990) and mediating the folding and aggregation of proteins, transmembrane transportation and stabilizing the cytoskeleton and nuclear matrix (Hendrick and Hartl, 1993; Yonehara et al., 1996), apoptosis (Arya et al., 2007), heat tolerance (Parsell and Lindquist, 1994) and antitumor responses (Neal et al., 2004). Heat shock proteins are classified into six types according to their relative molecular weights, structures and functions (Sugiyama et al., 2000). HSP90 is the most abundant protein in eukaryotes and is produced by almost all species in response to biotic and abiotic stresses (Buchner, 1996; Peter et al., 1998; Nollen and Morimoto, 2002; Whitesell and Lindquist, 2005). HSP90 are closely related with the duration of stress and could improve the ability of insects to withstand adversity (Sorensen et al., 2003; Wang et al., 2007; Jiang et al., 2012).

The Chinese oak silk moth, *A. pernyi* is an economically valuable silk-producing insect (Zhou and Han, 2006). Although the HSP90 gene has been found in a variety of insects (Rinehart and Denlinger, 2000; Sonoda et al., 2006; Wang et al., 2007), the HSP90 gene in *A. pernyi* and its role remains unclear. In this study, HSP90 gene was identified from *A. pernyi* and its expression were also investigated.

*Corresponding author. E-mail: cyschx@163.com. Tel/Fax: +86 551 5786201.

# These authors contributed to this work equally.

MATERIALS AND METHODS

Experimental insects

The experimental insects, *A. pernyi,* were provided by the

Table 1. Primers used for PCR.

Primer number	Primer sequence(5'- 3')
F1	CATCTCTCGTGARATGCTCC
R1	CTTGACCTTRTCTTCCTCAC
F2	TGCGGATCCATGCCTGAAGGGATGGAGAC
R2	CGCCTCGAGTTAATCCACTTCCTCCATTC
F3	CATCACCCAGGAGGAGTA
R3	TGCGAGGTACAAACAGTAAA
F18S	CGATCCGCCGACGTTACTAC
R18S	GTCCGGGCCTGGTGAGATT

Sericultural Research Institute of Henan and were reared on the leaves of oak under indoor conditions.

Cloning of HSP90 cDNA

Total RNA was isolated from fat bodies with TRIzol reagent (Invitrogen, USA) and the First-Strand cDNA was obtained by TransScript Synthesis SuperMix (TransGen, Beijing, China). The sequences of HSP from various animals were aligned by Clustal W (http://www.ebi.ac.uk/Tools/ClustalW). Degenerate oligonucleotide primers, F1 and R1 (Table 1) were designed with Primer premier 5.0 software package. PCR was performed using the amplification program consisted of 5 min at 94°C followed by 35 cycles of 94°C for 30 s, 53°C for 35 s, 72°C for 30 s and a final elongation step of 72°C for 5 min. Based on the obtained fragment, the rapid amplification of cDNA ends PCR (RACE–PCR) was performed using the SMART RACE cDNA Amplification Kit (Clontech). PCR products were analyzed by 1% agarose gel electrophoresis and sequencing at Invitrogen, Shanghai.

Semi-quantitative PCR analysis

Total RNA from hemocytes, fat bodies, midgut, ovaries and testes of five pupae were reverse transcribed into cDNA. Semi-quantitative PCR was carried out with specific primers F2 and R2 to determine the expression levels of HSP90 in different tissues. The 18S rRNA gene was used as an internal reference (with primers F18S and R18S). The amplification program used for semi-quantitative PCR was 95°C for 4 min followed by 35 cycles of 94°C for 30 s, 57°C for 40 s and 72°C for 90 s. The agarose gel electrophoresis was used to analyze the PCR products.

Real-time PCR analysis

The primers F3, R3, F18S and R18S are used for real-time quantitative PCR. Total RNA was extracted from hemocytes, fat bodies, midgut, ovaries and testes of oak pupae which had been treated at 25°C or 42°C for 30 min. Real-time PCR was performed in a StepOnePlus Real-Time PCR System using the SYBR® Premix Ex Taq™kit (TaKaRa). Reaction mixtures (20 µL) contained 10 µL 2× SYBR® Premix Ex Taq™ buffer, 1 µL forward and reverse primers, 1 µL cDNA, and 7 µL RNase-free H_2O. The PCR procedure was as follows: 95°C for 10 s followed by 40 cycles each at 95°C for 15 s and 55°C for 60 s. At the end of the reaction, a melting curve was produced by monitoring the fluorescence continuously while slowly heating the sample from 60 to 95°C. Each independent experiment was conducted in triplicate and the relative expression level of gene was determined using the method (Livak and Schmittgen, 2001). Student's t-test was used to analyze the data and differences were considered statistically significant when p values were less than 0.05.

Prokaryotic expression of Ap-hsp90

After purification by agarose gel electrophoresis, the PCR products were digested with *Bam*HI and *Xho*I enzymes and then were ligated to the PET-28 (a+) vector (Invitrogen). The recombinant plasmids (PET-hsp90) were identified by sequencing, then transformed into competent *E. coli* BL21 (DE3) cells (Invitrogen) and induced by different concentrations of IPTG. The recombinant fusion proteins were analyzed with 12% sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE).

Purification of recombinant Ap-hsp90 protein

After induced by IPTG at 37°C for 4 h, cells were harvested by centrifugation at 5,000 × *g*. The cell pellets were suspended in lysis buffer (50 mM NaH_2PO_4, 300 mM NaCl, pH 8.0,1 mg/ml imidazole), stirred on ice for 30 min and then disrupted by sonication. After centrifugation at 10,000 × *g* for 30 min at 4°C, the recombinant protein was purified by affinity chromatography through the nickel-nitrilotriacetic acid agarose resins (Qiagen) following the manufacturer's protocol. Purity and identity of the eluted fractions were analyzed by SDS-PAGE.

RESULTS AND DISCUSSION

Cloning and sequence analysis of HSP90 cDNA

A cDNA fragment of 2,482 bp was obtained by conventional PCR and RACE–PCR. Nucleotide sequence analysis revealed that HSP90 cDNA contained a 122 bp 5'-untranslated sequence, a putative ORF of 2,154 bp, a 206 bp 3'-untranslated region and a putative polyadenylation signal predicted by ExPASy (http://us.expasy.org). The inferred amino acid sequence for HSP90 of *A. pernyi* (Figure 1) was compared with those of HSP90s of other species and the result show that this protein had 94.5% identity with *A. yamamai* HSP90 and 73.1% identity with *Bombyx mori* HSP90. A phylogenetic tree was constructed based on the amino acid sequences of HSP90s from 10 species by MEGA 4.0 using the neighbor-joining algorithm method.

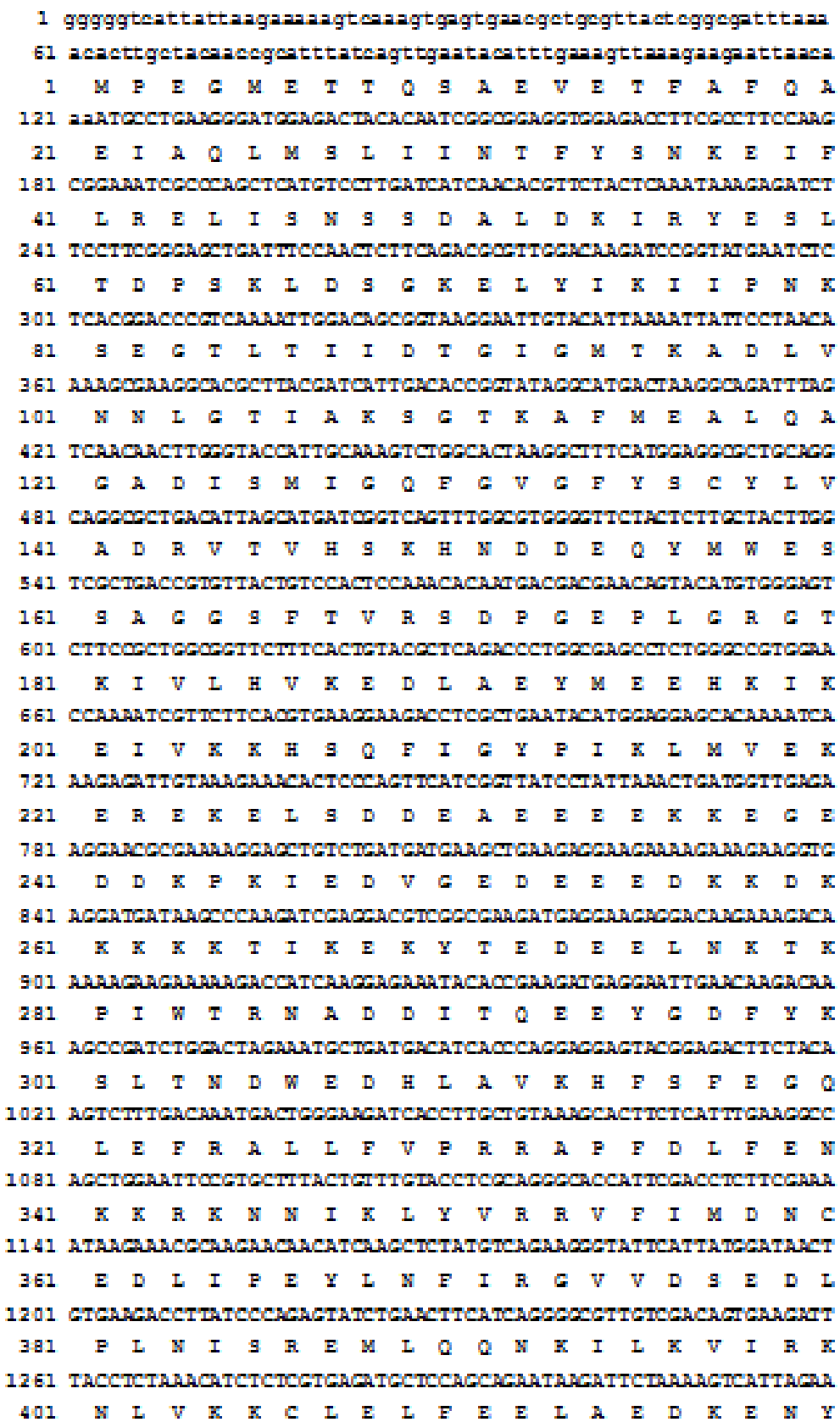

Figure 1. Nucleotide and inferred amino acid sequences of HSP90 from *A. pernyi*. Asterisk indicates the termination codon.

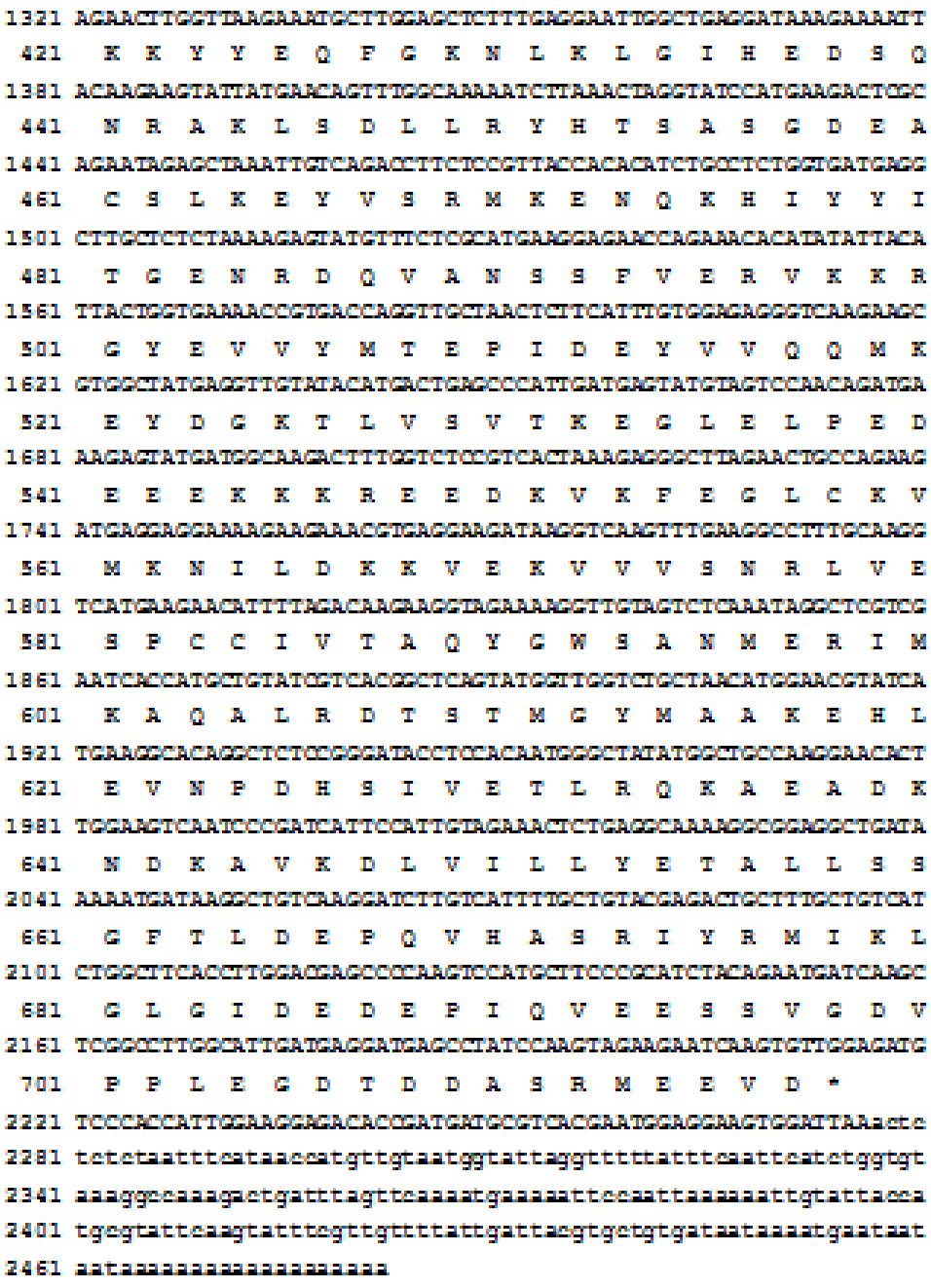

Figure 1. Continued.

Phylogenetic analysis indicated that the *A. pernyi* HSP90 was most closely related with the HSP90s from Lepidoptera insects (Figure 2).

Expression of Ap-HSP90 in different tissues

Total RNA was extracted from hemocytes, fat bodies, midgut, ovaries and testis and assessed semi-quantitatively using 18S rRNA expression as an internal control. HSP90 was expressed at significantly higher levels in the testis, but there were no significant differences among the other four tissues (Figure 3).

Expression of HSP90 in pupa stimulated by high temperature

Real-time quantitative PCR was performed to determine the expression levels of HSP90 mRNAs under standard

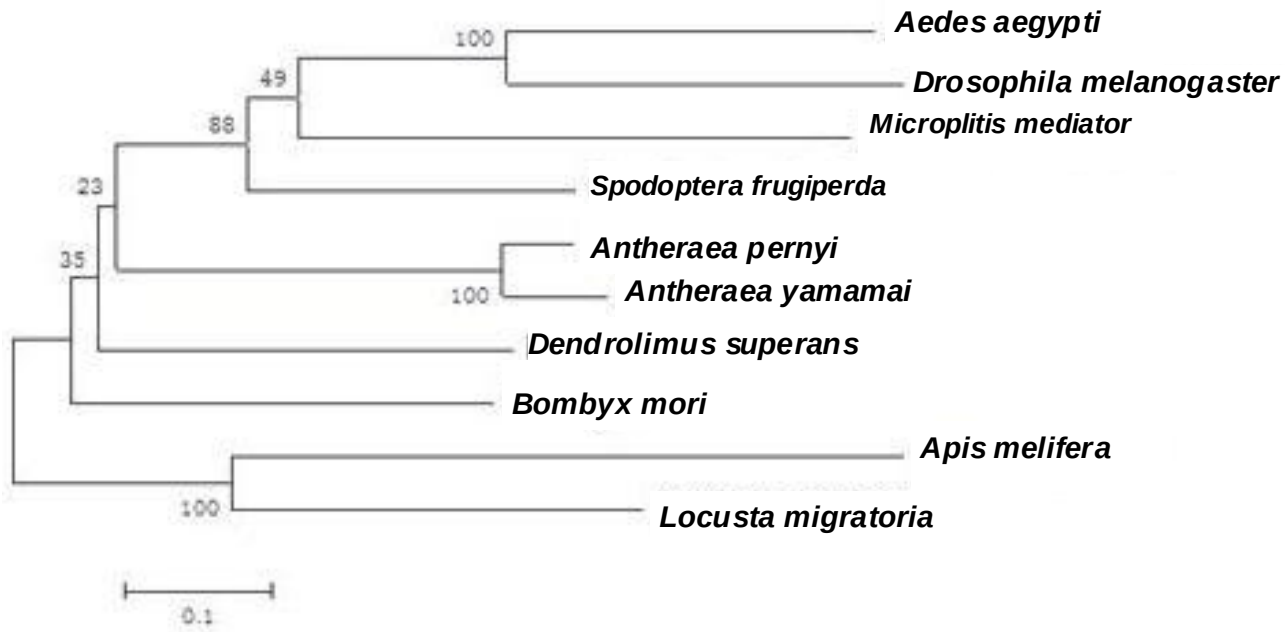

Figure 2. Phylogenetic tree of HSP90 amino acid sequences from 10 species. The phylogenetic tree was constructed using the neighbor joining algorithm method and bootstrap values (1000 repetitions) of the branches indicated. The HSP90 sequences deposited in the GenBank database are: *Aedes aegypti* (XP_001649752), *Drosophila melanogaster* (NP_523899.1), *Microplitis mediator* (ABV55506.1), *Spodoptera frugiperda* (AAG44630.1), *Antheraea yamamai* (BAD15163.1), *Dendrolimus superans* (ABM89112.1), *Bombyx mori* (NP001036876.1), *Apis mellifera* (NP_001153536.1) and *Locusta migratoria* (AAS45246.2).

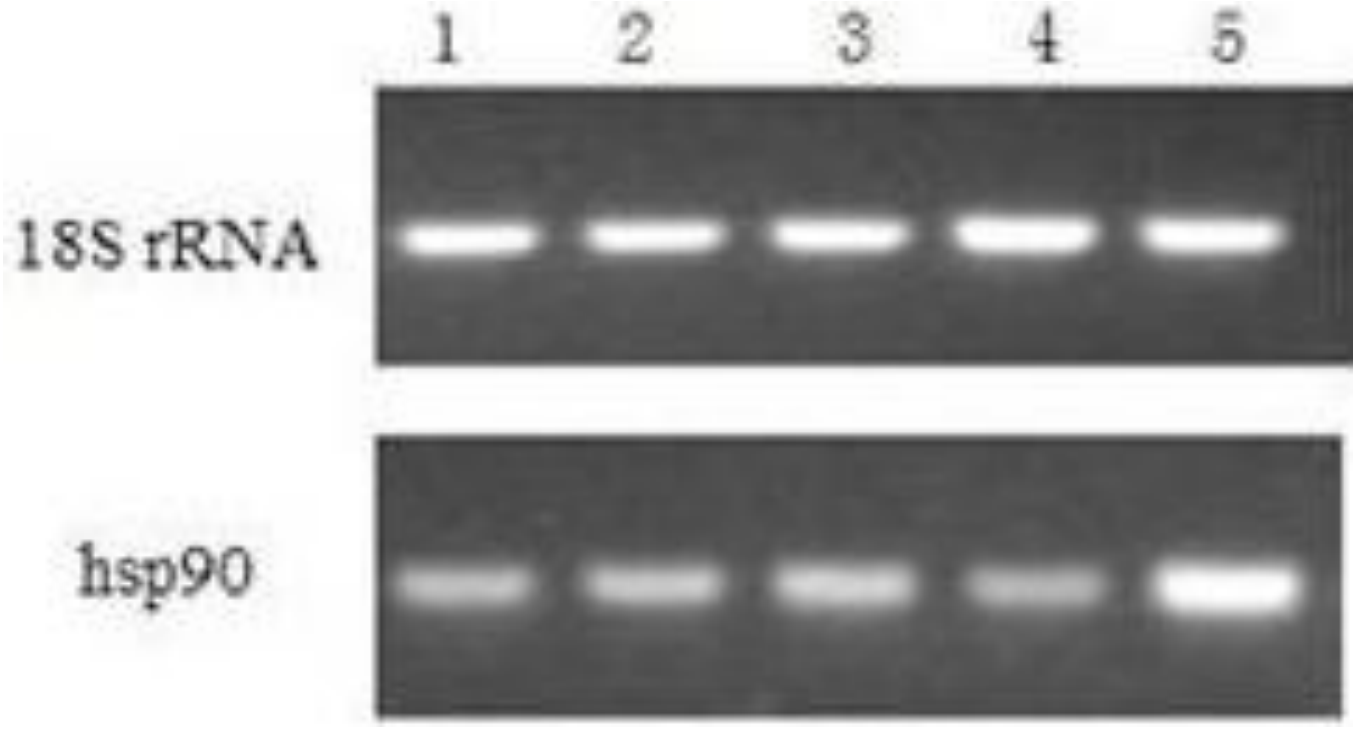

Figure 3. 1% agarose gel of semi-quantitative PCR amplification products of the *A. pernyi* HSP90 gene in different tissues. The expression of Ap-18S rRNA was used as a control. Lanes 1 to 5 show expression of HSP90 in hemocytes, fat bodies, the midgut, ovaries and testis, respectively.

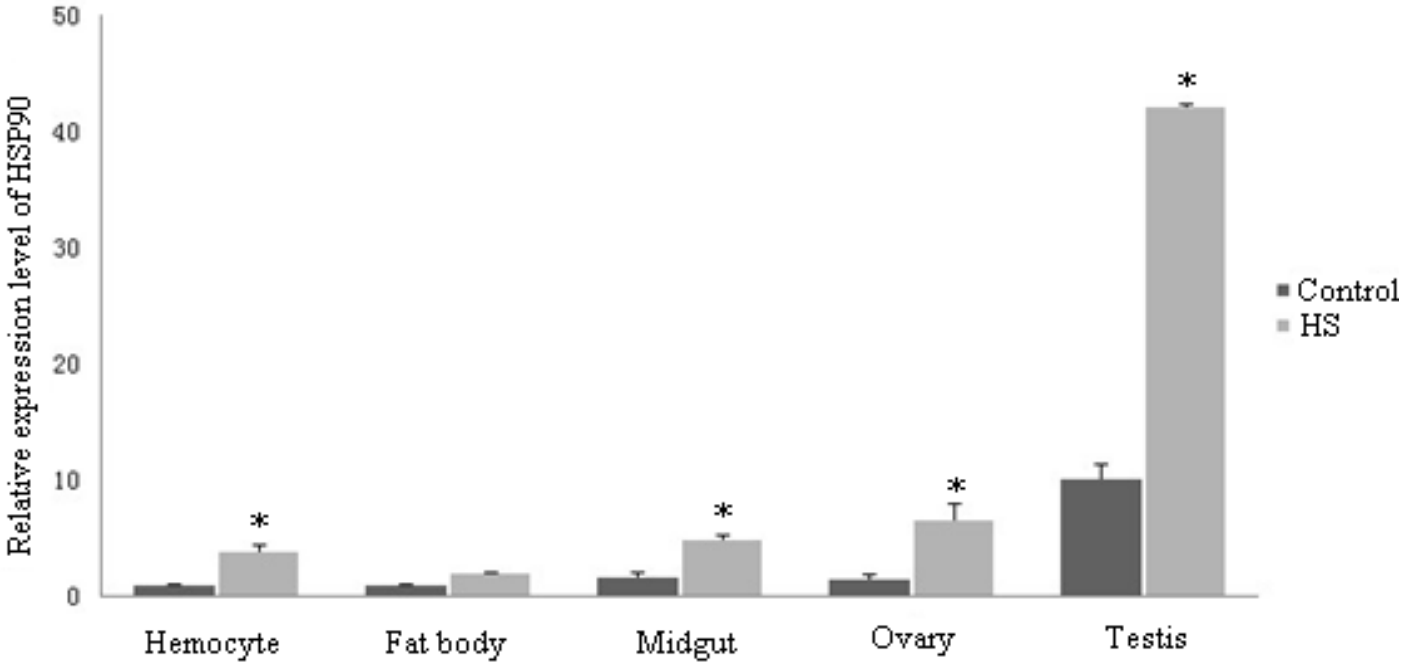

Figure 4. Ap-HSP90 expression levels in different tissues. HS indicates the treatment with 42°C and the treatment with 25°C used as a control. Expression levels were assessed using 28SrDNA gene for normalization. The data were analyzed by Student's t-test and presented as mean±SE of independent experiments done in triplicates and the asterisk represents the significant differences.

(25°C) and high (42°C) temperature treatments. The constitutively-expressed gene 18S rRNA was used as an internal control. The expression levels of HSP90 among different tissues treated at 25°C are not significantly different from one another with the exception of the testis, which had significantly higher levels (Figure 4). Except for fat bodies, the expression levels in the other examined tissues increased significantly compared with the control.

Prokaryotic expression of Ap-hsp90

When induced by IPTG, a recombinant protein with a molecular weight of about 86 kDa consistent with the theoretical value for HSP90 was detected (Figure 5) and its expression was not influenced by different IPTG concentrations (Figure 6).

Purification of Ap-hsp90 recombinant protein

The recombinant fusion proteins were purified by affinity chromatography and the extracts were measured by SDS-PAGE. A protein band that corresponded to the predicted molecular weight of 86 kDa was obtained according to the result (Figure 7).

In this study, a 2,482 bp cDNA sequence of HSP90 from *A. pernyi* was identified and the conserved proteinwas highly homologous to HSP90s from other Lepidoptera insects based on the phylogenetic analysis. As the result show, the *Ap*-HSP90 was extensively expressed in all examined tissues with a higher level in testis; this was consistent with the conclusion that HSP90 was highly conserved protein and has extensive biological functions (Hendrick and Hartl, 1993; Huang and Ma, 2004).

The expression level of Ap-HSP90 was obviously changed under high temperature treatment in the examined tissues, and this indicates that Ap-HSP90 is related with the tolerance to heat stress. However, the expression of Ap-HSP90 was not obviously changed in fat bodies by high temperature treatment; it is considered as the result of differences in the expression pattern of various HSPs (Li et al., 2012), species (Joy and Gopinathan, 1995; Singh and Lakhotia, 2000) or treatment (Xu et al., 2011). In addition, the recombinant Ap-HSP90 protein was successfully expressed and purified and the interaction between Ap-HSP90 and its chaperones will be further studied.

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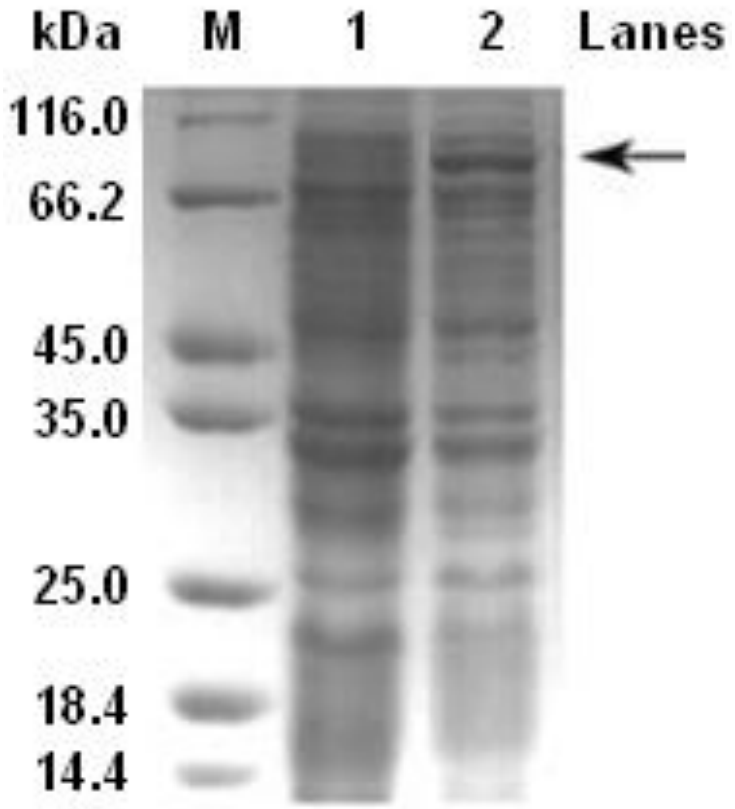

Figure 5. 12% SDS-PAGE electrophoresis of prokaryotic expression of *A. pernyi* HSP90. M, molecular weight marker; lane 1, before induction; lane 2, after induction.

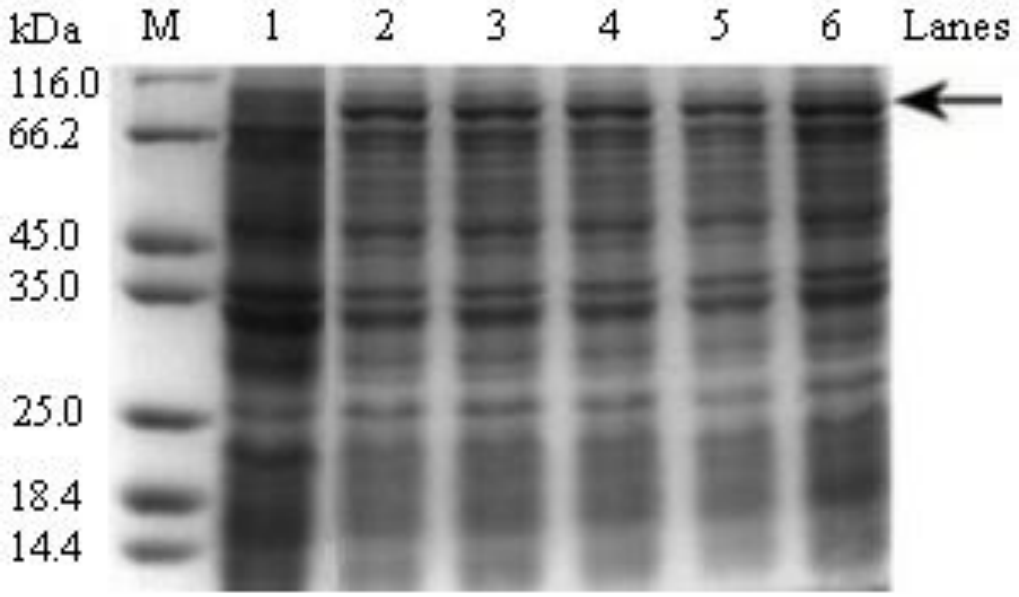

Figure 6. SDS-PAGE electrophoresis of prokaryotic expression of *A. pernyi* HSP90 induced by different IPTG concentrations. M, molecular weight marker; lane 1, before induction; lanes 2-6, after induction by 0.2, 0.4, 0.6, 0.8, 1.0 mM IPTG, respectively.

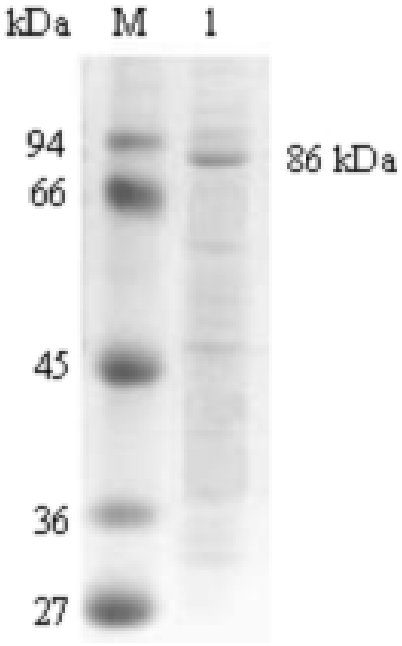

Figure 7. SDS-PAGE analysis of purified recombinant proteins. M, low molecular weight protein marker; lane 1, purified recombinant proteins.

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