Biotechnology and Bioprocess Engineering 2008, 13: 624-631
DOI/10.1007/s12257-008-0049-9

Proteome Analysis on Differentially Expressed Proteins of the Fat Body of Two Silkworm Breeds, *Bombyx mori*, Exposed to Heat Shock Exposure

S. Hossein Hosseini Moghaddam[1,2], Xin Du[1], Jun Li[1], Jinru Cao[3], Boxiong Zhong[1], and YuYin Chen[1]*

[1] College of Animal Sciences, Zhejiang Univerisy, Hangzhou 310029, China
[2] Agriculture Faculty, University of Guilan, Rasht, Iran
[3] Sericultural Research Institute, Zhejiang Academy of Agricultural Sciences, Hangzhou 310021, China

Abstract Proteomes of heat tolerant (multivoltine) and heat susceptible (bivoltine) silkworms (*Bombyx mori*) in response to heat shock were studied. Detected proteins from fat body were identified by using MALDI-TOF/TOF spectrometer, MS/MS, and MS analysis. Eight proteins, including small heat shock proteins (sHSPs) and HSP70, were expressed similarly in both breeds, while 4 protein spots were expressed specifically in the bivoltine breed and 12 protein spots were expressed specifically in the multivoltine breed. In the present proteomics approach, 5 separate spots of sHSP proteins (HSP19.9, HSP20.1, HSP20.4, HSP20.8, and HSP21.4) were identified. Protein spot intensity of sHSPs was lower in the multivoltine breed than in the bivoltine breed after the 45°C heat shock treatment, while the difference between two breeds was not significant after the 41°C heat shock treatment. These results indicated that some other mechanisms might be engaged in thermal tolerance of multivotine breed except for the expression of sHSP and HSP70. There were visible differences in the intensity of heat shock protein expression between male and female, however, differences were not statistically significant. © KSBB

Keywords: proteome analysis, heat shock proteins, silkworm, 2D electrophoresis, mass spectrometry

INTRODUCTION

The sericulture industry has contributed significantly to the economic development of many countries. This has resulted in enriched silkworm germplasm resources due to the efforts of silkworm breeders in the research of genetics and breeding of silkworm, *Bombyx mori*. *B. mori* is used widely in basic research, in biotechnology and as a model insect. Hundreds of geographical races and genetically improved strains are maintained in different countries where sericulture was/is in vogue. These races and strains are not only in well-characterized Mendelian traits, but also in not so well studied quantitative traits, such as body size, feeding duration, thermal tolerance, and disease resistance [1]. Thermotolerance is particularly emphasized in silkworm breeding programs for the selection of silkworm varieties with better adaptation to varied environmental conditions, especially in tropical regions such as Southern India, or summer and autumn season in China.

A set of proteins known as heat shock proteins (HSPs) appears to be involved in tolerance against adverse growth condition in many organisms [2]. HSPs are induced at various temperatures in different organisms, and the number of proteins and the relative importance of each HSP family in stress tolerance vary from organism to organism [3]. The concentration of inducible HSPs and other molecular chaperones within cells vary [4]. It has been well established that prior exposure to stress induces tolerance and cross-tolerance to subsequent stress [5]. HSP expression can be correlated with resistance to stress [6]. Within the HSPs, small heat

*Corresponding author
Tel: +86-571-86971657 Fax: +86-571-86971657
e-mail: chenyy@zju.edu.cn

shock proteins (sHSP) range from 12 to 42 kDa. They are synthesized ubiquitously in eukaryotic and prokaryotic cells in response to heat and other stresses induced thermotolerance in some organisms [2,7,8]. A strong correlation has been found between HSP70 expression and thermotolerance [4]. Over-expression and introduction of exogenous HSP70 increases the thermotolerance of various types of mammalian cells in culture, protects cells against ultraviolet radiation, protects whole mammalian hearts against post-ischemic trauma, and increases the inducible thermotolerance of *Drosophila* cells in culture, embryos, and larvae [4,9,10]. Induced thermotolerance is mediated by increased expression of heat shock proteins in a wide variety of cells and organisms [3,7,11]. Wang and Kang [10] studied the genetic basis of thermotolerance in tropical and temperate populations of the migratory locust, *Locusta migratoria*, by measuring expression of HSP70 and HSP90 mRNA at low (0°C) and high temperatures (40°C) and suggested that thermotolerance of locust eggs had a complex genetic basis, and heat shock proteins might be involved in differences in thermotolerance between locust populations. It should be mentioned that pre-conditioning insects can confer thermal tolerance to a subsequent higher thermal treatment, but such an effect is not necessarily related to HSPs, other factors may participate in thermal tolerance [12].

Manipulation or engineering of genes related to thermotolerance was reported for the copy number of HSP70, which was sufficient to affect inducible thermotolerance at some life stages of *Drosophila melanogaster* [13]. The *Drosophila* heat shock protein 70 (HSP70) promoter was introduced as driver for inducible expression of exogenous genes in insects, and successful transfection driven by the *Drosophila* HSP70 promoter was carried out for developing a heat shock inducible and an inheritable RNA interference (RNAi) system in the silkworm (*B. mori*) [14].

Lohmann and Riddiford [15] indicate that the heat shock response of *B. mori* was similar to that of other insects, in which produced were three groups of heat shock proteins, including the HSP82, HSP70, and sHSP, according to molecular weight markers (one-dimensional gel electrophoresis). They also concluded that the heat shock response of *B. mori* was different than that of *Drosophila*, in which the repression of non-heat shock protein synthesis during heat shock was not a prominent feature of the response. By studying the heat shock response of the different races of silkworm including the multivoltine breeds C.Nichi and Pure Mysore, and the bivoltine breed NB4D2, Joy and Gopinathan [16] showed that the presence of new proteins in response to heat shock was different among different tissues, and that both multivoltine and bivoltine silkworms responded to heat shock as evidenced by the presence of additional proteins. It was reported that expression of heat shock proteins in silkworm might vary in different developmental stages based on experiments using SDS-PAGE electrophoresis [17]. Li *et al.* [18] analyzed the expression of the small heat-shock gene BmHSP19.9 in silkworms by RT-PCR and found varying levels of this protein in tissues. It was most abundant in testis, ovary, silk gland, and pupae. Song *et al.* [19] found that the heat shock 70 kDa protein cognate was one of the up-regulated hemocytic proteins when silkworm larvae responded to the inoculation of heat-inactivated bacteria (*Bacillus megaterium*).

"Structural proteomics" maps out the structure of protein complexes or the proteins presenting in a specific cellular organelle, "functional proteomics" is a broad term for many specific, directed proteomics approaches and "expression proteomics" is the quantitative study of protein expression in samples that differ in some variable [20]. There have been no publications to date reporting the proteomics approach to heat shock proteins in silkworm. In the present work, proteome patterns of fat body from heat shocked silkworms was compared with control silkworms in both resistant and susceptible breeds and expression patterns of the differential protein were targeted for identification. The fat body tissue of insects (a homologue of mammalian liver) has important functions as a storage tissue and as a key center of metabolism and biochemistry.

MATERIALS AND METHODS

Genetic Materials and Silkworm Rearing

Two breeds of silkworm (*B. mori* L.), the heat-tolerant Nistari (a multivoltine breed) and the heat-susceptible Jingsong (a bivoltine breed from China), were selected based on previous evaluations for heat tolerance by the Sericultural Research Center of China. Both breeds were provided by the Silkworm Genetics and Breeding Laboratory, College of Animal Sciences, Zhejiang University. Nistari is from tropical regions where the field temperatures often reaches 40°C or higher in summer, and it has exhibited high tolerance to high temperatures [16].

Experimental silkworm larvae were reared using standard techniques and conditions in May 2007. To compare the effect of exposure to elevated temperature on males and females independently, sex identification was carried out by observing silkworm larval sex marks (imaginal buds on posterior abdominal section of silkworm larva) before heat shock. Multivoltine females, multivoltine males, bivoltine females, and bivoltine males were used in the present experiment. To evaluate survival rate of the heat-exposed silkworms, extra larvae were exposed to heat in each treatment and then kept at normal rearing condition. The amount and duration of exposure in our experiment were with the same as Li *et al.* [18] and Joy and Gopinathan [16].

Thermal Treatments and Sampling

Because the fat body is located under the cuticle in larvae, heat stress can easily reach the tissue when the larva is exposed to heat. On the fourth day of the fifth instar, 9 silkworm larvae of each gender were exposed to either 45°C for 35 min or 41°C for 1 h in controlled growth chambers. After the heat exposure, the silkworms were returned to the standard rearing temperature (24°C) and allowed to recover. The

fat body was removed 2 h after heat exposure and placed in ice-cold insect physiological salt solution (0.7% NaCl) [17]. Three fat body samples were pooled to minimize variation and to get enough tissue for analysis. Water was removed from samples by short time centrifugation. Control samples of the fat body were prepared from larvae that were not exposed to heat. All larvae were genetically similar (from a single moth family). All samples were stored at 72°C until analysis.

Protein Extraction and 2D Electrophoresis

A 30 mg sample of fat body was homogenized by grinding it in 450 μL lysis solution (7 M urea, 2 M thiourea, 4% CHAPS, and 1 mM PMSF). The sample mixture was vortexed, and then incubated for 10 min in ice. Sixty mM dithiothreitol (DTT) and 2% IPG buffer (pH range, 3~10) was then added. After centrifugation (30 min at 15,000 g, 4°C), the soluble protein fraction was removed, and the protein concentration was determined using the Bradford method [21]. Isoelectric focusing was carried out with 80 μg of protein sample in 450 μL solution (8 M urea, 2% CHAPS, 12 μL/mL DeStreak™ Reagent and 0.5% IPG buffer, pH 3~10). Protein was loaded onto IPG DryStrips (pH range, 3~10) by the in-gel rehydration method, and subjected to electrophoresis using an Ettan IPGphor IEF unit (Amersham Pharmacia Biotech) at 30 V for 12 h, 500 V for 1 h, 2,000 V for 1 h, 4,000 V for 1 h, and 8,000 V for 10 h. After IEF separation, the strips were immediately equilibrated 2 × 15 min in 50 mM Tris-HCl buffer (pH 8.8) containing 6 M urea, 2% SDS, and 30% glycerol. For the sample without reduction and alkylation, DTT (1%) was added in the first equilibration step, and 2.5% iodoacetamide was added in the second equilibration step. The strips were subjected to the second-dimensional electrophoresis using an Ettan DALTsix multiple-gel electrophoresis unit (GE Healthcare) on top of 12.5% polyacrylamide gels for SDS-PAGE. The electrophoresed proteins were stained with a silver stain. Eight gel replicates of each breed (heat exposed group and control group) were repeated twice.

DeStreak™ Reagent, IPG buffers and IPG DryStrips were purchased from GE Healthcare Bio-sciences AB (Sweden), CHAPS and DTT were purchased from USB corporation (Canada), iodoacetamide was purchased from GE Healthcare (Buckinghamshire, UK), and urea and thiourea were purchased from Amesham Biosciences (EU) and Sigma, respectively. Electrophoresis were purchased from Amresco (OH, US). Deionized water (Millipore, France) with resistance of 18.2 MΩ cm was used throughout.

Image Acquisition, Data Analysis, and Protein Identification

Spots were scanned using a high-resolution image scanner (Amersham Bioscience; 300 pixels/gel) and analyzed by ImageMaster 2D software (version 6). Molecular mass and p*I* were calculated from digitized 2-D images using standard molecular mass marker proteins. Each selected spot which met the criterion that it was repeatedly present in two gels was compared in both treatments and sexes. In order to measure protein expression levels, the spot volume was calculated as a percentage relative to the total volume of all the spots in the gel as normalized data to quantify gel spots and used to evaluate protein expression differences between gels. Normalized volumes of some spots were analyzed using analysis of variance (ANOVA) by SPSS software with three factors including thermal treatment, breed and sex.

Protein samples were distained and trypsin-digested, and peptides were extracted as described elsewhere [22]. MS and MS/MS spectra were obtained using the ABI 4700 Proteomics Analyzer MALDI-TOF/TOF mass spectrometer (Applied Biosystems). Proteins were analyzed using MS/MS or PMF analysis and were identified with the database search program MASCOT Daemon (Matrix Science) against NCBInr/Swiss-prot database using the following parameters: enzyme, trypsin; fixed modification, carbamidomethyl (C); variable modifications, oxidation (M); no restriction on protein mass; one missed cleavage; peptide charge +1; monoisotopic; a peptide mass tolerance of 100 ppm. Protein identification with a confidence interval (C.I. %) protein score greater than 95% ($P < 0.05$) was accepted in both MS/MS and PMF results. Biological and molecular functions were found by using UniProt Knowledgebase (Swiss-Prot and TrEMBL) (http://www.expasy.org/sprot).

RESULTS

Qualitative Comparisons of 2D Protein Patterns

Comparison of the fat body proteomes of both sexes of heat exposed multivoltine and bivoltine breeds of silkworm and controls is shown in Fig. 1. In these proteome profiles, 534 and 744 spots were detected in bivoltine females and males, respectively, and 582 and 825 spots were detected in multivoltine females and males, respectively, by digital image analysis and using the same detection parameters (Smooth 2, Min Area 5, and Saliency 1500). The number of spots was higher in multivoltine silkworms than in bivoltine silkworms, and was higher in males than in females (Table 2). Fig. 1 shows 12 differentially expressed protein spots in bivoltine silkworms and 20 differentially expressed protein spots in multivoltine silkworms in response to heat exposure. There are two regions in the 2D gels which show altered expression of proteins in both multivoltine and bivoltine silkworms heat shock patterns. These are numbered 1 to 4 (region 1) and 5 to 8 (region 2) in Fig. 2. These 8 expressed spots are very similar for both breeds and give reproducible staining patterns ("common response spots"). Although protein spot distribution patterns differ between the two breeds, they are similar within each breed between the sexes and heat treatments (45 and 41°C). Therefore, sex and the two heat treatments can be pooled for this experiment. Besides the common response spots (spots 1~8), there are 4 spots (spots 11~14) in the bivoltine silkworms and 12 spots (spots 31~42) in multivoltine silkworms induced by heat shock and

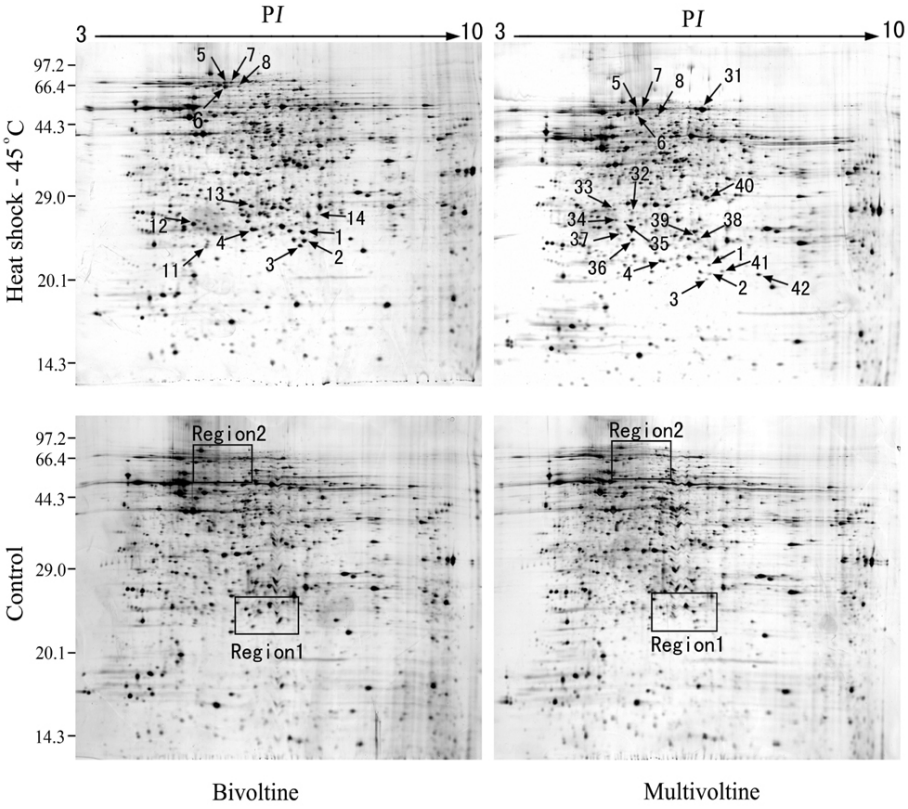

Fig. 1. 2D electrophoresis protein profiles of fat body of the control and heat exposed silkworm larvae from the thermo-susceptible breed (bivoltine) and thermo-tolerant breed (multivoltine). Two regions of interest in low and high molecular weight areas are outlined by the dashed boxes. All expressed heat shock proteins are numbered.

2 up-regulated spots (spots 34 and 35) were detected only in silkworms exposed to 45°C.

Mass Spectrometry of Differential Protein Spots

A total of 24 differential protein spots were selected for identification by comparing treatments and control gel profiles based on reproducibility among replicates. Two faint spots were rejected and the remaining 22 spots were selected for the analysis by peptide mass mapping. Of these, 9 could be identified by MS/MS and 5 could be identified by PMF. Table 1 lists the identified proteins with their intensity matched of MALDI-TOF/TOF spectra ranged from 20.54 to 60.43%. Nine matches of the spectra nicely matched heat shock proteins (HSPs), which belonged to 2 major families of HSP (sHSP and HSP70). Spots 1, 3, 4, 11, and 36 were identified as HSP20.4, HSP19.9, HSP20.8, HSP20.1, and HSP21.4, respectively, while spots 5, 6, 7, and 8 were identified as HSP70. Only a small portion of the genome sequence of silkworm (*B. mori*) is available. Since this is essential to a proteomic approach, some spots which we were unable to identify are not listed in Table 3. Among those identified successfully, spots 13 and 28 corresponded to a protein for zinc ion binding, spot 2 corresponds to a protein responsible for zinc ion binding and DNA binding and spots 34 and 40 corresponded to a protein responsible for transferase activity and protein amino acid phosphorylation.

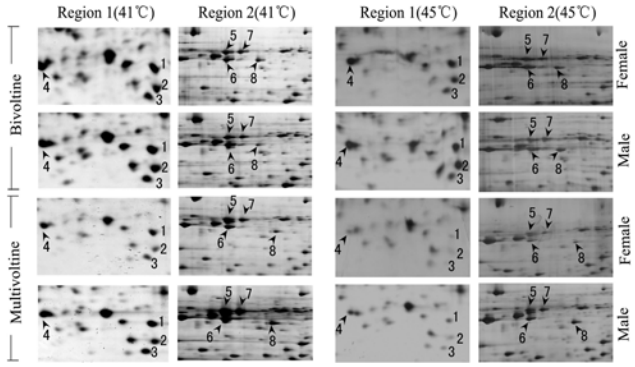

Fig. 2. Comparison of protein spot intensities of the regions of interest from Fig. 1, including temperature (45 and 41°C), breed (bivoltine and multivoltine) and sex.

Quantitative Comparisons of 2D Protein Patterns

Quantitative comparisons of protein expression patterns with the control patterns were performed by eye and by Ima-

Table 1. List of identified silkworm fat body proteins in responses to high heat exposure

Spot no.	Protein name (Matched organism)	Accession GI no.	No. of peptides (coverage)	Protein score (C.I. %)	*Mr* calcd/obsd (*P*I calcd/obsd)	Ontology
	Common response spots					
1	Heat shock protein HSP20.4 (*Bombyx mori*)	49036077	9 (49.86%)	148 (100)	26 / 20.41 (7.10 / 6.54)	Response to stress
2	DNA-formamidopyrimidine glycosylase* (alpha proteobacterium BAL199)	163793016	7 (31.00%)	90 (99.42)	25 / 32.87 (7.05 / 8.57)	Zinc ion binding, DNA binding; catalytic activity
3	Heat shock protein HSP 19.9 (*Bombyx mori*)	56378317	7 (29.24%)	120 (100)	24 / 19.88 (6.23 / 6.53)	Response to stress
4	Heat shock protein HSP20.8 (*Bombyx mori*)	11120618	7 (46.09%)	177 (100)	26 / 20.79 (5. 80 / 5.98)	Response to stress
5	Heat shock protein HSP70 (*Antheraea yamamai*)	47232576	16 (40.28%)	98 (99.96)	85 / 69.55 (5.80 / 5.7)	Response to stress ATP binding
6	Heat shock protein HSP70 (*Antheraea yamamai*)	47232576	12 (25.87%)	110 (99.98)	80 / 69.55 (5.9 / 5.7)	Response to stress ATP binding
7	Heat shock protein HSP70 (*Antheraea yamamai*)	47232576	13 (29.65%)	102 (99.98)	85 / 69.55 (5.9 / 5.7)	Response to stress ATP binding
8	Heat shock protein HSP70 (*Antheraea yamamai*)	47232576	15 (45.40%)	86 (99.44)	79 / 69.55 (6.15 / 5.7)	Response to stress ATP binding
	Specific response spots (Bivoltine)					
11	Heat shock protein HSP20.1* (*Bombyx mori*)	112983134	7 (33.00%)	84 (97.80)	25 / 20.18 (5.51 / 5.46)	Response to stress
13	PRETICTED: similar to zinc finger protein 436 (*Canis familiaris*)	57048379	7 (20.54%)	80 (97.74)	30 / 55.30 (6.3 / 8.94)	Zinc ion binding
	Specific response spots (Multivoltine)					
34	PREDICTED: similar to CG10504-PA* (*Tribolium castaneum*)	91079909	14 (33.00%)	82 (96.20)	33 / 51.45 (5.45 / 7.77)	Transferase activity; protein amino acid phosphorylation
36	Heat shock protein HSP21.4 (*Bombyx mori*)	56378321	8 (60.43%)	120 (100)	29/2139 (5.74 / 5.79)	Response to stress
38	PREDICTED: similar to zinc finger protein 46* (*Canis familiaris*)	57048379	13 (27.00%)	96 (99.84)	29/55.30 (6.91 / 8.94)	Zinc ion binding
40	PREDICTED: similar to CG9935-PA isoform 1* (*Apis mellifera*)	66507549	11 (27.00 %)	86 (98.50)	37/61.90 (7.04 / 6.14)	Transferase activity; protein amino acid phosphorylation

C.I. %: confidence interval of protein score.
*Identification of protein by PMF analysis.

geMaster 2D software. These analyses revealed that expression intensity of some spots were different in multivoltine and bivoltine breeds. Among the 4 identified proteins of the bivoltine breed, spots 11 and 12 showed a specific up-regulation under both heat exposure treatments. Among the 12 identified proteins of multivoltine breed, spots 40 and 42 were up-regulated in response to both heat exposure treatment, and spots 36 and 41 showed up-regulation in a 41°C treatment alone. These differences showed that in the multivoltine breed, the number of expressed protein spots increases in response to an increase in the heat exposure temperature, however, in the bivoltine breed, no differences were observed between the heat exposure treatments. Spot 37 was up-regulated only in females of the multivoltine breed. There are differences in the volume of protein expression between bivoltine and multivoltine breeds in both heat exposure treatments, as well as between sexes. Table 1 presents the means of the normalized volumes (volume percentage) of common response spots, including 4 sHSPs (region 1) and 4 HSP70 (region 2), separated by treatment, breed, and sex. Significant differences between the two breeds and the temperature exposures were determined by ANOVA (Table 2). Data in Table 1 and ANOVA in Table 2 revealed that common response protein spots were expressed in each of the samples. However, the quantity of HSPs differed. Moreover, protein expression intensity of sHSPs (region 1) differed significantly ($P < 0.01$) between the two heat exposure treatments and the silkworm breeds. The expression of sHSPs in the multivoltine breed was lower than in the bivoltine breed after the 45°C heat exposure treatment, while there was no significant difference

Table 2. The mean of normalized volumes (%) of 8 protein spots, including 4 sHSP (region 1) and 4 HSP70 (region 2), in different treatments, breeds, and sexes

Breed	Sex	Heat treatment (45°C)			Heat treatment (41°C)	
		Number of spot*	sHSP	HSP70	sHSP	HSP70
Bivoltine	Female	534	0.353 (± 0.102)	0.215 (± 0.086)	0.322 (± 0.067)	0.218 (± 0.063)
	Male	744	0.332 (± 0.091)	0.225 (± 0.070)	0.332 (± 0.069)	0.221 (± 0.135)
Multivoltine	Female	582	0.072 (± 0.043)	0.151 (± 0.050)	0.282 (± 0.063)	0.278 (± 0.221)
	Male	825	0.077 (± 0.040)	0.225 (± 0.079)	0.235 (± 0.042)	0.302 (± 0.040)

*Total number of spots in 2D electrophoresis image pattern.

Table 3. ANOVA on normalized volumes of 8 protein spots including 4 sHSP (region 1) and 4 HSP70 (region 2)

Source	df	sHSP		HSP70	
		M.S.	P	M.S.	P
Heat treatment	1	0.057	0.008	0.021	0.125
Breed	1	0.226	0.000	0.003	0.554
Sex	1	0.001	0.657	0.006	0.400
Error	28	0.007	–	0.008	–

in the intensity of protein expression between breeds after the 41°C heat exposure treatment. In other words, at lower heat exposure treatments, silkworm breeds did not differ significantly in their response, while, at higher temperature exposure treatments, the thermotolerant breed expressed significantly lower sHSPs ($P < 0.01$). The differences between the two temperatures were not significant for HSP70 in the multivoltine breed. Lower expression of sHSPs and stable expression of HSP70 suggested that HSP70 and sHSP may play different role in thermal tolerance at higher temperature in the thermotolerant breed. Comparison of the volume between the two sexes indicates that there are some differences in protein expression, although it was not significant (Table 2).

DISCUSSION

Thermal sensitivity and heat shock response of different races of *B. mori* can be measured by observing the survival rate of larva, pupa, moth and egg and by observing cocoon characteristics [16,23-26]. But the study of the heat shock response on the molecular level gives more information about heat shock proteins and biomarkers. Identification of protein markers will also provide breeders with a mean for more efficient and correct selection of heat-tolerant traits [27]. We identified 14 proteins that are differentially expressed after heat exposure, of which 9 are known HSPs and 5 are predicted to be involved in heat-shock responses. The methods we used in these experiments, including high-resolution 2D gel electrophoresis of fat body using silver staining combined with MS/MS and MS analysis of mass spectrometry, proved to be a successful strategy in the study of HSPs in different silkworm varieties. The changes in protein expression as a result of heat shock response were not identical in the two breeds. This suggests some clear candidate marker proteins for identifying heat-tolerant and heat-susceptible silkworm larvae. Generally, the multivoltine breed has shown higher survival rates than the bivoltine breed in response to heat shock [16,26]. Koundinya *et al.* [28] showed that the Nistari breed, which is the multivoltine breed used in the present experiment, is the most tolerant breed among 11 multivoltine breeds. Specific response proteins, including spots 11~14 and 31~42, may serve as marker proteins for heat-susceptible and heat-tolerant, respectively. In particular, protein spots 11 and 13 in bivoltine breed and spots 34, 36, 38, and 40 in multivoltine breed can be considered as protein markers related to tolerance. Skylas *et al.* [27] observed that 7 protein spots were expressed in a heat shock tolerant cultivar of wheat after heat shock using proteome analysis. Süle *et al.* [29] also used proteomic analysis (2D-PAGE, MS) to detect the effects of heat shock on an abiotic stress-tolerant and an abiotic stress-susceptible cultivar of barley. They found two proteins spots unique to the stress-susceptible cultivar.

In this work, we identified 5 low molecular weight HSP proteins (20.4, 19.9, 20.4, 20.1, and 21.4) which were expressed after heat exposure. Sakano *et al.* [30] reported that *B. mori* had six sHSPs, including the above described sHSPs and sHSP23.7. Skylas *et al.* [27] reported that the majority of the heat shock proteins in both the heat-susceptible and the heat-tolerant cultivars of wheat had low molecular weight. Four protein spots in region 2 of both breeds were HSP70. Increases in HSP70 can protect intact larvae against the thermal inactivation of alcohol dehydrogenase and against thermal inhibition of feeding [13]. HSP70 plays a central role in stress tolerance, including promoting growth at moderately high temperatures and protecting organisms from mortality at extreme temperatures, by chaperoning unfolded proteins [3]. Once folded properly, these proteins are less sensitive to denaturation and aggregation. Three expressed proteins, which are similar to zinc finger protein identified in this research, are likely involved in the folding process of

proteins, because zinc fingers are involved in folding of proteins.

The expression of sHSPs in the multivoltine breed is significantly ($P < 0.01$) lower than in the bivoltine breed when exposed to the 45°C treatment, but there is no difference between the breeds when exposed to the 41°C treatment (Table 2 and Fig. 2). This demonstrates that the thermotolerant silkworm breed was not characterized by a higher level of sHSP synthesis under severe heat shock as compared to the thermosensitive breed. But HSP70 expression was not significantly reduced at the higher temperature treatment. This suggests the sHSP and HSP70 may play different role in thermotolerance of silkworms. Based on the available research, the authors concluded that other mechanisms might be involved with thermotolerance other than the sHSPs and the HSP70. The number of specific proteins involved in the tolerance of multivoltine breed might also have an important role, as we had detected 12 spots in the multivoltine breed compared with 4 spots in the bivoltine breed when exposed to heat. Shilova *et al.* [7] concluded that thermotolerance required several alternative molecular mechanisms, and that HSP40, sHSPs, and other unidentified factors played an important role in this process along with HSP70 in *D. melanogaster*. Previous research has shown that, in thermotolerant breeds of *D. melanogaster*, HSP70 synthesis is maintained at low levels. The most thermotolerant strain, T (isolated in Central Africa), has a lower level of HSP70 synthesis under moderate heat exposure (37.5°C) compared to the less thermotolerant Oregon R strain [7,31].

Fig. 2 and Table 2 show that male silkworm larva expressed slightly more HSP70, especially in the multivoltine breed, but the difference is not significant ($P < 0.01$). The number of protein spots detected by image analysis software is also higher in males than in females. To the best of our knowledge, no other publication discusses the differences between female and male silkworms in responding to heat tolerance. Further experimentation is required to determine the difference in thermotolerance between the sexes of silkworm larvae.

In order to identify more protein markers and to enhance our understanding of the relationship between silkworm breeds and their different thermal tolerances and their expressions of different kinds of HSPs, it is necessary to search for more differential spots using more thermotolerant and susceptible silkworm breeds. Additional methods for the other silkworm tissues should also be explored. In the future, therefore, we will investigate the effects of excessive heat shock on the proteome of different breeds and sexes.

Furthermore, there are many successful experiments on transgenic silkworm. However, it is only recently that scientists are technically capable of targeting endogenous genes when engineering transgenic silkworm [14]. Therefore, manipulation of genes related to robustness and thermotolerance of silkworm is not too far away. An understanding of the molecular mechanisms of thermal tolerance is essential for attaining any results in this direction, particularly in the understanding the differential expression pattern of various HSPs in bivoltine and multivoltine breeds. The importance of HSP70, which was confirmed for silkworm larvae thermotolerance in the present research, would greatly facilitate this research.

Acknowledgements We thank Jian-ying Li, Zhong-hua Zhou, and Fang Yu for their technical help in experiments. Financial support by the Department of Science and Technology, Zhejiang Province, China (2006C12056) and National Basic Research Program of China (2005CB121003) is gratefully acknowledged.

Received March 25, 2008; accepted July 8, 2008

REFERENCES

1. Nagaraju, J. (2002) Application of genetic principles for improving silk production. *Curr. Sci*. 83: 409-414.
2. Ferreira, A. S., M. R. Tótola, M. C. M. Kasuya, E. F. Araújo, and A. C. Borges (2005) Small heat shock proteins in the development of thermotolerance in *Pisolithus* sp. *J. Therm. Biol.* 30: 595-602.
3. Parsell, D. A. and S. Lindquist (1993) The function of heat-shock proteins in stress tolerance: degradation and reactivation of damaged proteins. *Annu. Rev. Genet*. 27: 437-496.
4. Krebs, R. A. and M. E. Feder (1998) Hsp70 and larval thermotolerance in *Drosophila melanogaster*: how much is enough and when is more too much? *J. Insect Physiol*. 44: 1091-1101.
5. Newman, A. E. M., M. Foerster, K. L. Shoemaker, and R. M. Robertson (2003) Stress-induced thermotolerance of ventilatory motor pattern generation in the locust, *Locusta migratoria*. *J. Insect Physiol.* 49: 1039-1047.
6. Feder, M. E. and G. E. Hofmann (1999) Heat-shock proteins, molecular chaperons, and the stress response: evolutionary and ecological physiology. *Annu. Rev. Physiol.* 61: 243-282.
7. Shilova, V. Y., D. G. Garbuz, M. B. Evgen'ev, and O. G. Zatsepina (2006) Small heat shock proteins and adaptation of various *Drosophila* species to hyperthermia. *Mol. Biol.* 40: 235-239.
8. Sun, W., M. Van Montagu, and N. Verbruggen (2002) Small heat shock proteins and stress tolerance in plants. *Biochim. Biophys. Acta* 1577: 1-9.
9. Krebs, R. A. and B. R. Bettencourt (1999) Evolution of thermotolerance and variation in the heat shock protein, HSP70. *Am. Zool.* 39: 910-919.
10. Wang, X. H. and L. Kang (2005) Differences in egg thermotolerance between tropical and temperate populations of the migratory locust *Locusta migratoriam* (Orthoptera: Acridiidae). *J. Insect Physiol*. 51: 1277-1285.
11. Robertson, R. M. (2004) Thermal stress and neural function: adaptive mechanisms in insect model systems. *J. Therm. Biol*. 29: 351-358.
12. Neven, L. G. (2000) Physiological responses of insects to heat. *Postharvest Biol. Technol.* 21: 103-111.

13. Feder, M. E. and R. A. Krebs (1998) Natural and genetic engineering of the heat-shock protein Hsp70 in *Drosophila melanogaster*: Consequences for thermotolerance. *Am. Zool.* 38: 503-517
14. Dai, H., R. Jiang, J. Wang, G. Xu, M. Cao, Z. Wang, and J. Fei (2007) Development of a heat shock inducible and inheritable RNAi system in silkworm. *Biomol. Eng*. 24: 625-630.
15. Lohmann, C. M. F. and L. M. Riddiford (1992) The heat-shock response and heat sensitivity of *Bombyx mori*. *Sericologia* 32: 533-537.
16. Joy, O. and K. P. Gopinathan (1995) Heat shock response in mulberry silkworm races with different thermotolerances. *J. Biosci.* 20: 499-513.
17. Chavadi, V. B., A. H. Sosalegowda, and M. H. Boregowda (2006) Impact of heat shock on heat shock proteins expression, biological and commercial traits of *Bombyx mori*. *Insect Sci.* 13: 243-250.
18. Li, B., Q. Y. Xia, H. Fujii, Y. Banno, and C. Lu (2005) Expression of the small heat-shock protein Bmhsp19.9 gene in silkworm (*Bombyx mori*). *Chin. J. Agr. Biotechnol*. 13: 195-201.
19. Song, K. H., S. J. Jung, Y. R. Seo, S. W. Kang, and S. S. Han (2006) Identification of up-regulated proteins in the hemolymph of immunized *Bombyx mori* larvae. *Comp. Biochem. Physiol. Part D Genomics Proteomics* 1: 260-266.
20. Graves, P. R. and T. A. J. Haystead (2002) Molecular biologist's guide to proteomics. *Microbiol. Mol. Biol. Rev*. 66: 39-63.
21. Bradford, M. M. (1976) A rapid and sensitive method for the quantitation of microgram qualities of proteins utilizing the principle of protein-dye binding. *Anal. Biochem.* 72: 248-254.
22. Jin, Y. X., Y. Y. Chen, Y. H. Jiang, and M. K. Xu (2006) Proteome analysis of the silkworm (*Bombyx mori.* L) colleterial gland during different development stages. *Arch. Insect Biochem. Physiol.* 61: 42-50.
23. Umadevi, K. and D. R. Rao (2005) Evaluation and selection of multivoltine breeding resource materials of silkworm, *Bombyx mori* L. for high temperature and humidity conditions. *J. Exp. Zool*. 8: 329-336.
24. Kumar, N. S., H. K. Basavaraja, G. V. Kalpana, N. M. Reddy, and S. B. Dandin (2003) Effect of high temperature and high humidity on the cocoon shape and size of parents, foundation crosses, single and double hybrids of bivoltine silkworm, *Bombyx mori* L. *Indian J. Sericulture* 42: 35-40.
25. Kato, M., K. Nagayasu, W. Hara, and O. Ninagi (1998) Effect of exposure of the silkworm, *Bombyx mori*, to high temperature on survival rate and cocoon character. *Jpn. Agr. Res. Q*. 32: 61-64.
26. Hsieh, F. K., S. J. Yu, S. Y. Su, and S. J. Peng (1995) Studies on the thermotolerance of the silkworm, *Bombyx mori. Chin. J. Entomol.* 15: 91-101.
27. Skylas, D. J., S. J. Cordwell, P. G. Hains, M. R. Larsen, and D. J. Basseal (2002) Heat shock of wheat during grain filling: proteins associated with heat-tolerance. *J. Cereal Sci.* 35: 175-188.
28. Koundinya, P. R., P. Kumaresan, R. K. Sinha, and K. Thangavelu (2003) Screening of promising germplasm of polyvoltine silkworm (*Bombyx mori* L.) for thermotolerance. *Indian J. Sericulture* 42: 67-70.
29. Süle, A., F. Vanrobaeys, G. Hajós, J. Van Beeumen, and B. Devreese (2004) Proteomic analysis of small heat shock protein isoforms in barley shoots. *Phytochemistry* 65: 1853-1863.
30. Sakano, D., B. Li, Q. Xia, K. Yamamoto, H. Fujii, and Y. Aso (2006) Genes encoding small heat shock proteins of the silkworm, *Bombyx mori*. *Biosci. Biotechnol. Biochem.* 70: 2443-2450.
31. Zatsepina, O. G., V. V. Velikodvorskaia, V. B. Molodtsov, D. G. Garbuz, D. N. Lerman, B. R. Bettencourt, M. E. Feder, and M. B. Evgenev (2001) A *Drosophila melanogaster* strain from sub-equatorial Africa has exceptional thermotolerance but decreased Hsp70 expression. *J. Exp. Biol.* 204: 1869-1881.