

## RESEARCH

# BmHSP20.8 is Localized in the Mitochondria and has a Molecular Chaperone Function In Vitro

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**ABSTRACT.** Heat shock proteins (HSPs) are abundant and ubiquitous in almost all organisms from bacteria to mammals. BmHSP20.8 is a small (sHSP) in *Bombyx mori* that contains a 561 bp open reading frame that encodes a protein of 186 amino acid residues with a predicted molecular mass of 20.8 kDa. The subcellular localization prediction indicated that BmHSP20.8 is likely distributed in the mitochondria with a 51% probability. To identify the subcellular localization of BmHSP20.8, three recombinant vectors were constructed and used to transfect BmN cells. The cytoplasmic and mitochondrial proteins were extracted 72 h after transfection. The Western blot showed that recombinant BmHSP20.8 exists only in the mitochondria. To locate the mitochondrial localization signal domain of BmHSP20.8 more accurately, we cloned four truncated recombinant vectors. The Western blot analysis of the cytoplasmic and mitochondrial proteins showed that the mitochondrial localization signal domain of BmHSP20.8 is located between amino acids 143 to 186. We constructed the pETduet-HIS-SUMO-BmHSP20.8 vector and a soluble BmHSP20.8 was expressed. In a citrate synthase (CS) thermal aggregation experiment, we found that the recombinant BmHSP20.8 protein can protect CS from aggregating at 43 and 48°C and thus exhibited molecular chaperone activity. Taken together, the results showed that BmHSP20.8 could be a mitochondrial protein and has a molecular chaperone activity, suggesting an important role in mitochondria.

**Key Words:** *Bombyx mori*, BmHSP20.8, mitochondria, molecular chaperone, subcellular localization

The heat shock protein (HSP) was first observed in *Drosophila* (Ritossa 1962). Subsequent studies suggested that HSPs are abundant and ubiquitous in almost all organisms from bacteria and unicellular algae to higher organisms such as humans (Kim et al. 1998; Waters and Rioflorida 2007; Aebermann and Waters 2008; Waters et al. 2008). HSPs are either expressed under extreme physiological conditions (Haslbeck et al. 2005) or induced in response to chemical or physical stressors, such as high temperature (Vierling and Kimpel 1992; Nover and Scharf 1997; Narberhaus, 2002). HSPs are usually divided into several classes, including HSP100, HSP90, HSP70, HSP60, and small HSP (sHSP), according to their molecular weight and homologous relationship. Among these HSP families, little is known about the sHSP class due to its wide molecular mass range of 15 to 42 kDa and low homology. However, the primary structure of an sHSP can be dissected into three major parts of functional importance: an N-terminal region, the conserved  $\alpha$ -crystallin domain, and a C-terminal region (Van Montfort et al. 2001a). The  $\alpha$ -crystallin domain represents the signature motif of the sHSP class and is necessary for dimer formation. This domain is a basic building block of oligomers. Both flanking regions promote the formation of higher-order structures (Van Montfort et al. 2001a). The N-terminal regions are highly variable in both sequence and length. The diverse N-terminal sequences may be associated with the diverse expression, function, and evolutionary patterns observed among sHSPs. These appear to be flexible, and variants lacking the N-terminal regions are, in most cases, defective in the formation of higher oligomers (Haslbeck et al. 2004; Stromer et al. 2004; Giese et al. 2005). In all crystal structures, the C-terminal region that follows the  $\alpha$ -crystallin domain is involved in stabilizing the oligomer (Van Montfort et al. 2001b). The comparison of the assemblies indicates that, although the contact site of the C-terminal region with a neighboring  $\alpha$ -crystallin domain appears to be conserved, the orientation of the

C-terminal region remains flexible (Carver et al. 1995; Lindner et al. 2000). The conserved C-terminal sequence plays a significant role in sustaining the chaperone functions and other roles of sHSPs in cells or organisms.

HSPs are evolutionarily conserved in all organisms that have been studied to date and are found in all cellular compartments. The functions of HSP are the following: (1) interaction with nascent proteins that are not fully modified as molecular chaperones to maintain their folding/unfolding status and to regulate their appropriate cellular compartmentalization; (2) minimization or aggregation of nonnative proteins or targeting of damaged proteins for hydrolysis and removal from the cell (Becker and Craig 1994; Haslbeck 2002; Stengel et al. 2010); (3) stabilization of cytoskeletal structure; (4) maintenance of normal cell function (Bruemmer-Smith et al. 2001; Wettstein et al. 2012); (5) protection of organelle structure and function (in particular chloroplasts and mitochondria) (Sun and MacRae 2005); (6) maintenance of cell membrane stability (Torok et al. 2001; Vigh et al. 2007); (7) improvement of bodily tolerance against a variety of stresses (Rinehart et al. 2006; Seo et al. 2006); (8) involvement in cell growth, development, and differentiation; and (9) protection of RNA from processing and splicing (Jakob and Buchner 1994).

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 varies depending on the specific sHSP. For example, HSP22 of *Drosophila melanogaster* and HSP23.5 of *Arabidopsis thaliana* are localized in the mitochondria (Visioli et al. 1997), whereas HSP23 and HSP26 of *D. melanogaster* are found in the cytoplasm, and HSP27 is accumulated in the nucleus (Michaud et al. 1997). The localization of sHSPs in the membrane is unusual. The presence of sHSPs in membranes can regulate membrane fluidity and preserve membrane integrity during thermal

fluctuations (Michaud et al. 1997; Tsvetkova et al. 2002). The prediction of its subcellular localization indicated that BmHSP20.8 is likely distributed in the mitochondria with a 51% probability. In the present work, we further studied the subcellular localization regulation and molecular chaperone activity of BmHSP20.8. A clear understanding of the mechanism of sHSP action is, therefore, critical to many aspects of cell function in normal, stressed, or diseased states.

## Materials and Methods

**Cells and Materials.** The silkworm ovary cells of line BmN were cultured in our laboratory. Restriction endonuclease, T4 DNA ligase, and Taq and T4 DNA polymerases were obtained from TaKaRa Biotechnology (Dalian, China). Goat antimouse IgG (HRP) antibody was obtained from Biosharp (USA). FuGENE 6 transfection reagent and mouse antiHis6 antibody were purchased from Roche Diagnostics (Mannheim, Germany). Sf-900II medium and fetal bovine serum (FBS) were obtained from Gibco (USA). The cell culture plates were purchased from Corning Incorporated (New York). The Ni-NTA purification system was obtained from Invitrogen (USA). The cytoplasmic and mitochondrial protein extraction kit was purchased from Sangon Biotech (Shanghai, China).

### Construction of Eukaryotic and Prokaryotic Expression Vectors

**Construction of the pIEx-1-BmHSP20.8/BmHSP20.8-EGFP/EGFP-BmHSP20.8 Eukaryotic Expression Vectors.** The recombinant vectors pIEx-1-BmHSP20.8 and pIEx-1-EGFP-BmHSP20.8 were constructed by inserting the *BmHSP20.8* gene fragment into the *Pst* I/*Xho* I sites of pIEx-1 and pIEX-1-EGFP, which were constructed in-house (Yang et al. 2012). *BmHSP20.8* was amplified from silkworm DNA complementary to RNA (cDNA) by polymerase chain reaction (PCR) using the primers 5'-AACTGCAGATGTCTCTTCTACCA TTC-3' and 5'-CCCTCGAGCTACTTATTTTCGGC-3' (the *Pst* I and *Xho* I sites are underlined, respectively). The recombinant vector pIEx-1-BmHSP20.8-EGFP was generated by inserting the *BmHSP20.8* gene fragment into the *Xcm* I/*Pfi* MI sites of pIEx-1-EGFP. *BmHSP20.8* was amplified by PCR using the primers 5'-CCATCACTCTTCTGGTTAT GTCTCTTCTACCATTC-3' and 5'-CCAGCACATGGCTTATTTTCGGC-3' (the *Xcm* I and *Pfi* MI sites are underlined, respectively). PCR was performed under the conditions in Supp Material Table 1 (online only).

**Construction of Truncated Eukaryotic Expression Vectors.** To identify the location of the mitochondrial localization signal of BmHSP20.8, we constructed four truncated recombinant vectors: pIEx-1-EGFP-(41-186)BmHSP20.8, pIEx-1-EGFP-(77-186)BmHSP20.8, pIEx-1-EGFP-(112-186)BmHSP20.8, and pIEx-1-EGFP-(143-186)BmHSP20.8. These truncated fragments of *BmHSP20.8* were amplified using the primers shown in Table 1, which contained *Pst* I and *Hind* III sites. PCR was performed under the conditions in Supp Material Table 1 (online only).

**Construction of pETduet-HIS-SUMO-BmHSP20.8 Prokaryotic Expression Vector.** The recombinant vector pETduet-HIS-SUMO-BmHSP20.8 was constructed by inserting the *BmHSP20.8* gene fragment into the *Bam* HI/*Xho* I sites of pETduet-HIS-SUMO. *BmHSP20.8*

was amplified by PCR using the primers 5'-CACGGATCC ATGTCTCTTCTACCATTCGTG-3' and 5'-CCCTCGAGCTACTTA TTTTCGGC-3' (the *Bam* HI and *Xho* I sites are underlined, respectively). PCR was performed under the conditions in Supp Material Table 1 (online only).

**Eukaryotic Expression of Enhanced Green Fluorescent Protein (EGFP), BmHSP20.8, and Their Fusion Proteins in BmN Cells.** To express enhanced green fluorescent protein (EGFP), BmHSP20.8, and their fusion proteins (BmHSP20.8-EGFP and EGFP-BmHSP20.8), the constructed plasmids, pIEX-1-EGFP, pIEx-1-BmHSP20.8, pIEx-1-BmHSP20.8-EGFP and pIEx-1-EGFP-BmHSP20.8, were transfected into BmN cells, respectively. Three microliters of FuGENE 6 transfection reagent and 5 µg of each plasmid were added to 97 µl of serum-free medium, and the mixture was incubated for 15 min at room temperature and added to BmN cells in serum-free medium for 8 h. The cells were then cultured at 27°C for 72 h in Sf-900 II SFM medium containing 10% (v/v) FBS. The transfected BmN cells were observed with a fluorescence microscope and then harvested. The expressed EGFP, BmHSP20.8, and fusion proteins were identified through Western blot using the HIS monoclonal antibody.

### Identification of Mitochondrial Localization of BmHSP20.8 in BmN Cells.

The BmN cells transfected with the above-described constructed vectors were harvested after culture at 27°C for 72 h. The cells were washed with PBS twice. Cytoplasmic extraction buffer (1 ml) was added to the harvested cells, and the cells were then homogenized in a glass homogenizer 40–50 times. The homogenate was then transferred into a new centrifuge tube, vortexed vigorously for 15 s, incubated for 30 min on ice, and centrifuged at 3,000 rpm and 4°C for 10 min. The supernatant was transferred into a new centrifuge tube and centrifuged at 12,000 rpm and 4°C for 30 min to pellet the mitochondria, leaving the cytoplasmic proteins in the supernatant. The collected mitochondria were lysed with mitochondria dissolution buffer for 30 min on ice. The lysate was then centrifuged at 13,000 rpm and 4°C for 10 min to retain the mitochondrial proteins in the supernatant. The cytoplasmic and mitochondrial protein preparations were subjected to Western blot with the HIS monoclonal antibody to determine the localization of BmHSP20.8 in BmN Cells.

### Identification of the Mitochondrial Localization Signal Domain of BmHSP20.8.

The four truncated eukaryotic expression vectors were transfected into BmN cells. The cells were harvested after culture at 27°C for 72 h, and the cytoplasmic and mitochondrial proteins were then extracted. The cytoplasmic and mitochondrial protein preparations were subjected to Western blot with the HIS monoclonal antibody to determine the mitochondrial localization signal domain of BmHSP20.8.

### Analyses of the Molecular Chaperone Activity of BmHSP20.8.

The recombinant expression vector pETduet-HIS-SUMO-BmHSP20.8 was used to transform *Escherichia coli* BL21 cells. The expression of the recombinant protein was induced using IPTG (Sanland-chem, USA) at a final concentration of 0.1 mM. The bacterial pellets were thawed, resuspended in lysis buffer, and then lysed by pulsed sonication on ice. The lysates were centrifuged at 14,000 rpm and 4°C for 10 min. After centrifugation, the supernatant was electrophoresed by SDS-PAGE. The purification of the protein was conducted using the Ni-NTA kit according to the manufacturer's instructions. The purified SUMO-BmHSP20.8 was digested by ULP1 to obtain BmHSP20.8.

To determine whether or not BmHSP20.8 has molecular chaperone activity, a thermal aggregation experiment using citrate synthase was performed. Citrate synthase (CS, 0.5 mg/ml) and BmHSP20.8 were mixed together in 20 mmol/l Tris-Cl (pH 8.0) buffer, and the mixture was incubated at 48°C for 15 min. CS in the absence of BmHSP20.8 was used as a negative control. After incubation, the mixture was centrifuged at 15,000 rpm for 15 min. The supernatant and pellet were electrophoresed using SDS-PAGE. To verify the inhibition efficiency, we also incubated the mixture at 43, 53, and 58°C for 15 min.

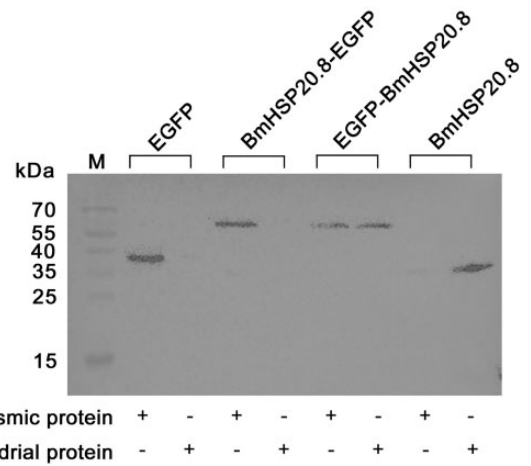
**Table 1. Primers for PCR amplification of truncated *BmHSP20.8* genes**

Truncated genes	Primers	Sequences
(41-186) <i>BmHSP20.8</i>	Forward	5'-AACTGCAGATGGTGCCTGTCCAGTG-3'
	Reverse	5'-CCCAAGCTTCTACTTATTTTCGGCATCCTG-3'
(77-186) <i>BmHSP20.8</i>	Forward	5'-AACTGCAGATGGTCAATCTGGACGTGCAG-3'
	Reverse	5'-CCCAAGCTTCTACTTATTTTCGGCATCCTG-3'
(112-186) <i>BmHSP20.8</i>	Forward	5'-AACTGCAGATGGGGTATATTTCAAGGCAG-3'
	Reverse	5'-CCCAAGCTTCTACTTATTTTCGGCATCCTG-3'
(143-186) <i>BmHSP20.8</i>	Forward	5'-AACTGCAGATGCTCACCATCACCAGC-3'
	Reverse	5'-CCCAAGCTTCTACTTATTTTCGGCATCCTG-3'

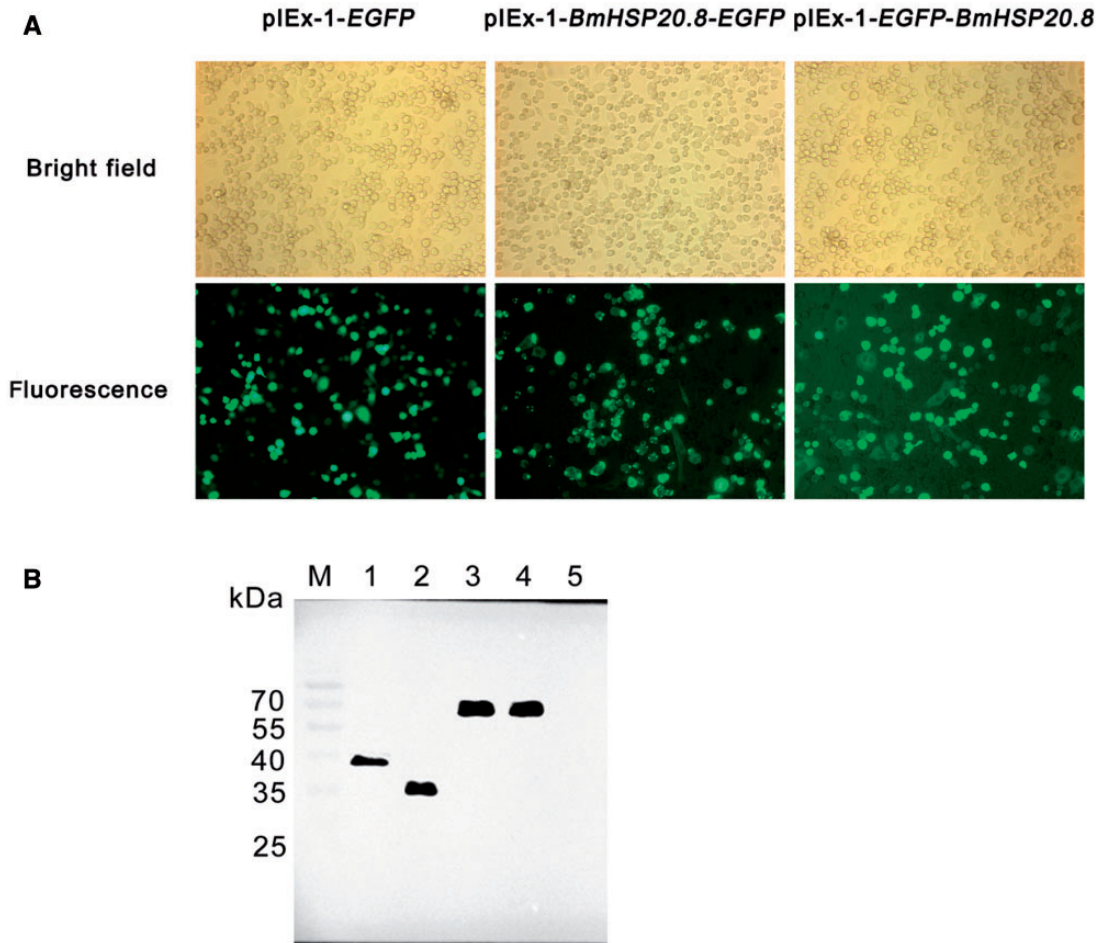
**Results**

**Construction of Eukaryotic and Prokaryotic Expression Vectors.** A series of expression vectors were successfully constructed in the present study. The eukaryotic expression vectors pIEx-1-BmHSP20.8, pIEx-1-BmHSP20.8-EGFP, and pIEx-1-EGFP-BmHSP20.8 were generated for the identification of the subcellular localization of BmHSP20.8 by inserting *BmHSP20.8* gene fragments into the corresponding sites of the pIEx-1 and pIEx-1-EGFP vectors (Supp Material Fig. 1 [online only]). The truncated eukaryotic expression vectors were generated for the identification of the localization sequence by inserting the truncated *BmHSP20.8* gene fragments into the corresponding sites of the pIEx-1-EGFP vector (Supp Material Fig. 2 [online only]). The prokaryotic expression vector pETduet-HIS-SUMO-BmHSP20.8 was generated for the expression of soluble BmHSP20.8 (Supp Material Fig. 3 [online only]). The above-mentioned recombinant plasmids were all verified by PCR amplification and double digestion with the corresponding restriction enzymes.

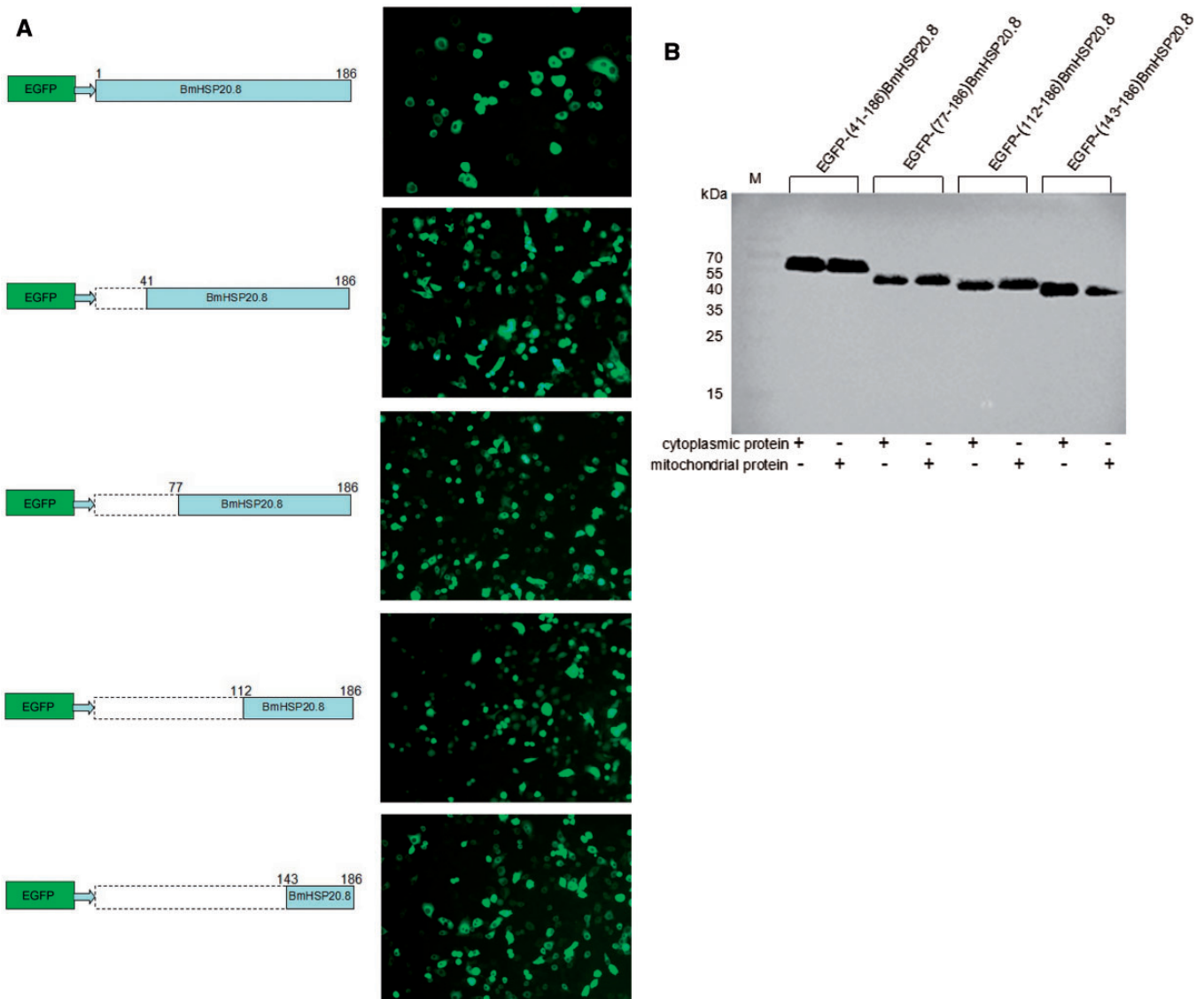
**Eukaryotic Expression of EGFP, BmHSP20.8, and Their Fusion Protein in BmN Cells.** EGFP, BmHSP20.8, and their fusion proteins BmHSP20.8-EGFP and EGFP-BmHSP20.8 were successfully expressed at a high level in BmN cells. A strong green fluorescence was observed in BmN cells transfected with the pIEx-1-EGFP, pIEx-1-EGFP-BmHSP20.8, and pIEx-1-BmHSP20.8-EGFP plasmids (Fig. 1A). Their expression was further investigated using Western blot with the HIS monoclonal antibody (Fig. 1B).



**Fig. 2.** The determination of mitochondrial localization of BmHSP20.8 protein in BmN cells. EGFP, BmHSP20.8 and their fusion protein, BmHSP20.8-EGFP and EGFP-BmHSP20.8, were identified in the cytoplasmic or mitochondrial portion of BmN cells by western blotting using the HIS monoclonal antibody. The recombinant BmHSP20.8 and EGFP-BmHSP20.8 but not BmHSP20.8-EGFP could be identified in mitochondrial extracts, suggesting mitochondrial localization and a localization signal domain in the C-terminal of BmHSP20.8.



**Fig. 1.** The eukaryotic expression of EGFP, BmHSP20.8, and their fusion proteins. (A): Fluorescence microscopy images of EGFP and EGFP-fused proteins expressed in BmN cells; (B): Expression of EGFP, BmHSP20.8 and their fusion proteins BmHSP20.8-EGFP and EGFP-BmHSP20.8, in BmN cells as identified by western blotting. M: Prestained Marker; 1: HIS-EGFP protein; 2: HIS-BmHSP20.8 protein; 3: HIS-BmHSP20.8-EGFP fusion protein; 4: HIS-EGFP-BmHSP20.8 fusion protein; 5: normal BmN cells.

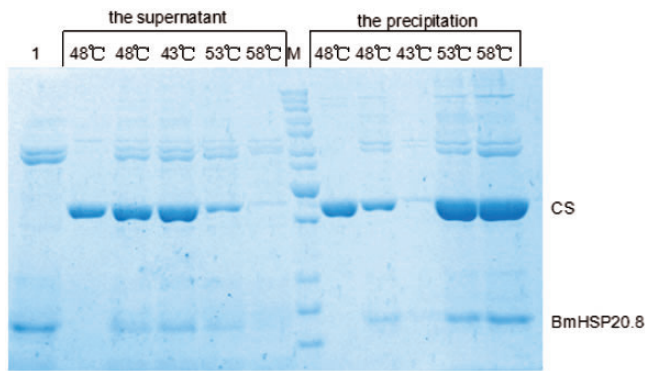


**Fig. 3.** The determination of mitochondrial localization signal domain of BmHSP20.8. **(A):** The expression cassette of EGFP-fused truncated BmHSP20.8 and their expression in BmN cells. The green fluorescence of EGFP-truncated BmHSP20.8 proteins could be observed under the fluorescence microscope. **(B):** The mitochondrial localization of EGFP-truncated BmHSP20.8 proteins in BmN cells identified by western blotting. The EGFP-(41-186)BmHSP20.8, EGFP-(77-186)BmHSP20.8, EGFP-(112-186)BmHSP20.8, and EGFP-(143-186)BmHSP20.8 protein were all identified in the mitochondrial portion of BmN cells suggesting a localization signal domain in the range of amino acids from 143 to 186 in BmHSP20.8.

**Identification of the Mitochondrial Localization Sequence of BmHSP20.8 in BmN Cells.** The prediction of the subcellular localization of BmHSP20.8 using bioinformatic method indicated that BmHSP20.8 may be distributed in the mitochondria with a 51% probability. Therefore, we extracted the cytoplasmic and mitochondrial proteins of BmN cells in which recombinant BmHSP20.8, BmHSP20.8-EGFP, and EGFP-BmHSP20.8 were overexpressed. The Western blot results showed that the BmHSP20.8 and the fusion protein EGFP-BmHSP20.8 were detected in the mitochondrial protein extracts. However, the expressed EGFP (control) and BmHSP20.8-EGFP were detected in the cytoplasmic extract and not in the mitochondrial extract, suggesting that BmHSP20.8 may be localized in the mitochondria and that the mitochondrial localization signal domain of BmHSP20.8 is near the C terminus of BmHSP20.8 (Fig. 2). It is possible that the fusion protein BmHSP20.8-EGFP was unable to localize to the mitochondria due to the effect of EGFP on the localization signal domain in the C terminus of BmHSP20.8.

**Determination of the Mitochondrial Localization Signal Domain of BmHSP20.8.** To identify the mitochondrial localization signal domain of BmHSP20.8, we constructed four EGFP-fused truncated BmHSP20.8 expression vectors and used to transfect BmN cells. EGFP was fused to the N terminus of the truncated BmHSP20.8 and successfully expressed in BmN cells (strong green fluorescence was observed; Fig. 3A). The cytoplasmic and mitochondrial proteins of these cells were extracted and identified by Western blot using the HIS monoclonal antibody. The results showed that the fragment encoding the mitochondrial localization signal domain of BmHSP20.8 is located between 427 to 561 bp of the open reading frame (ORF) of this gene (Fig. 3B), which corresponds to a location between amino acids 143 to 186 of the resulting protein.

**Analyses of the Molecular Chaperone Activity of BmHSP20.8.** To identify the molecular chaperone activity of BmHSP20.8, the protein was first isolated. To prepare the protein, we constructed the pETduet-HIS-SUMO-BmHSP20.8 prokaryotic expression vector and



**Fig. 4.** Molecular chaperone activity analysis of BmHSP20.8. Thermal aggregation of CS was used to identify whether BmHSP20.8 has molecular chaperone activity in vitro. SDS-PAGE analysis of CS thermal aggregation in the presence of BmHSP20.8 at 43, 48, 53 and 58°C.

an expressed recombinant SUMO-BmHSP20.8 in *E. coli*. BmHSP20.8 was obtained from the fusion protein SUMO-BmHSP20.8 through digestion using ULP1 (Supp Material Fig. 4 [online only]). The fused SUMO peptide was used to achieve soluble BmHSP20.8.

A thermal aggregation experiment employing citrate synthase was used to identify whether or not BmHSP20.8 has molecular chaperone activity. Compared with the negative control, more CS was found in the supernatant, and only a slight amount of CS was found in the precipitate at 43 and 48°C, which indicates inhibition of the heat-induced aggregation of CS. However, after incubation at 53 and 58°C, the CS in the precipitate increased as compared with the negative control (Fig. 4), suggesting that BmHSP20.8 may be inactivated at 53°C or higher temperatures due to the observed loss of its molecular chaperone activity.

## Discussion

Recently, *Bombyx mori* sHSP genes, such as sHSP19.9, sHSP20.1, sHSP20.4, sHSP20.8, sHSP21.4, sHSP23.7, sHSP24.3, and sHSP25.4 have been analyzed in studies that mainly focused on bioinformatic analyses, gene cloning, molecular chaperone analyses, and gene expression profiling (Sakano et al. 2006; Hossain et al. 2010; Sheng et al. 2010; Li et al. 2009, 2012). The analysis of the subcellular localization indicated that BmHSP20.8 is likely localized in the mitochondria with a 51% probability. However, the subcellular localization and the localization mechanism of BmHSP20.8 in silkworm have not been reported. In the present work, a series of eukaryotic expression vectors were constructed and used to show that BmHSP20.8 localization and to identify the protein's localization signal domain. We found that the function of the localization signal domain, which is located in the C terminus (143–186 amino acids) of BmHSP20.8, may be lost when a new foreign protein is fused with the C terminus. The members of the sHSP family are diverse and exist in many organisms. However, in some organisms, many sHSPs localize in specific cell components, suggesting their functional diversity. For example, *D. melanogaster* HSP22 localizes in the mitochondria (Visioli et al. 1997), HSP23 and HSP26 are found in the cytoplasm, and HSP27 accumulates in the nucleus (Michaud et al. 1997). The *Synechocystis* HSP17 localizes to the membrane, regulates membrane fluidity, and preserves membrane integrity during thermal fluctuations (Tsvetkova et al. 2002) due to its “lipid chaperone” function. The sequence of the  $\alpha$ -crystallin domain in the C terminus of sHSPs is highly conserved. This conserved region may be the result of the strongly functional constraint of sHSPs as molecular chaperones. In the present work, we discovered that recombinant BmHSP20.8 can effectively inhibit the thermal aggregation of CS, suggesting a molecular chaperone activity of BmHSP20.8 in vitro. The mitochondrial localization and chaperone activity suggest that BmHSP20.8 may bind to other mitochondrial proteins during thermal

and other extreme stress events to protect these proteins from denaturation, which would ensure continued functioning of the “power house” of BmN cells; however, this mechanism requires further study.

## Supplementary Data

Supplementary data are available at *Journal of Insect Science* online.

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*Conflict of interests:* none declared.

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