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Cloning, Expression, and Cell Localization of a Novel Small Heat Shock Protein Gene: *BmHSP25.4*

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Abstract Using molecular approaches, a new member of the *Bombyx mori* small heat shock protein family was cloned and characterized. The isolated gene contains an open reading frame of 672 bp, encodes a polypeptide of 223 amino acid residues with a predicted molecular mass of 25.4 kDa, and is therefore named *BmHSP25.4*. The gene codes for a protein homologous to the previously characterized HSP20.4 and HSP19.9. Western blotting analysis revealed that BmHSP25.4 existed in the fifth-instar larva's fatty body and blood tissues. Immunohistochemistry assay also showed that BmHSP25.4 was located in the fifth-instar larva's fatty body. The results of above studies have indicated constitutive expression of *BmHSP25.4* in fatty body, blood tissues, and Bm5 cells. Finally, we examined the effect of heat stress on localization of BmHSP25.4 using anti-BmHSP25.4 polyclonal antibody by immunofluorescence. Under normal conditions, BmHSP25.4 was mostly found in the cytoplasm. However, after heat treatment, most of BmHSP25.4 distributed in the cell membrane. After 3 h of recovery following the heat shock treatment, the localization of BmHSP25.4 was the same as that under normal conditions.

Keywords BmHSP25.4 · Prokaryotic expression · Purification · Polyclonal antibody preparation · Tissue localization · Subcellular localization

Introduction

Small heat shock proteins (sHSPs) exhibit a low degree of conservation with the exception of an α-crystallin domain consisting of 80–100 amino acid residues. sHSPs with molecular

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weights ranging from 12 to 43 kDa have been found in almost all organisms studied [1, 2]. Virtually all sHSPs form high molecular weight aggregates with a molecular mass of approximately 200–800 kDa or more under conditions of cellular stress [3]. Multimerization is the result of the interaction of sHSP subunits and is believed to be crucial for chaperone activity [4–6]. As molecular chaperone, sHSP binds to the unfolded protein and maintains it in a soluble and folding competent state so that the target protein can be refolded to its native state by other ATP-dependent molecular chaperones [4, 7, 8]. sHSPs appear to be involved in many biological processes including cell proliferation and differentiation, actin polymerization, and modulation of redox parameters [7–9].

In many organisms, several members of the sHSP family are present in one cell compartment, suggesting their functional diversity. However, in some organisms, the intracellular localization varied with the specific sHSP. As examples, HSP22 of *Drosophila melanogaster* was localized to the mitochondria, while its HSP23 and HSP26 were found in the cytoplasm and HSP27 accumulated in the nucleus [10].The membrane localization of sHSPs is unusual. As examples, α-crystallin from the vertebrate lens can be also associated with plasma membrane [11]. The presence of sHSPs in membranes can regulate membrane fluidity and preserve membrane integrity during thermal fluctuations [12].

So far six *Bombyx mori* sHSPs have been recognized: HSP19.9, HSP20.1, HSP20.4, HSP20.8, HSP21.8, and HSP23.6 [13]. In this study, we identified BmHSP25.4, a new member of the sHSP family, and examined its subcellular localization in Bm5 cells and histological localization in the fifth-instar *B. mori* larvae. This research lays the foundation for further function study.

Materials and Methods

Bioinformatics Analysis

The cDNA of *BmHSP25.4* was compared to different expressed sequence tags (ESTs) and genomic databases by BLAST algorithm. Alignment of the *BmHSP25.4* cDNA and related ESTs to genomic sequences was performed with LocusLink and the UniGene EST ProfileViewer all provided by the National Center for Biotechnology Information (NCBI; Bethesda, MD, USA). The EditSeq (DNAstar) program was used to find putative open reading frames (ORFs). Multiple amino acid sequence alignment of BmHSP25.4 to its homologs was carried out with ClustalX1.8. Protein hydrophobicity profiles were determined with the ProtScale program from the Expasy server of the Swiss Institute of Bioinformatics using the Kyte and Doolittle amino acid hydropathicity scale [14]. Analysis of the potential signal peptide was performed using the SignalP prediction, and the subcellular localization was predicted by TargetP program.

Cloning and Expression of *BmHSP25.4*

The entire sequence of a cDNA containing the complete ORF of *BmHSP25.4* in pHelix was amplified by means of polymerase chain reaction (PCR) through which a *Bam*HI restriction endonuclease site was created immediately upstream of the start codon using the primer 5′-GGGGGATCCATGATCGCCTTAGTGTTGT-3′ and a *Xho*I restriction endonuclease site immediately downstream of the translational stop codon using the primer 5′-GGGCTCGAGTTAATATGGAATCGGCAAG-3′. The resulting 672-bp fragment was digested with *Bam*HI and *Xho*I (Promega, USA) at 37 °C for 2 h [15], cloned into

pET28a(+) expression vector (Invitrogen, Carlsbad, CA, USA) and verified by DNA sequencing. Recombinant BmHSP25.4, which had six histidines at the N-terminal end, was expressed in *Escherichia coli* BL21(DE3) cells and purified by nickel affinity column chromatography [16]. The purified recombinant protein was determined by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and Coomassie Brilliant Blue R-250 staining.

Antibody

A male New Zealand white rabbit was immunized with 400 μg of purified recombinant protein emulsified with Freund's complete adjuvant (Sigma) [16]. Three booster doses were given at intervals of 7 days with the same amount of antigen and Freund's incomplete adjuvant (Sigma). After 1 month, blood was collected, and the serum fraction was isolated. The antibody was purified by protein A chromatography. This purified antibody was used for immunoblotting, immunohistochemistry, and immunofluorescence.

Western Blot Analysis

Head, blood, Malpighian tubule, epidermis, silk glands, midgut, fatty body extracts from the fifth-instar larvae of *B. mori* and Bm5 cells extracts were analyzed by Western blot. The extracts were loaded onto 10% SDS-polyacrylamide gel, electrophoresed, and transferred to Immobilon-P membranes (Millipore) electrophoretically. The blots were incubated in blocking buffer containing 5% skim milk, and 0.05% Tween 20 in TBS (TBST; pH 7.5) at room temperature for 1 h or 4 °C overnight and then were incubated with anti-BmHSP25.4 antibody at 1/50 in blocking buffer at room temperature for 1 h or 4 °C overnight. The blots were washed with 0.05% TBST three times of 5 min and reacted with horseradish-peroxidase-conjugated goat antirabbit immunoglobulin G (IgG; Bio-Rad Laboratories) at 1/500 in blocking buffer for 1 h. The blots were washed with TBST four times and then stained with diaminobenzidine method.

Immunohistochemistry

The fifth-instar larvae of *B. mori* on day 3 was embedded in ornithine transcarbamylase freezing medium, frozen immediately in liquid nitrogen, sectioned into 10-μm thickness with a cryostat microtome and thaw-mounted on a glass slide. The slides were fixed in acetone for 30 s at room temperature, washed three times in phosphate-buffered saline (PBS), incubated in blocking buffer containing 3% bovine serum albumin (BSA) and 0.1% Tween 20 in PBS (PBST; pH 7.4) at room temperature for 30 min, then were incubated with anti-BmHSP25.4 antibody at 1/1,000 in blocking buffer at room temperature for 1 h or 4 °C overnight, washed with PBST three times for 5 min, reacted with Cy3-conjugated antirabbit IgG (Proteintech) at 1/500 in blocking buffer at room temperature for 40 min, and washed with PBST three times for 5 min. The stained sections were observed by fluorescence microscopy.

Cell Culture and Heat Treatment

Bm5, silkworm ovary cell line, was maintained at subconfluent densities (60–70%) in TC-100 (Sigma, USA) supplemented with 10% fetal calf serum (Sigma) at 27 °C in a humidified atmosphere. For heat stress, Bm5 cells were incubated at 39 °C in a water bath for 3 h. For

recovery study, heat-stressed cells were incubated at 27 °C in humidified chamber for 3 h. Cell viability, as assessed by Trypan blue staining, was greater than 95%.

Indirect Immunofluorescence Assays

Bm5 cells grown on confocal dishes were given treatment as described above. Normal cells and the treated cells were washed two times with PBS and then fixed in 4% formaldehyde for 30 min and permeabilized with 0.1% Triton X-100 in PBS for 10 min. After being washed three times with PBS, cells were blocked with 3% BSA and 0.05% PBST at 37 °C for 30 min, then were incubated with anti-BmHSP25.4 antibody at 1/200 in blocking buffer at 37 °C for 1 h or 4 °C overnight, washed with 0.05% PBST three times for 5 min, reacted with Cy3-conjugated antirabbit IgG (Proteintech) at 1/500 and DAPI nuclear counterstain at 1/1,000 in blocking buffer at 37 °C for 30 min, and washed with PBST three times for 5 min. Confocal imaging was performed with a Nikon C1si confocal microscope. To excite the DAPI fluorochrome (blue), a 353-nm laser line generated by PMT1 was used, and for the Cy3 fluorochrome (red), a 550-nm laser line from PMT2 was used. The localization of BmHSP25.4 in Bm5 cells was determined by the distribution of fluorescence.

Results

Bioinformatics Analysis

The cDNA sequence of *BmHSP25.4* (accession no. EU401668) contained an ORF of 672 bp encoding a polypeptide of 223 amino acids with a predicted molecular mass of 25.4 kDa and a theoretical pI of 4.995 (Fig. 1). GenBank searches revealed that its ESTs mainly existed in *B. mori* fatty body, rarely in testis and ovary. Structure analysis of the cDNA of *BmHSP25.4* was found to contain one exon. By sequence alignment, BmHSP25.4 had 97% identity with HSP20.4 (*B. mori*), 80% identity with HSP19.9 (*B. mori*). By domain search, we could identify its partial structure highly similar to the alpha-crystallin domain. Consequently, we aligned BmHSP25.4 with six *B. mori* sHSPs and discovered its reasonably high homology with them (Fig. 2). Analysis of the potential signal peptide and subcellular localization indicated that BmHSP25.4 contained a signal peptide at the N-terminal, and it mostly centralized in secretory pathway.

Production of Recombinant Protein

The complete ORF of *BmHSP25.4* was amplified by PCR and inserted into the pET28a(+) expression vector. Following transformation of *E. coli* BL21(DE3) cells with the pET28a-*BmHSP25.4* construct, isopropyl β-D-1-thiogalactopyranoside (IPTG) was added to the culture medium to induce the expression of recombinant protein. Recombinant protein was detected in bacterial lysates by SDS-PAGE after 4 h (Fig. 3). Bacterial lysates were subjected to nickel affinity column chromatography to purify recombinant protein (Fig. 3, lane 5).

Western Blot Analysis

Rabbit antiserum was raised against purified recombinant protein, and the anti-BmHSP25.4 antibody was purified by protein A chromatography. Purified anti-BmHSP25.4 antibody

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      M  I  A  L  V  L  C   G  L  L   A  A  V   S  A  A  P   Q  Y  Y
1     ATGATCGCCT TAGTGTTGTG CGGACTGCTG GCGGCGGTCT CGGCCGCGCC ACAGTACTAC
      H  G  S  S  H  W  P   Y  H  H   Y  D  P   F  S  P  Y   V  R  E
61    CATGGCTCGT CACATTGGCC GTATCACCAT TACGACCCCT TCAGTCCTTA CGTTCGGGAA
      S  M  L  D  T  H  S   L  W  S   N  L  A   N  E  M  Q   H  L  D
121   AGCATGTTGG ACACACATTC GCTTTGGTCC AACCTTGCCA ACGAAATGCA ACACTTGGAC
      N  M  M  K  E  L  S   L  K  F   P  S  I   I  N  E  G   R  V  E
181   AACATGATGA AGGAGCTGTC GTTGAAGTTC CCCAGCATTA TAAACGAAGG ACGCGTGGAA
      G  D  K  Y  Q  I  S   I  H  L   P  G  Y   E  Q  K  D   I  N  V
241   GGCGACAAGT ATCAGATATC TATTCACCTG CCTGGTTACG AACAGAAAGA CATCAACGTG
      K  A  K  N  G  V  L   M  V  Q   A  N  S   A  F  N  H   Y  L  K
301   AAAGCGAAAA ATGGAGTGCT GATGGTGCAG GCTAACAGTG CTTTTAATCA TTACTTGAAA
      I  Q  N  L  P  W  D   V  N  S   E  G  S   W  V  Y  E   K  D  V
361   ATACAGAACC TTCCCTGGGA TGTGAATTCC GAAGGCAGCT GGGTTTACGA GAAAGACGTG
      L  K  I  T  F  P  L   K  Q  K   Q  P  E   D  S  K  R   P  V  A
421   TTGAAAATCA CCTTCCCGCT GAAGCAAAAG CAGCCAGAGG ATAGCAAGAG GCCAGTTGCA
      E  P  T  E  T  T  S   T  N  V   S  R  E   E  M  E  F   T  T  E
481   GAGCCCACTG AGACGACCTC TACGAATGTA AGTCGTGAAG AGATGGAGTT CACCACCGAG
      S  N  V  R  D  V  D   V  G  L   E  T  A   Q  K  T  N   E  I  A
541   AGCAACGTGC GGGACGTTGA CGTCGGCTTG GAGACAGCCC AGAAGACCAA TGAGATCGCG
      K  A  V  E  A  T  T   Y  A  V   N  I  R   D  D  A  E   F  L  P
601   AAAGCTGTAG AAGCGACCAC GTACGCTGTC AACATCAGAG ACGATGCGGA GTTCTTGCCG
      I  P  Y  *
661   ATTCCATATT AA
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Fig. 1 Nucleotide and putative amino acid sequence of *BmHSP25.4* cDNA. Nucleotide numbering is shown on the *left-hand side*

was used to confirm the expression of BmHSP25.4 in different tissues (head, blood, Malpighian tubule, epidermis, silk glands, midgut, fatty body) of fifth-instar larvae of *B. mori* by immunoblotting (Fig. 4). Positive signals were only found in fatty body and blood. And BmHSP25.4 could be also detected in Bm5 cell extracts by using anti-BmHSP25.4 antibody (Fig. 5). The anti-BmHSP25.4 antibody recognized a single band by immunoblotting, indicating that the purified antibody is highly specific.

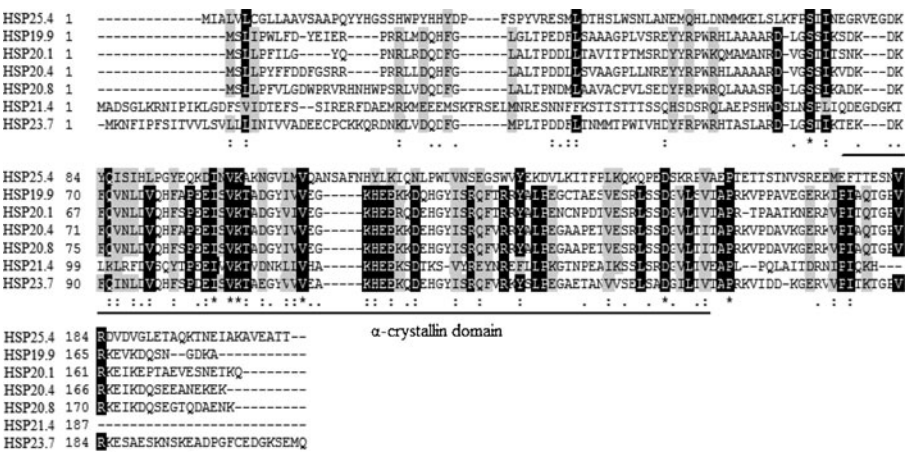

Fig. 2 Alignment of BmHSP25.4 with six known *B. mori* sHSPs. Amino acid sequences of HSP19.9 (accession no. BAD74195.1), HSP20.1 (accession no. BAD74196.1), HSP20.4 (accession no. AAG30945.2), HSP20.8 (accession no. AAG30944.1), HSP21.4 (accession no. BAD74197.1), HSP23.7 (accession no. BAD74198.1), and BmHSP25.4 were aligned with ClustalX1.8. *Black-shaded* amino acids indicate identical residues; *gray-shaded* amino acids indicate similar residues

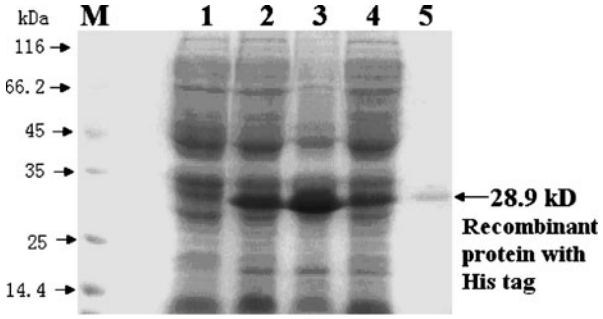

Fig. 3 Expression and purification of recombinant protein. Lysates prepared from *E. coli* cells overexpressing BmHSP25.4 were passed over a nickel affinity column. Proteins were analyzed by SDS-PAGE and visualized by Coomassie Brilliant Blue R-250 staining. M contains molecular weight markers with the masses shown, in kilodalton, to the *left* with *arrows*. *Lanes 1* and *2* show proteins isolated from uninduced and induced (4 h after IPTG induction) *E. coli* cells. *Lanes 3* and *4* show the lysate precipitation and supernatant after induction with IPTG for 4 h. *Lane 5* shows the purified protein

Immunohistochemistry

As shown in Fig. 4, the anti-BmHSP25.4 antibody positively stained the fifth-instar larvae of *B. mori*. So immunohistochemistry assay and cryosectioning method were utilized to find its histological localization in various tissues. The fatty body was strongly stained. The normal rabbit serum showed negative staining (Fig. 6).

Immunolocalization of BmHSP25.4

Immunostaining with anti-BmHSP25.4 antibody shows that BmHSP25.4 was mostly found in the cytoplasm under normal conditions (Fig. 7a). To determine the localization of BmHSP25.4 upon heat shock and subsequent recovery, we have incubated Bm5 cells at 39 °C for 3 h, followed by recovery at 27 °C for 3 h. Cells were fixed immediately after heat stress or recovery and immunostained with anti-BmHSP25.4 antibody. It is evident from Fig. 7b that, after the heat stress, BmHSP25.4 exclusively presented in the cell membrane with little or no staining in the cytoplasm, indicating that BmHSP25.4 translocated from cytoplasm to cell membrane upon heat shock. As shown in Fig. 7c, BmHSP25.4 was detected in cytoplasm after 3 h of recovery from heat shock, exhibiting a staining pattern similar to that in unstressed cells.

Discussion

Normally, small heat shock proteins genes do not have intron. This essential characteristic guarantee to produce mature mRNA without splicing after transcription initiation can prevent the effect of serious heat shock on pre-mRNA splicing and meet the need of rapid

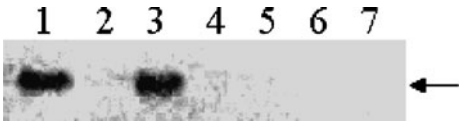

Fig. 4 The expression level of BmHSP25.4 in different tissues of fifth-instar larva of *B. mori*. *Lane 1* fatty body; *lane 2* epidermis; *lane 3* blood; *lane 4* silk glands; *lane 5* midgut; *lane 6* Malpighian tubule; *lane 7* head. The *arrow* indicated the position of BmHSP25.4

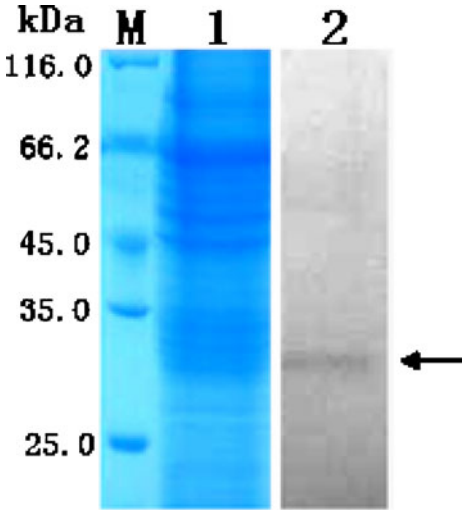

Fig. 5 Western blotting of Bm5 cell extracts. Protein extracts from Bm5 cells were analyzed. *Lane 1*: Bm5 cell extracts (SDS-PAGE); *lane 2*: Bm5 cell extracts (Western blotting). The *arrow* indicated the target bands

and large-scale expression of small heat shock proteins [17]. *BmHSP25.4* selected from silkworm pupa cDNA library has only one extron, but no intron. And no nucleotide sequences similar to *BmHSP25.4* were found in NCBI. To sum up, we believe that BmHSP25.4 is a new member of the sHSP family.

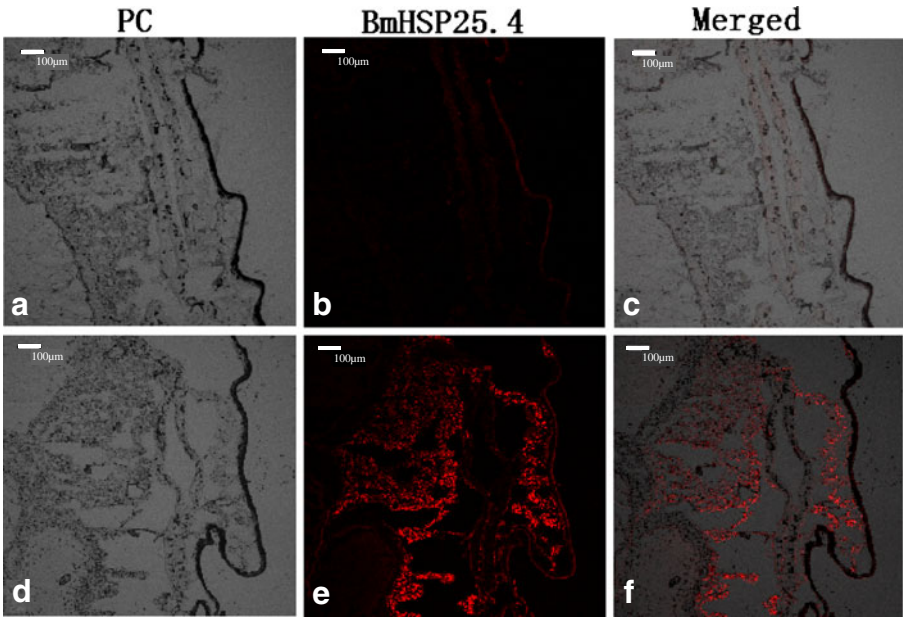

Fig. 6 Immunohistochemical localization of BmHSP25.4 in fifth-instar larvae of *B. mori*. Frozen sections of larvae were stained with anti-BmHSP25.4 antibody and then with Cy3-conjugated goat antirabbit IgG. **a**–**c** No positive staining by normal rabbit serum. **d**–**f** The positive staining at fatty body by rabbit antiserum. The *red color* indicates the positive reaction. *PC* phase-contrast images. *Scale bar* 100 μm

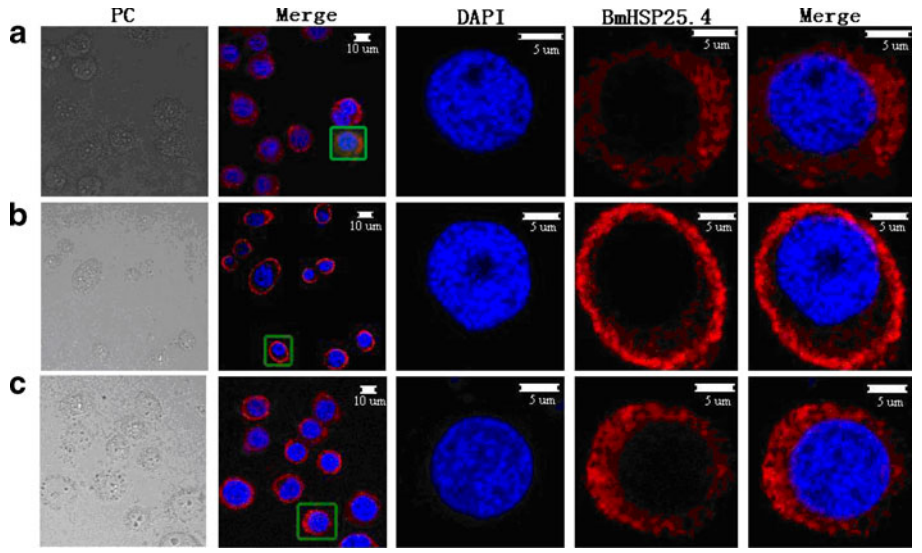

Fig. 7 Indirect immunofluorescence localization of BmHSP25.4 in Bm5 cells. Bm5 cells were stained with anti-BmHSP25.4 antibody (*red*) to evidence localization of BmHSP25.4 as well as DAPI (*blue*) to visualize nuclei. **a** Unstressed cells (under normal conditions), **b** stressed cells (39 °C, 3 h), and **c** 3 h after recovery at 27 °C. *PC* phase-contrast images. *Scale bar* 10 μm, 5 μm

In this study, we made some experiments on *BmHSP25.4* to analyze its characteristics and functions.

Proteins from head, blood, Malpighian tubule, epidermis, silk glands, midgut, and fatty body from the fifth-instar larvae of *B. mori* were extracted and analyzed by Western blotting. The positive signals were detected in larva's fatty body and blood tissues but not in head, Malpighian tubule, epidermis, silk glands, and midgut. One possibility is that the expression of *BmHSP25.4* in these tissues is too little to be detected, or there is no expression in these tissues. We are apt to the latter because the result of the expression profile analysis showed that fatty body is the specific tissue for *BmHSP25.4* expression. Some researches have shown that most proteins expressed in fatty body are secretory proteins which could be secreted into blood [18]. Bioinformatics analysis showed that BmHSP25.4 may be a secretory protein. We presumed BmHSP25.4 expressed in fatty body can be secreted into blood to exert its function.

The expression of small heat shock proteins is very important for heat tolerance, and small heat shock proteins can act as molecular chaperones on the processes of protein folding, aggregation, membrane transportation, and degradation under stresses. But not all heat shock proteins have the same expression pattern, since some heat shock proteins can express under normal condition which is called heat shock cognate (HSC) proteins. The result of Western blotting showed that BmHSP25.4 can express under normal conditions, so it is a HSC. This implies that BmHSP25.4 could perform the function of molecular chaperone under normal condition.

Finally, we carried out subcellular localization of BmHSP25.4 in Bm5 cell. Under normal conditions, BmHSP25.4 was mostly found in cytoplasm. However, after Bm5 cell had been treated with heat shock of 39 °C for 3 h, most BmHSP25.4 distributed in the cell membrane. After 3 h of recovery following the heat shock treatment, the localization of BmHSP25.4 was the same as under normal conditions. The experimental results indicated

that BmHSP25.4 can migrate from cytoplasm to membrane under heat stress. According to the previous studies [4, 7, 8, 11, 12], we inferred that BmHSP25.4 can associate onto cell membrane and participate in membrane protein refolding, maintain membrane fluidity, and keep the integrity of cell membrane to play an important role in biological process. Through the experiments, the distribution of BmHSP25.4 is observed in Bm5 cells under heat stress, and this will be beneficial to understand the cellular responses to heat stress. The biological function of BmHSP25.4 should be further studied.

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