2. REVIEW OF LITERATURE
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Though extensive studies on heat shock proteins expressed in response to imposed temperature stress have made from "bacteria to man", in recent years, relatively great interest been growing in silkworm, *B. mori*. Hence, the information’s on structural and functional properties of major HSPs in general and heat shock response, heat shock proteins, thermotolerance and role of HSPs in biological and commercial traits of *B. mori* in particular was reviewed and presented in this chapter.

Since the legendary fall, unraveling of a cocoon in the teacup of Chinese princess Xi Ling Shi, the mulberry silkworm has been intimately associated with human. The only insect *B. mori* that truly domesticated over 5000 years is completely dependent on human and the trade of silk enriched human endeavor through art and culture, contributing to an early form of globalization for nearly 2000 years during the silk route.

Exposure of an organism or tissue to the temperature higher than optimal growth temperature alters the normal biological, biochemical and physiological functions and ultimately resulted in death. But an early consequence of heat shock facilitated in synthesis of set of proteins, which are considered to confer protection against the adverse effect of heat shock (Hightower, 1991). Thus, functional properties of major HSPs are presented here.

2.1 Heat shock proteins (HSPs)

The cellular stress response was described for the first time by Ritossa in *Drosophila melanogaster* (1962) and the protein expressed in
elevated temperature was termed as "heat shock protein" (Tissieres et al., 1974). Primarily, HSPs are identified based on molecular weight between 19 and 110 kDa, which are broadly classified as large (major) HSPs and small HSPs (SmHSPs), now they can be identified and confirmed through various proteomic and bioinformatic approaches.

2.1.1 Important classes of HSPs

Among different HSPs - HSP110, HSP90, HSP70, HSP60, HSP40 and small HSPs families are considered as important classes of HSPs.

HSP90: the most abundant HSP, representing almost 1% of total cellular protein in unstressed cells, required for viability under all conditions in pro and eukaryotic systems. It interact with a variety of protein kinases and transcription factors required for growth and development, which includes - Src and Raf family of kinases, certain MAP kinases, members of the steroid receptor family, telomerase, the tumour suppressor p53 and heat shock transcription factors. While interacting with large number of co-chaperones, HSP90 appears to maintain its client proteins in a conformation that allows for their subsequent activation in response to appropriate growth signals (Pearl and Prodromou, 2006; Richter and Buchner, 2006).

The HSP90 utilizes ATP binding and hydrolysis as part of its reaction cycle. Biochemical and structural analysis revealed a complex mechanism by which ATPase-coupled conformational changes in HSP90 dictates interactions with its myriad of co-chaperones. These co-chaperone interactions in turn influence how and when HSP90 interacts with and activates/inactivates its client proteins. For example, HSP90 along with one set of its co-chaperones (HSP70/HSP40 chaperone
machinery) binds to and stabilizes steroid hormone receptors in their inactive state within the cytosol (Picard, 2006).

Upon subsequent binding to the appropriate steroid hormone ligand, the receptor undergoes a conformational change in its acquisition of DNA binding and transcriptional activity. Similarly, HSP90 along with another set of co-chaperones binds to and stabilizes the newly synthesized forms of various protein kinases, maintaining them in a folded-competent conformation. Thus, via its utilization of numerous co-chaperones and ATP, the very abundant HSP90 chaperone functions in unstressed cells regulate client proteins, which are important for growth and development. Any changes in the level of HSP90 (via genetic means or manipulations with HSP90 inhibitory drugs) led to rapid alterations in cell signalling pathways and adaptation of new cellular phenotypes (Picard, 2006). Less well characterized members of the HSP90 family have been described in the

(Source: www.enzolifesciences.com)
mammalian endoplasmic reticulum (ER) and in plants. In the case of Grp94 (glucose regulated protein in mammalian ER), there is some evidence for its involvement in facilitating folding and transport of newly synthesized proteins destined for secretion or membrane insertion (Mahalingam et al., 2009).

**HSP70:** The HSP70 family represents one of the largest stress protein families with related members distributed throughout the cell. Genetic studies designed to detect host-bacteriophage interactions revealed a number of bacterial genes whose expression was required for proper phage growth. One such set of genes was shown to be important for both phage and host DNA replication and therefore were referred to as DnaK, DnaJ, and GrpE proteins. Subsequent biochemical studies revealed that the three proteins worked together in facilitating the disassembly of a large protein complex necessary for the commencement of DNA replication. These early studies indicated that the DnaK protein, later shown to be a relative of the eukaryotic HSP70 family, facilitated the disassembly of large protein complexes needed to initiate the early stages of DNA replication.

In contrast to the single DnaK protein in bacteria, eukaryotes express a multitude of DnaK homologues referred to as the HSP70 family. They appear to bind and hydrolyze ATP and interact with other proteins undergoing maturation and folding. For example, HSP70 family members bind to and stabilize nascent polypeptides as they emerge from the ribosome and as they are translocated across membranes into the endoplasmic reticulum or mitochondria. The interaction of the HSP70 chaperone with its unfolded protein target is mediated by ATP and a number of co-chaperones (Mayer and Bukau, 2005; Meimaridou et al., 2009).
The reaction cycle of HSP70/HSP40/Bag-1 machinery has been well characterized. In its "open" or ATP bound state, recognizes and binds to hydrophobic or unstructured sequences of amino acids within the substrate protein. Binding to the target stimulates the hydrolysis of ATP to ADP and a conformational change in the HSP70/HSP40/Bag-1 machinery to its closed state resulted in tight substrate binding. Subsequent exchange of bound ADP for ATP returns HSP70/HSP40/Bag-1 machinery to its open state releasing the substrate polypeptide. This cycling of HSP70/HSP40/Bag-1 machinery between the open and closed states is regulated by the co-chaperones HSP40 and Bag-1. Thus, through repeated cycles of binding and release to its target, HSP70/HSP40/Bag-1 machinery helps to prevent premature folding or aggregation, thereby facilitating high fidelity protein maturation throughout the cell. The various family members of HSP70 are distributed throughout different intracellular compartments like cytosolic/nuclear HSP70 proteins, HSC70 and HSP70 (also known as HSP73 and HSP72 respectively), Grp78 or Bip present within the lumen of the endoplasmic reticulum, and Grp75 (also called mortalin) localized within mitochondria. Normally, whenever the cell finds itself under conditions that are unfavourable for protein folding, members of the HSP70 family are expressed at higher levels. Increased expression of the chaperones help in the repair of proteins damaged by the particular stress event as well as guide the synthesis of new polypeptides needed to replace those irreparably damaged. In mammals, one particular HSP70 family member (e.g. HSP72) is expressed only in times of stress and therefore its appearance often serve as a critical indicator that a cell, tissue, or organ has undergone a stress response.
Since their initial identification and characterization many clinicians have focused their interest on the HSP70 family. Elevated levels of HSP70 proteins have been linked with inhibition of apoptosis as well as the resistance of cells to various chemotherapeutic agents. In addition, numerous studies continue to demonstrate that changes in the levels of the different HSP70 family members may prove clinically useful for the diagnosis of many important human diseases (Schlesinger, 1990)

**HSP60:** Members of the HSP60 (eukaryotes) and GroEL (bacteria) family of HSPs, participate in protein maturation events and also called as chaperonins. All members of this chaperonin family exhibit molecular masses of around 60 kDa, but are usually part of large oligomeric structures. For example, bacterial GroEL, initially named because of its essential role in bacteriophage growth, exists as a large
homo-oligomeric complex (~800 kDa). This large complex can discriminate between folded and unfolded proteins. In combination with its particular co-factor (HSP10 in eukaryotes or GroES in bacteria) the HSP60/GroEL proteins bind newly synthesized polypeptides and facilitate their folding to the native state in an ATP-dependent cycle. Chaperonins perform their chaperone role somewhat different from that of their HSP70 counterparts. Specifically, binding and sequestration of the substrate polypeptide occurs within the large central cavity of the chaperonin complex. It is thought that protection of the substrate protein within the central cavity of the chaperonin provides a sequestered protein folding environment, thereby reducing the probability of misfolding and aggregation of the target protein with other polypeptides. The HSP60 is localized within mitochondria and with its co-chaperone, HSP10, participates in the folding and assembly of newly synthesized proteins as they are transported into the mitochondria from the cytosol. In plants, the related Cpn60/Cpn10 function similarly within the chloroplast to orchestrate the folding and assembly of Rubisco (and therefore is also referred in the literature as the Rubisco-binding protein) and other chloroplast proteins. Finally, within the eukaryotic cytosol a number of proteins distantly related to HSP60 have been described but remain less characterized. These proteins, TRiC (TCP-1 ring complex, also called CCT for chaperonin-containing TCP-1) consist of at least 8 family members. These proteins again are arranged in a large complex similar to that formed by the GroEL or HSP60 proteins. So far, only a few protein substrates, notably the cytoskeletal proteins actin and tubulin appears to require the TRiC complex for their efficient folding and assembly. Curiously, no GroES/HSP10 like co-factor has been identified for this seemingly more specialized cytosolic chaperonin (Frydman, 2001; Horwich et al., 2007).
In addition to their prominent role as molecular chaperones, members of the GroEL and HSP60 families have long been recognized as highly immunogenic proteins. The related GroEL proteins from different pathogens elicit strong humoral and cellular immune responses. Finally, chaperonins are now proving its usefulness as it pertains to in vitro folding of recombinant proteins important for clinical medicine and therapeutic purposes (Gutsche et al., 1999; Spiess et al., 2004).

sHSP: The small HSPs are perhaps the most widespread but least conserved members of the HSP family while bacteria and single-cell eukaryotes express only one or two members, D. melanogaster expresses
Bombyx larva encodes 6, human 10, and plants as many as 19 (Hossain et al., 2010). Although diverse in sequence most members of the family share a number of properties including: a low molecular mass between 14-45 kDa with most in the 20 kDa range; sequence homology with the α-crystallin proteins; and the formation of large and dynamic oligomeric complexes. In the case of human low molecular weight HSP, collectively termed HSP27 family, the proteins are found in complexes of 400 to 500 kDa. Phosphorylation of HSP27, in response to different stimuli, may play a role in the oligomeric dynamics of the protein. The best characterized member of the family, α-crystallin is abundant in the eye lens where the overall protein concentration is quite high.

The α-crystallins (subunits A and B) are thought to prevent protein aggregation resulting from light damage and/or other
metabolic insults. Similarly, other members of the low molecular weight HSPs are now thought to function as ATP-independent molecular chaperones. Via their large surface and potential to recognize and bind exposed hydrophobic patches, HSP27 and its counterparts may act promiscuously to bind unfolded proteins and then present their substrates to the other ATP-dependent molecular chaperone machineries (e.g. HSP60, HSP70 or HSP90) for subsequent re-folding.

2.2 HSPs in B. mori

2.2.1 Heat shock response

Even though B. mori was derived from wild progenitor Bombyx mandarina (Arunkumar et al., 2006), lost its temperature-tolerance during the period of domestication over 5000 years. And silkworm races/strains are found to be diverged as tropical (polyvoltine) and temperate (Univoltine and bivoltine). Eventually, the silkworm strains grown in tropical environment became resistant to high temperature and diseases, while the strains reared in temperate condition became susceptible. Since, there is enough diversity existing among silkworm strains/races, loss of tolerance to environmental insults in B. mori need to be reinvestigated systematically. Towards this, Evgen'ev et al., (1987) suggested to check whether high thermo-resistance is inherent only in the cultured cells or cells behave in a similar way in vivo, when cells of B. mori and Antheraea pernyi exhibited resistant to high temperature (40 to 45°C).

Subsequently, the action of heat shock on B. mori cells infected by nuclear polyhedrosis virus (NPV) both in vitro and in vivo was studied. While infected cells exhibited their inability in synthesis of HSPs after temperature elevation, northern blot experiments revealed that NPV
infection does not interfere with the induction of transcription of hs- 
genesis, rather inhibits HSP synthesis at the level of translation (Titareka et al., 1990). Eventually, Kobayashi and Sudawan (1993) opined that 
rearing of silkworm on farms at sustained high temperature might be 
useful in preventing infectious viral diseases. Further, Lohman and 
Riddiford (1992) reported that heat shock response in B. mori differs 
from that of Drosophila but showed similarity with that of Manduca at 
42°C as the maximum tolerable temperature. The relationship between 
thermotolerance and heat stable esterase in B. mori varies with silkworm 
strains and it is positively related to the activity of the heat stable 
esterase (Wu and Hou, 1993). Later, HS response and thermal 
sensitivity of different silkworm strains was reported determining 43°C 
lethal to silkworm strains C. nichi, PM and NB4D2 (Joy and Gopinathan 
1995).

Interestingly, Hsieh et al., (1995) reported that polyvoltine strains 
were more tolerant to heat than the bivoltine strains at 4th and 5th 
instars, and pupal stage as high temperature treatment affected the 
urvival rate more seriously than the others. Another polyvoltine strain 
C. nichi proved to be tolerant than the bivoltine strain NB4D2 (Joy and Gopinathan, 1995). Among bivoltines, NB4D2 exhibited better tolerance 
to environmental fluctuation both at laboratory and field conditions 
compared to new bivoltine hybrids, CSR2, CSR4, NP2, KSO1, etc. 
(Vasudha et al., 2006) in India. Between two productive breeds CSR2 
and CSR4, based on percent of hatching, over expression and inhibition 
of some protein synthesis, it was inferred that CSR2 was comparatively 
more tolerant than CSR4 (Manjunatha et al., 2005). Due to its better 
tolerance to heat shock, Zamood and Manjunatha (2002) suggested to 
use CSR2 as male parent PM as female parent for production of F1
hybrids for production of cocoons and silk. Whereas, the Chinese race Feng found to be most tolerant strain than Japanese races Kuo and J-09, but the Chinese race C-54 was most susceptible (Hsieh et al., 1995). Since the range and significance of individual adaptive reactions differs in different species under different environmental conditions, the level of tolerance in elevated temperature varies between polyvoltine and bivoltine strains/races of B. mori. This diversity could be, the races (species) living in hot and desert conditions for many years modified the molecular-biological mechanisms of adaptation, which made possible their normal life and reproduction under extreme conditions (Evgen'ev et al., 2005). However, the thermal tolerance of economically important organisms like silkworm to environmental fluctuations attains significance in field rearing and their performance in field/nature mainly depends on inbuilt (native) adaptability to varied environmental conditions, which is governed by molecular mechanisms of the cell. Notably, the polyvoltine silkworm strains exhibited better survivability over bivoltine strains due to their adaptation to thermal stress.

Further, heat shock response in different developmental stages of five bivoltine breeds NB4D2, NP2, KSO1, CSR2, and CSR4 was reported for the first time by Vasudha et al., (2006). Surprisingly, of the five instars young age silkworms that includes first, second and third instars were found relatively sensitive to any given heat shock temperature while late age silkworms tolerated high temperature for relatively longer period as also opined by Joy and Gopinathan (1995). During larval development thermotolerance increased in the order of first instar > second instar > third instar > fourth instar > fifth instar (Vasudha et al., 2006). Evidently, highest mortality (21%) was observed in the first
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In the fifth instar larvae of CSR4, while NP2 exhibited comparatively least mortality in the first instar and also 100% survivability in the fifth instar. Comparatively, the fifth instar larvae of *Manduca sexta*, another lepidopteran model species, exhibited 100% survivability at 42°C but full mortality as the heat shock temperature increased to 48°C (Fittingoff and Riddiford, 1990). While, Lohmann and Riddiford (1992) reported no mortality at 42°C (for one hour) and 100% mortality at 46°C in the case of pure mysore a tropical multivoltine strain of *B. mori*. Hsieh *et al.*, (1995) observed 100% mortality at 46°C (one hour) among few Japanese and Chinese silkworm strains. Whereas, the threshold temperature that induced 100% mortality was 40°C in *Drosophila melanogaster* (Lindquist, 1986), 45°C in *B. mori* strains NB4D2, NP2, KSO1, CSR2, and CSR4 (Vasudha *et al.*, 2006), 46°C in Chinese, Japanese (Hsieh *et al.*, 1995) and Indian (pure mysore - Lohmann and Riddiford, 1992; Joy and Gopinathan, 1995) silkworm strains, 46°C in *Musca domestica* (Tiwari *et al.*, 1997), 48°C in *Manduca Sexta* (Fittingoff and Riddiford, 1990), 48°C in *Lucilia cuprina* (Tiwari *et al.*, 1995), and 50°C in *Locusta migrotoria* (Qin *et al.*, 2003). All these studies explicit that the heat shock treatment is employed to determine the level of thermostolerance based on mortality (Loeschcke and Sorensen, 2005) and demonstrated that it varies in different strains/races of silkworm and other allied insects as well. But no such informations are available from wild silk moths, *Antheraea mylitta* (Tasar), *A. assamensis* (Muga) and *Samia cynthia ricini* (Eri).

2.2.2 Expression and analysis of HSPs in *B. mori*

Evgen’ev *et al.*, (1987) for the first time, utilised the cells of *B. mori* and *A. pernyi* to study the effect of high temperature and synthesis of HSPs. While mulberry silkworm cells synthesized 83, 80, 74, 70, 68, 25,
and 23 kDa HSPs at temperature reaching 48°C, the gypsy moth cells synthesized no proteins at temperature 40°C and above and died under extreme conditions (Evgen'ev et al., 1987). Thus, the kinetics of HSP synthesis revealed distinct and reproducible differences between cell cultures of *B. mori* and the gypsy moth *Lymantria dispar*.

*B. mori* cells infected with NPV showed inability in synthesising HSPs under elevated temperature (Broude *et al.*, 1998). The HSP70 transcription initiation site was identified by cloning and sequencing of a gene encoding HSP70 in mulberry silkworm (Titareka *et al.*, 1990). Further, Lohman and Riddiford (1992) reported several HSPs, one approximately of 84 kDa, 2 proteins near 70kDa, 3 proteins of 31, 30 and 29 kDa and 3 proteins of near 26 kDa from epidermis of fifth instar larvae of *B. mori* at 42°C as the maximum tolerable temperature. Subsequently, Joy and Gopinathan (1995) have observed expression of 93, 46, and 28 kDa in PM, 93, 70, 46, and 28 kDa in *C. nichi* and 93, 89, 46, and 28 kDa from NB4D2. Further, tissue specific expression of HSP with molecular weight of 93, 89 and 70 kDa in fat body, 70 kDa in hemolymph and 93, 46 and 28 kDa in cuticle was reported from both polyvoltine and bivoltine silkworm strains (Joy and Gopinathan, 1995). In addition, Hsieh *et al.*, (1995) reported 70 kDa HSP from fat body cell and haemocytes of fifth instar larvae of Chinese and Japanese silkworm strains.

After a long gap of about ten years, Manjunatha *et al.*, (2005) have initiated the investigation on HSP in *B. mori* and observed expression of 30 kDa protein, which includes 27, 28, and 29 kDa HSP in the 3 day old embryos exposed to heat shock at 30°C. Thereafter, HSPs differentially expressed in response to heat shock in newly evolved bivoltine breed, NP2, KSO1, CSR2, and CSR4 was analysed in comparison with that of
NB4D2, which exhibited acclimation in the field over three decades (Vasudha et al., 2006). Only one set of HSP with a molecular mass of 90 kDa expressed in the first, second and third instars was reported for the first time. In addition, a HSP with molecular weight of 84 kDa found expressed only in fourth instar larvae of all breeds. Concomitantly, five different sets of 84, 62, 60, 47, and 33 kDa HSPs from the fifth instar larvae of NB4D2, KSO1, and CSR2 strains; three HSPs, 84, 47, and 33 kDa, in NP2 strain and only two HSPs, 84 and 47 kDa, in CSR4 strain heat shocked at 35 and 40°C for two hours were reported (Vasudha et al., 2006). Similarly, Rajesh et al., (2008) have reported induction of 90 kDa HSP in first, second and third instar larvae of four multivoltine (PM, Daizo, P2D1 and Nistari) and two bivoltine (NB4D2 and CSR2) silkworm strains heat shocked at 40°C. Expression of 84 kDa HSP in fourth instar larvae of all the silkworm strains and five different sets of HSPs 84, 62, 60, 52 and 33 kDa in fifth instar larvae of Daizo, CSR2 and NB4D2 were reported (Rajesh et al., 2008).

Landais et al., (2001) characterized the cDNA encoding 90 kDa HSP in B. mori and compared with Spodoptera frugiperda (both lepidopteran insects). These two cDNA encode 716 aa (amino acid) and 717 aa proteins in B. mori and S. frugiperda, respectively, with a calculated molecular mass of 83 kDa, which is similar to Drosophila. Unlike vertebrates, hsp90 is a unique gene both in B. mori and S. frugiperda genomes, which does not contain any introns. Comparison of aa sequences of B. mori and S. frugiperda with that of D. melanogaster, Homo sapiens, and S. cerevisiae revealed a high percentage of identity and phylogenetic relationship (Landais et al., 2001). In D. melanogaster, hsc70-4 (constitutive hsp gene family) was expressed at a high level in embryo, larvae, and adult, whereas, the hsc70-1 and hsc70-2 expression was
highest in adult but not detected in larvae. The hsc70-1 was expressed at a low level while no expression of hsc70-2 was observed in the embryo. In Chironomus tentans, hsc70 expression was obvious at all developmental stages but slightly lower in the embryo than older stages (Karouna-Renier et al., 2003).

The members of small heat shock protein (smHSPs or sHSPs) genes are less conserved when compared with those of major hsp gene families, but occur ubiquitously in a variety of organisms. These are involved in apoptosis besides protection against heat stress (Arrigo, 2005; Feder and Hofmann, 1999). In B. mori (strain p50), six genes encoding sHSP19.9, sHSP20.1, sHSP20.4, sHSP20.8, sHSP21.4, and sHSP23.7 were reported (Sakano et al., 2006) but their biological and commercial role is unknown. The B. mori sHSPs were classified into I, II, III and IV types based on the numbers of Cys residues and their positions (Hossain et al., 2010). The deduced amino acid residues of these sHSPs are quite similar to each other. The values of identity from the CLUSTALW multiple alignments are about 82, 80, and 80% between Pia25 and sHSP20.8, between sHSP20.8 and sHSP20.4, between sHSP20.4 and sHSP19.9, respectively. Besides the α-crystallin domain, the N-terminal XXLXDQXFG motif were commonly conserved in the sequences of these HSPs (Sakano et al., 2006). Further, reverse transcriptase - polymerase chain reaction (RT-PCR) revealed no difference in expression levels of smHSP genes in different organs (Sakano et al., 2006), but the increased amount of transcripts was obvious upon heat shock in B. mori strains p50 (Sakano et al., 2006), Nistari and NB4D2 (Velu, et al., 2008) which is found to be strain specific. The structural properties of sHSP19.9 and sHSP20.8 were studied in B. mori. The sHSP19.9 found to be aggregated while
sHSP20.8 was not aggregated by itself during incubation at 60°C. This aggregation was suppressed in the presence of DTT and at high ionic strength. This clearly demonstrated that sHSP19.9 could easily be differentiated from sHSP20.8 at low ionic strength in the absence of DTT, though they have some similar properties (Hossain et al., 2010).

2.2.3 Analysis of HSPs in B. mori by proteomic methods

Since most of the above studies were carried out following 1-DE for analysis of HSPs and to determine their size based on their molecular weight, the advances in proteomics tools and techniques necessitated for the identification and molecular analysis of HSPs in B. mori. Though, HSPs were identified in different tissues of normal silkworm larvae employing proteomic approach, the information's on HSPs expressed in response to heat shock either in cell/tissues or whole body of B. mori is scarce.

To date, there was only one literature available on induced HSP expression in B. mori. Here, Moghaddam et al., (2008) have identified about 20 and 12 differently expressed protein spots in response to heat shock (45°C for 30 mins) given to fifth instar larvae of Nistari (MV) and Jing-song (BV) strains respectively using 2-DE and mass spectrometry. Among them, 8 expressed protein spots were similar for both the breeds, 4 protein spots in BV and 12 protein spots specifically found expressed in MV breeds. These were identified as sHSP (HSP19.9, HSP20.1, HSP20.4, HSP20.8 and HSP21.4) and HSP70. The intensity of protein spots of sHSPs lower in the MV breed than in the BV breed at 45°C, while the difference between two breeds was not significant after 41°C HS (Moghaddam et al., 2008).
Kajiwara et al., (2005) used p50 silkworm strain grown under optimum environmental conditions (means that without heat shock exposure) for identification and analysis of proteins present in the midgut tissue of B. mori following 2-DE and mass spectrometry. They have identified a total of 158 proteins including HSP70 cognate 3 precursor, HSP70Bb, HSP cognate-1 and HSP70 cognate. Proteome analysis of silk gland in the silkworm strain with genotype of Y:C and phenotype of yellow blood and yellow cocoon of B. mori was performed. About 93 protein spots were analysed by 2-DE and MALDI-TOF MS and three protein spots were identified as HSP90, HSP70 and HSP60 along with other proteins in the silk glands of B. mori (Zhang et al., 2006). Further, shotgun strategy based proteome profiling was employed to uncover HSP21.4, HSP90, HSP60 and HSP1 from the head of B. mori silkworm strain p50 (Li et al., 2010). In addition, concerted efforts have been made to establish protein map for silk glands (Jin et al., 2004; Zhang et al., 2006), and midgut (Kajiwara et al., 2005) of B. mori larva using advanced proteomic methods and the silkworm proteome database was constructed (Kajiwara et al., 2006), which is accessible through KAIKO-2D database (http://kaiko2d.db.dna.affrc.go.jp/).

2.2.4 Biological and commercial traits of silkworm

Lohmann and Riddiford (1992) reported 100 % survivability from the HS induced silkworm larvae at 42°C, which spun normal cocoons. Whereas, 66% mortality was observed at 44°C HS and 100% mortality at 46°C as none of the silkworm larvae spun cocoons after heat shock exposure of fifth instar larvae of B. mori.

Exposure of newly hatched larvae of Chinese and Japanese silkworm races to HS at 44, 45, 45.5 and 46°C for 1hr showed 0, 2, 87 and
100 percent mortality respectively. However, polyvoltine strains were more tolerant to heat than bivoltine strains at the fourth and fifth instars. Among different BV strains investigated the Chinese race ‘Feng’ was the most tolerant followed by Kuo and J-09. Whereas, the cocoon shell weight percentage was not significant in PV strains but varied in BV strains. However, reduction in silk yields was observed in response to high and fluctuated temperatures (Hseih et al., 1995).

The Indian MV races (C. nichi and PM) showed better survivability compared to BV (NB4D2) strain after heat shock exposure at 41°C and above for 1 hr. Fifth instar larvae and pupae were more tolerant than early larval instars, adults or the eggs. Treatment of larvae at 41°C for 1 hr resulted in a variety of physiological alterations including increased heart beats, differential haemocyte count, enlargement of granulocytes and presence of additional proteins in tissues and hemolymph. Further, heat shock at 43°C and above proved to be lethal for all the developmental stages and strains of B. mori. Whereas, Joy and Gopinathan (1995) opined that there is no impact of HS on rearing parameters.

Manjunatha et al., (2005) adopted a new strategy for evaluation of thermotolerance in tropical silkworm strains wherein the whole eggs were exposed to different heat shock temperatures and recorded varied hatching percentage as an embryonic development index. Heat shock temperature between 35 and 45°C on early embryos reduced the hatchability, but 45°C and above acts as lethal by reducing the hatching percentage less than 50%. Contrastingly, the increased hatching over control was also recorded upon heat shock at 30°C for 1 hr at blastokinesis stage.
Further, Vasudha et al., (2006) examined thermotolerance of new bivoltine silkworm strains NB4D2, KSO1, NP2, CSR2 and CSR4 of *B. mori* after different instars larva were exposed to heat shock at 35°C, 40°C and 45°C for 2 hr followed by 2 hr recovery. Comparatively, better survivability was observed in NP2 than other strains. Resistance to heat shock at different instar larvae was analysed for the first time and reported in the order of first instar > second instar > third instar > fourth instar > fifth instar in all the silkworm strains. Impact of heat shock on commercial traits was also reported for the first time. Increased cocoon and shell weight to 17.52% and 19.44% respectively in heat shock induced population over control of NB4D2 was observed.

2.3 Purification of HSPs

Since, purification of HSP in *B. mori* has not been attempted so far, we have reviewed here the literatures available for other organisms in brief.

In 1982, for the first time, Welch and Feramisco described a purification protocol, which involved a series of ion-exchange and gel-filtration chromatographic steps (DEAE-cellulose, Sepharose 6B-CL and Sephacryl S-300 columns) for purification of HSP90, HSP72 and HSP70 from the soluble fraction of stressed HeLa cells. Subsequently, a new and rapid method was developed for purification of HSP70 from mammalian cells. Purification by DE52-cellulose ion-exchange chromatography followed by affinity chromatography on ATP-agarose resulted in considerable amount of HSP72 and HSP73. Localization of HSP70 was detected in the cellular system and reported the shift in HSPs localization from cytoplasm to nucleus during heat shock (Welch and Feramisco, 1985). Arrigo and Welch (1987) purified and
characterized sHSP28 from HeLa cells, which were heat shocked at 42.5°C for 90 min by ion exchange and gel filtration chromatography. Naoto et al., (1988) purified the HSP90 from Lymphoma tissues by DEAE-cellulose equilibrated with GTP, gel filtration through a Sephacryl S-300 and Mono Q columns.

HSP90 (Ullrich et al., 1986) and Tumor derived gp96 (Srivastava, 1997) were the first HSP preparations used in vaccination against cancer followed by the usage of HSP70 and calreticulin.

Itoh and Tashima (1990) successfully purified 105 kDa protein from bovine testis and brain tissue, wherein 40 - 80% saturated ammonium sulfate precipitate was applied onto DEAE-cellulose column followed by hydroxyapatite column. HSP90 was purified from yeast over producing plasmid by using DE52 and S-Sepharose (Ursula, 1995). Peng et al., (1997) by replacing ATP-affinity chromatography with ADP-affinity chromatography successfully purified immunogenic HSP70-peptide complex from tumor cell line. Although ATP-affinity chromatography was widely been used to purify molecules of the HSP70 family, but this procedure leads to dissociation of peptides from HSP70 molecules that resulted in HSP70 without immunological activity. Chu et al., (1997) purified HSP90 from porcine brain using DEAE cellulose HPLC column and 98% purity was achieved, which was confirmed by western blotting using monoclonal antibody raised against human HSP90. This observation suggested that small scale HPLC-purification of HSP90 from porcine brain tissue can be readily accomplished, with high yield, using convenient one step purification method.
The HSP60 and HSP10 were purified from *Methylobacillus glycogens* using DEAE-toyopearl (anion-exchange) and Sephacryl S-400 (gel-filtration) columns (Kawata *et al.*, 1998). The native molecular weights of each chaperonin protein as determined by HPLC-gel filtration were 8,20,000 for cpn 60 and 65,000 for cpn10. Antoine and Bell (2000) have purified multiple HSPs from single tumor sample in one step using heparin agarose chromatography. Following this procedure they have purified HSP40, HSP60, HSC70, HSP84, HSP86 and gp96 (grp94) and this procedure could separate HSP70 isoforms HSP70 and HSC70, but not the HSP90 isoforms HSP84 and HSP86. The three main immunogenic HSPs, gp96, HSP86/84, and HSC70 were further isolated to homogeneity using additional purification methods.

Recently, Skarga *et al.*, (2009) developed a simple and efficient method for the purification of HSP90 and a procedure for the simultaneous purification of several HSPs (HSP70/HSC70, HSP90 and HSP96) using thiophilic interaction chromatography from mammalian tissues and cells. One step thiophilic interaction chromatography of proteins resulted in a more than 80% purity and 85% yield of HSP90. The HSP90 revealed much higher affinity towards the T-gel than the other HSPs. All the HSPs were recovered with high yield and purity (90-99%). These results suggested that the thiophilic gel shall be highly efficient affinity matrix for the purification of HSP90 from cells and tissues of various animal species.

In view of the advances in the field of protein purification and biomedical importance, purification of HSPs from silkworm larvae has high significance and applications.
2.4 Proteases and their activity in silkworm, *B. mori*

Generally, varied classes of protease are known to possess different activities such as digestion, defense mechanism, histolysis in moulting, metamorphosis, homeostatic adjustments and behavior, digestion of cocoon shell for moth emergence (cocoonase) and growth and development of insects including silkworm (Kafatos et al., 1976 a & b; Hruska et al., 1973; Law et al., 1977; Sasaki and Suzuki, 1982; Eguchi and Kuriyama 1983; Yoshida and Ashida, 1986).

In recent years, the gut, particularly midgut, has been considered as a potent organ for studying function which acts as the major interface between the insect and its environment (Terra and Ferreira, 1994). The efficiency of utilization, absorption and conversion of food substances was determined by set of enzymes, their concentration and activity, which are involved in digestion and metabolism and facilitate normal growth and development of silkworm as in the other insects. The proteolytic activity in the digestive fluid was relatively high when compared to gut tissue or peritrophic membrane (Eguchi et al., 1982). These enzymes are mainly responsible for the breakdown of proteins from the ingested food into smaller compounds needed by the organism followed by degradation into cellular proteins for metabolic requirements. Alkaline protease activity in regurgitated digestive juice of fifth instar silkworm larvae was studied in detail by Sasaki and Suzuki (1982). In addition, the vitelline protease observed in the midgut of 8.5 to 9.5 day old embryo of *B. mori* was found to disappear completely in the larva while reappeared in the adult stage (Ikeda et al., 1991).
It is evident from the experimental evidences that enzyme activity differs in different silkworm races/breeds/strains. Comparatively, the alkaline protease activity in the midgut of BV was 2 to 3 times higher than the MV larvae (Sarangi, 1985). Whereas, high protease activity was reported in female larvae than male larvae of BV silkworm strains (Maribashetty et al., 2001). However, the F1 hybrids of MV and BV exhibited increased protease activity than its MV parent that resulted in improvement in silk output but decreased in F2 generation due to segregation (Sarangi, 1986). Even the sodium ions showed positive effect on midgut protease that facilitated for the release of enzymes from the gut lumen (Subramanya and Sarangi, 1989). Further, the protease activity in tissues of fifth instar larvae was influenced by age, sex and number of feeding/day given to silkworm *B. mori* (Jadhav and Kallapur 1988). In addition, starvation induced changes in midgut protease activity during V instar was evident while silkworm larvae starved for longer duration showed decreased protease activity, which increased significantly as the duration of starvation prolonged (Roopa et al., 2001). Isaiarsu et al., (2003) was also noticed that the dietary supplementation of amino acid glycine increased the protease activity in the fifth instar larvae of BV hybrid CSR18 x CSR19. The protease activity was found high in the mid gut of silkworm larvae fed with coarse leaves compared to medium coarse and tender leaves (Kalle and Sarangi, 2005). Besides, the level of protease activity was also influenced by season while high protease activity reported in post monsoon followed by monsoon, low activity was observed during pre monsoon seasons in BV and MV breeds of *B. mori* (Anil Kumar and Kalpana, 2008; 2009).
Hence, the proteolytic enzyme has been used as marker to determine the productivity of different silkworm strains (Krylova, 2005).

However, till date about fourteen proteases were reported from silkworm, *B. mori* following conventional methods but not by zymography. Katsumi *et al.*, (1995) identified the serine protease in zymogen form in silkworm body. Later vittellin degrading protease, which plays an important role in vittellin utilization during embryo development, was reported from the egg of *B. mori* (Ikeda *et al.*, 1997). Concomitantly, 30 kDa yolk degrading protease from silkworm egg was reported by Maki *et al.*, (1997). Kotani *et al.*, (1999) characterized a highly basic protease from silkworm digestive juice and showed that this enzyme was responsible for fast degradation of leafy material during larval stage. 35K protease with molecular weight of 35 kDa was identified in the digestive juice of *B. mori* by Jiang (2000).

Further, Maki *et al.*, (2001) purified a protease 30KP, which was different from that of proteases reported in the silkworm egg. Nakazawa *et al.*, (2004) purified serine protease 2 (BmSP-2) with molecular mass of 24271 Da from the silkworm digestive juice, which showed strong antiviral activity against *B. mori* nucleopolyhedrovirus. The BmSP-2 gene was not expressed in moulting and wandering stages indicating that the gene was hormonally regulated.

Based on the available literature, it was believed that metaloproteases were absent in insects including *B. mori*. But, Ote *et al.*, (2005) demonstrated the presence of ADAM type of metaloproteases domain in silkworm. These proteases were found distributed in different parts of the body and varied in different developmental stages.
like those of proteases that existed at egg stage but not seen in adult body. Similarly, proteases that are necessary for digestion of leaf may not be present in egg and pupae.

Keeping these findings and gap in view, zymography technique was employed in the present investigation not only for identification of proteases present in the whole body of *B. mori* but also to elucidate its activity under normal conditions and due to heat shock as a consequence of fluctuated environmental conditions that silkworm larvae experiences during rearing in the field.