CHAPTER 3

IDENTIFICATION AND SEQUENCING OF STRESS PROTEIN (HSP) IN ARTEMIA PARTHENOGENETICA

3.1. Introduction

Living organisms respond at the cellular level to unfavorable conditions such as heat shock or other stressful situations of many different origins by the rapid and transient acceleration in the synthesis of class of proteins known as heat shock proteins (Hsps) or stress proteins. Originally they were termed the heat-shock response because of the synthesis of these proteins following hyperthermia. Now, these proteins are known to be induced by diverse classes of physical and chemical agents (including metals Cd, Zn, Hg, Pb and Cu), oxidizing agents and drugs affecting respiration and energy metabolism (arsenite, anoxia, H₂O₂ and KCN). These Hsps are highly conserved and found in all organisms, from archaebacteria to eubacteria, yeasts, plants and vertebrates, including humans (Morimoto et al., 1990; Sanders, 1993). Some Hsps are constitutively expressed under normal physiological conditions; others are expressed in response to stress and are induced by temperatures approximately 5°C to 10°C greater than the optimum growth temperature of an organism (Lindquist, 1986).

Larvae of the brine shrimp *A.franciscana* serve as important feed in fish and shellfish larviculture. Combined hypothermic/hyper thermic shock followed by recovery ambient temperature induced Hsp70 synthesis in *Artemia* larvae. Exposure to abiotic stress was shown to shield *Artemia* larvae against infection by pathogenic *Vibrio*. The data support a casual link between Hsp70 accumulation induced by abiotic stress and enhanced resistance to infection by *Vibrio campbelli* (Sung *et al.*, 2008).
During the last decade more and more details of the biochemical function of Hsps have emerged. Hsps are the very important biochemical constituents because they are molecular chaperones and play primary roles in protein folding and biosynthesis. Molecular chaperones are proteins that fold other proteins but are not part of the final product (Ellis and Van der Vries, 1991). In addition, the molecular chaperones i.e., Hsps confer thermotolerance to cells and organisms by preventing denaturations (Luders et al., 1998) and aggregation of cellular proteins (Parsell and Lindquist, 1993). Hsps are associated with the normal folding of newly synthesized proteins (Kim et al., 1998). They are needed to protect cells from heat damage and they assist in normalization of functions during recovery (Nover, 1991; Vierling, 1991; Parsell and Lindquist, 1993; Morimoto et al., 1994; Nover and Scharf, 1997). As molecular chaperones, they help other proteins to maintain or regain their native conformation by stabilizing partially unfolded states (Craig et al., 1993; Hendrick and Hartl, 1993; Ellis and Hemmingsen, 1994; Martin and Hartl, 1997). Under normal non stressful conditions, molecular chaperones assist in the routine folding and compartmentation of newly synthesized proteins (Ellis, 1990; Hartl, 1996; Fink, 1999). During thermal stress, heat induced chaperones (Hsps), bind to thermally denatured proteins, thereby preventing their aggregation and providing an opportunity for them to re-fold into native functional states following restoration of normal body temperatures. Although the temperatures that trigger impression of Hsps vary among species according to their adaptation and acclimation temperatures (Hofmann and Somero, 1996; Feder and Hofmann, 1999; Tomanek and Somero, 1999) patterns of expression of Hsps show striking levels of conservation among species.
Three major aspects in the life cycle of a protein invoke chaperone proteins and are:

i. They ensure that nascent polypeptides emerging from the ribosome are kept in a folding competent state until the whole sequence information is available (Bukau et al., 1997).

ii. Since fully folded proteins cannot be translocated through membranes, chaperones are needed to maintain or create a partially unfolded form of proteins destined for the transport into mitochondria or plastids (Hohfeld and Hartl, 1994; Moore et al., 1994; Lubeck et al., 1997; Pfanner et al., 1997 and)

iii. They stabilize damaged proteins generated as a result of chemical or physical stress and thus facilitate renaturation and/or degradation in the recovery period.

Earlier studies indicate that members of different Hsp families act together in multisubunit complexes, so called chaperone machines (Hartl, 1996; Bukau et al., 1997; Bukau and Horwich, 1998) and different chaperone complexes may interact to generate a network for protein maturation, assembly and targeting (Frydman and Hohfeld, 1997; Johnson and Craig, 1997).

Under adverse environmental conditions, synthesis of these proteins increases and they act to repair and protect cellular proteins from stressor-induced damage and to minimize protein aggregation (Ellis, 1990; Martin et al., 1992). Stress proteins also facilitate the transport of badly damaged proteins to the lysosomes for break down.
At the cellular level, the regulation of the synthesis of stress-protein is closely linked to protein damage (Schlesinger et al., 1982). The extent of cellular damage to a particular tissue is dependent upon:

i. The distribution of the contaminant among tissue,

ii. The ability of each tissue type to detoxify the contaminant and thus minimize cellular damage, and

iii. The molecular mechanisms by which the contaminant exerts its toxic effects.

Since some Hsps assist in folding newly synthesized proteins to different cellular compartments, the house keeping functions of Hsps most likely account for the maintenance of these molecular chaperones in *Artemia parthenogenetica* despite a lack of environmental temperature.

The relative concentrations of stress proteins synthesize by tissues may differ significantly with the stressor used to elicit the stress response as a consequence of the interplay between these biological processes (Sanders et al., 1992). The differences in the synthesis of stress proteins may be useful in identifying tissues which are particularly vulnerable to damage by a specific stressor and in evaluating the extent of that damage.

Discovering the structures and functions of proteins in living organisms is an important tool for understanding cellular processes, and allows drugs that target specific metabolic pathways to be invented more easily. A wealth of information about a protein's function and evolutionary history can often be obtained from the primary structure (Berg et al., 2002). Protein structure is very complex and determining a protein's structure involves first protein sequencing.
The ability to determine the amino acid sequence of a protein was a major advancement in understanding protein structure and function relationships. In the era of biotechnology, sequence analysis still occupies a position of primary importance. Common uses for protein sequence data include design of DNA probes and/or PCR primers, verification of the identity of expressed recombinant proteins, production of synthetic peptide antigens and characterization of post-translational protein modification (Berg et al., 2002).

The two major direct methods of protein sequencing are mass spectrometry and the Edman degradation reaction. Edman sequencing is the preferred technique for determination of the N-terminal sequencing of proteins blotted to PVDF and analysis of radioactive samples. Edman Sequencing is a well-known technology with ever increasing sensitivity, speed and ease of operation (Berg et al., 2002).

Considering the above provided information, the present study was undertaken to identify and sequence the stress induced protein (Hsps) in *Artemia parthenogenetica* exposed to environmental, chemical and ayurvedic food stressors.
3.2. Materials and methods

Experimental design

Adult *A. parthenogenetica* collected from the stock culture were transferred to the laboratory culture tank and were acclimatized at 80 ppt salinity for a period of 5 days. During acclimation and experimental periods, they were fed with rice bran twice daily at *ad libitum*. Animals with well developed brood pouches ranging from 8 to 10 mm in size and 10 to 12 mg in weight were chosen for the experiments. These animals were segregated in four experimental groups for different stress treatments. Parallelly a control group was also maintained. Each group consisted of 150 animals. The first group was exposed to different salinities (20, 40, 120, 160 and 200 ppt) for 1 h stress. The second group was given the temperature shock at 17°C in 80 ppt and 120 ppt and also at 42°C in 40 ppt and 80 ppt for 1 h. The third group of animals was subjected to different concentrations of formalin (2% at 20 ppt and 80 ppt for 6 h; 3% at 20 ppt and 40 ppt for 1 h) and the fourth group animals were fed with *Aswagandhathı churanam* as the supplementary food in addition to rice bran at 3% in 80 ppt and 120 ppt for 1 h treatment. Considering the maximum survival and cyst induction of *A. parthenogenetica* at chosen stress factors, the experimental duration was fixed for assessing the synthesis of stress protein.

Sample preparation

After the experimental period, the stressed animals were collected from each group and were homogenized at low temperature with a hand homogenizer in 400 µl of PBS (Phosphate buffer saline). Simultaneously sample from the control group was also withdrawn and homogenized with 400 µl PBS. The homogenized samples were then centrifuged (Sigma 3K30 refrigerated laboratory centrifuge) at 10,000 rpm for 5 minutes and the resulting supernatant was collected and then stored at –20°C until use.
Estimation of protein

The total protein concentration in both control and experimental samples *A. parthenogenetica* were determined by adopting the method of Bradford (1976) with bovine serum albumin as standard. To 1 µl of the protein extract distilled water was added to make up the volume to 100 µl. Then 2.5 ml of Bradford reagent was added to the extract and mixed thoroughly and were allowed to stand for 5 minutes. The absorbency was read at 595 nm in a spectrophotometer (SHIMADZU UV-Visible Double beam spectrophotometer).

SDS-PAGE

SDS-PAGE is one of the most commonly used techniques in protein purification and for estimation of the molecular weight of polypeptide chains (Hames and Rickwood, 1984). Sodium Dodecyl Sulphate – Poly Acrylamide Gel Electrophoresis (SDS-PAGE) was preferred to separate the proteins extracted from the control and experimental samples (PBS, pH 7.2) by adopting the method of Laemmli(1970). A sandwich was made with two glass plates separated by spacer trips (1.5mm thickness). A 12% linear resolving gel and 2% stacking gel were used to separate various proteins. The resolving gel was prepared by adding the following.

Resolving gel

<table>
<thead>
<tr>
<th>Chemicals</th>
<th>Quantity</th>
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</thead>
<tbody>
<tr>
<td>30% Acrylamide solution</td>
<td>2.6 ml</td>
</tr>
<tr>
<td>Upper Tris Buffer – pH 6.8</td>
<td>1.6 ml</td>
</tr>
<tr>
<td>Double distilled water</td>
<td>2.2 ml</td>
</tr>
<tr>
<td>10% APS</td>
<td>40 µl</td>
</tr>
<tr>
<td>TEMED</td>
<td>10 µl</td>
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The above solutions were mixed gently but thoroughly and slowly poured into the glass plate sandwich. A fine mist of 0.1% SDS was layered onto the gel to exclude oxygen from inhibiting polymerization and to ensure an uniform flat gel surface and presence of sharp interface between the polymerization. At this time, the overlying SDS was decanted.

**Stacking gel**

A 2% stacking gel was prepared by mixing the following solutions and poured over the separating gel.

<table>
<thead>
<tr>
<th>Chemicals</th>
<th>Quantity</th>
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</thead>
<tbody>
<tr>
<td>30% Acrylamide solution</td>
<td>0.3 ml</td>
</tr>
<tr>
<td>Lower Tris Buffer – pH 6.8</td>
<td>0.45 ml</td>
</tr>
<tr>
<td>Double distilled water</td>
<td>0.72 ml</td>
</tr>
<tr>
<td>10% APS</td>
<td>14 µl</td>
</tr>
<tr>
<td>TEMED</td>
<td>4 µl</td>
</tr>
</tbody>
</table>

A Teflon comb of 1.5 mm thickness was inserted to form the wells. After polymerization, the comb was removed and the wells were rinsed with 0.1% SDS. After removing the basal strip, the glass plates with polymerized gel were fixed to the electrophoretic apparatus (Broviga, Balaji Scientific Services, Chennai, India). Care was taken to prevent trap of air bubbles while casting the gel. Protein samples were mixed with equal volume of sample loading buffer of pH 6.8 and kept in boiling water bath for 3 minutes and then loaded onto each well so that 100 µg of protein was loaded onto each well. Simultaneously molecular weight marker proteins were loaded for comparison. Electrophoresis was carried out for nearly 6 hours at a constant voltage of 30V in the region of stacking gel and 60V in the region of resolving gel (The Bio-Rad model 1000/500 constant voltage / constant current source). After the electrophoresis was completed the gel was removed.
from the sandwiched glass plates and was stained for detection of protein bands.

**Coomassie Blue staining**

After electrophoresis, the gels were stained with 0.2% Coomassie Blue R-250 overnight. Then, the gels were destained with destaining solution (NEO-LAB Gel shaker). Subsequently, they were stored in 7% acetic acid for the purpose of photography.

**Standard marker proteins**

Standard molecular weight marker proteins were purchased from Dalton Marker Proteins, Servo Company, USA. The polypeptide markers were run simultaneously to identify the different polypeptides and these markers are in the order of increasing migration i.e.

- β Galactosidase (MW: 116,000)
- Bovine Serum Albumin (MW : 66,000)
- Ovalbumin (MW : 45,000)
- Lactose Dehydrogenase (MW : 35,000)
- Restriction Endonuclease (BSP 981) (MW : 25,000)
- β Lactoglobulin (MW: 18,400)
- Lysozyme (MW : 14,000)

**Western blotting**

The development of protein transfer to nitro cellulose (NC) membranes, first described by Towbin et al. (1979) has lead to the characterization of many biologically important molecules. Western blot has been used to purify proteins. Western blot analysis of the desired proteins was carried out according to the method of Towbin et al. (1979). Protein extracted from the adult *A.parthenogenetica* (exposed to formalin induced stress and
temperature stress) was separated on a 10% SDS-PAGE. Immediately after
the electrophoretic separation of proteins, the gels were washed in distilled
water and soaked in Towbin’s transfer buffer pH 8.3 for 30 minutes. Simultaneously, a NC membrane of the same dimensions as the gel was
soaked and made wet from beneath in the Towbin’s transfer buffer for 30
minutes. The gel and NC membrane were then sandwiched between two extra
thick filter papers as follows:

Anode (Bottom) : Pre-wet filter paper
                Pre-wet membrane
                Equilibrated gel
Cathode (Top) : Pre-wet filter paper

The setup was then placed in the transfer cell (BIORAD POWERPAC
200 and Trans Blot semidry Transfer cell) and care was taken so that there
was no air bubble between the layers of the sandwich and alternatively the
bubbles were removed by rolling a pipette over the layers. The safety cover
was closed and the transfer was carried out for 15 minutes at 15V. After
transfer, the NC membrane was removed from the assembly and air-dried.
The non-specific binding of antibodies to proteins was blocked by incubating
the NC membrane in block solution containing 0.2% non fat dried milk
powder and 0.005% Tween – 20 in Tris buffer saline (TBS) of pH 7.5 for 3
hours with gentle shaking in a platform shaker. The membrane was then
washed twice in TBS- Tween – 20 for 10 minutes each with gentle shaking.
The proteins were probed with primary antibody to Hsp70 by incubating the
NC membrane with the antibody in 1 : 100 dilution with TBS in 0.2% milk
powder for about 12 hours. The excess antibody was removed by washing
twice with TBS-Tween – 20 for 10 minutes each with gentle shaking. The
primary antibody was again probed with the secondary antibody, goat anti-rat
IgG (1: 1000 dilution) tagged to the marker enzyme Hydrogen peroxidase.
The incubation was carried out for 3 – 4 hours with gentle agitation. The membrane was then washed twice in TBS- Tween –20 and twice again with TBS, each for 10 minutes with gentle shaking. The NC membrane was allowed to develop colour by the addition of the chromogenic substrate solution (4-Chloro-1-Naphthol) and was observed for the development of the purple colour. The NC membrane was transferred to distilled water to arrest reaction and photographed for further interpretation.

**Densitometric scanning**

After the identification and confirmation of the protein of interest, the quantity of stress proteins was measured using the scanner and the area of the respective peaks was marked down.

**Reagents**

**Phosphate – buffered saline: pH 7.2**

- Sodium dihydrogen phosphate : 780 mg
- Disodium hydrogen phosphate : 709.8 mg
- Sodium chloride : 876 mg
- Ethylene diamine tetra acetic acid : 30 mg
- Dithiothreitol : 1.54 mg
- Distilled water : 100 ml

**Bradford’s reagent**

- Coomassie Blue G 250 : 0.025 g
- 95% Ethanol : 12.5 ml
- 85% Ortho Phosphoric acid : 25 ml
- Distilled water : 212.5 ml
### 10% SDS

### 30% Acrylamide solution

<table>
<thead>
<tr>
<th>Component</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acrylamide</td>
<td>14.6 g</td>
</tr>
<tr>
<td>Bis-acrylamide</td>
<td>0.4 g</td>
</tr>
<tr>
<td>Distilled water</td>
<td>50 ml</td>
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</table>

### Upper Tris : pH 6.8

<table>
<thead>
<tr>
<th>Component</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris hydroxy methyl amino methane</td>
<td>3.025 g</td>
</tr>
<tr>
<td>10% SDS</td>
<td>2 ml</td>
</tr>
<tr>
<td>Distilled water</td>
<td>50 ml</td>
</tr>
</tbody>
</table>

### 10% APS

### Lower Tris : pH 8.8

<table>
<thead>
<tr>
<th>Component</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris hydroxy methyl amino methane</td>
<td>9.08 g</td>
</tr>
<tr>
<td>10% SDS</td>
<td>2.00 ml</td>
</tr>
<tr>
<td>Distilled water</td>
<td>50.00 ml</td>
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</tbody>
</table>

### Tank Buffer

<table>
<thead>
<tr>
<th>Component</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>SDS</td>
<td>1.00 g</td>
</tr>
<tr>
<td>Glycine</td>
<td>14.4 g</td>
</tr>
<tr>
<td>Tris buffer</td>
<td>3.00 g</td>
</tr>
<tr>
<td>Distilled water</td>
<td>1000 ml</td>
</tr>
</tbody>
</table>

### Sample loading buffer

<table>
<thead>
<tr>
<th>Component</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Upper Tris</td>
<td>1.25 ml</td>
</tr>
<tr>
<td>Glycerol</td>
<td>1.00 ml</td>
</tr>
<tr>
<td>β-Mercapto ethanol</td>
<td>0.50 ml</td>
</tr>
<tr>
<td>SDS</td>
<td>150 mg</td>
</tr>
<tr>
<td>Bromophenol blue</td>
<td>pinch</td>
</tr>
<tr>
<td>Distilled water</td>
<td>7.25 ml</td>
</tr>
</tbody>
</table>
Coomassie blue staining solution

Coomassie Blue r 250 : 1.00 g
Ethanol : 250 ml
Glacial acetic acid : 35.00 ml
Distilled water : 500 ml

Destaining solution

Acetic acid : 35 ml
Ethanol : 150 ml
Distilled water : 315 ml

Towbin transfer buffer

25 mM Tris : 3.03 g
192 mM Glycine : 14.4 g
Methanol : 200 ml
Double distilled water : 800 ml
Check the pH (not adjusted) 8.3

Tris buffered saline (TBS): pH 7.5

Tris : 1.21 g
Sodium chloride : 9.00 g
Double distilled water : 1000 ml

2% blocking solution

Skimmed Milk powder : 2 g
TBS : 100 ml
Substrate solution

a. 30 µg of 4-chloro-1-naphthol dissolved in 10 ml of methanol.

b. 30 µl of hydrogen peroxide (H₂O₂) dissolved in 40 ml of TBS.

Both a and b were mixed at the time of experiment.

Secondary antibody

(Anti-rat-IgG) was purchased from Gene Chemicals, Bangalore.

Isolation of Hsp proteins and N-terminal analysis

The control and experimental tissue samples of *A. parthenogenetica* were collected and subjected into 10% SDS-PAGE. After completion of electrophoresis cut two longitudinal strips from the sides of the slab gel and stained these with Coomassie blue, while keeping the rest of the gel on a glass plate in a refrigerator. After this, the stained side strips were lined up along the edges of the unstained gel and used as guide to cut out bands of interest from unstained gel as described in Hames and Rickwood (1984). After the bands of interest were localized, they were cut out with razor blade and washed (three times for 5 min.) with 2 ml of 250 mM Tris-EDTA buffer pH 7.2, and followed by three rinses of 5 min. with ultra-pure water (the gel slices were chopped finely or homogenized with a spatula in pieces of 2 – 4 mm). Then 1 ml of 7 mM Tris buffer pH 7.2 containing 0.1% v/v SDS (the ratio of buffer volume to gel piece volume was approximately 2: 1) was added. The samples were sonicated (Retamal et al., 1999) for 3 minutes in an ice bath (five and six passes of 30s), with a 3.0 mm probe sonicator (high intensity ultrasonic processor, 50 W model). To separate the sonicated gel from the extraction buffer, 1.5 ml of sample was applied to centrifuge (14,000 rpm for 10 minutes), obtaining a final volume of 1 ml protein free of gel matrix. Aliquots were removed for protein estimation. The partially isolated proteins (20 µg) were re-running and confirmed with Hsp specific antibody.
Isolated Hsp protein from *A.parthenogenetica* was subjected to 10% SDS-PAGE. Proteins were electrophoretically transferred to a PVDF (polyvinylidene difluoride) membrane. The membrane was lightly stained with Coomassie Brilliant Blue R-250 (0.025% in methanol : water 40 : 60), and de-stained with methanol : water (50 : 50). Individual bands were excised and sent for N-terminal sequence determination by automated Edman degradation to the Applied Bio-system sequencer at the University of Geyongsang National University, Jinju, South Korea. For internal sequencing larger amount of proteins were needed. A total of 0.5 mg of protein was loaded into sixteen wells of the 10% SDS-PAGE gel. Following electrophoresis, the gel was stained and destained as described above, and individual gel bands were cut out and digested with endoprotease Lys-C in situ. The resulting peptides were separated using an ABI 173A Microblotter Capillary HPLC and then collected onto a PVDF membrane for subsequence Edman sequencing. The obtained N-terminal sequence of *A.parthenogenetica* was further analysis with available data.
3.3. Results

Protein samples of *A. parthenogenetica* exposed to different stress treatment were analyzed on SDS-PAGE and stress proteins were identified. Western blot analysis and scanning densitometry were also performed to determine the relative amounts (quantities) of heat shock protein in each sample.

3.3.1. Salinity stress and protein synthesis

Plate 3.3.1. shows the pattern of electrophoretic profile of proteins of *A. parthenogenetica* stressed at experimental salinities.

Salinity stress resulted in the expression of new poly peptides with varied range of molecular weight. Compared to control (80 ppt) and low salinity (20 ppt), the number of poly peptides expressed was relatively less in *A. parthenogenetica* stressed at 40, 120 and 200 ppt salinities. In those organisms stressed at 160 ppt salinity, the number of poly peptides was little low when compared to those individuals exposed to control (80 ppt) and 20 ppt salinities. The densitometry analysis of protein profile of *A. parthenogenetica* also indicated the variation in poly peptides between organisms stressed at different salinities (Fig. 3.3.1).

Table 3.3.1. provides the data on Relative Front (RF), molecular weight (MW) and Raw Volume (RV) of protein profile of adult *A. parthenogenetica* stressed at low and high salinities. In control organisms maintained at 80 ppt salinity, fifteen poly peptides were noticed with the molecular weight range of 11.49 to 98.26 kDa. The corresponding Relative Front (RF) and Raw Volume (RV) values were respectively ranged from 0.99 to 0.102 and 168.75 to 25.82. In low salinity (20 ppt) stressed individuals, sixteen poly peptides were recorded with the molecules weight range of 11.36 to 98.82 kDa. Here the range of RF and RV registered were from 1.0 to 0.101 and from 165.62 to
26.52 respectively. In those individuals stressed at 40 ppt and 120 ppt ten poly peptides were recorded and their molecular weight was ranged from 11.49 to 149.23 kDa and from 11.45 to 141.76 kDa respectively. In these salinities the RF and RV were respectively ranged from 0.99 to 0.007 and from 159.41 to 35.21 in 40 ppt; from 0.997 to 0.018 and from 172.10 to 80.62. A.parthenogenetica stressed at 160 ppt salinity registered 14 poly peptides with the molecular weight range of 11.36 to 101.80 kDa. At this salinity the RF and RV of the poly peptides were ranged from 1.0 to 0.094 and from 171.83 to 38.77 respectively. In those individuals stressed at 200 ppt only eight poly peptides were recorded and the molecular weight was ranged from 12.13 to 48.90 kDa. Here the RF and RV values recorded were ranged from 0.98 to 0.32 and from 185.96 to 11.32.

3.3.2 Temperature shock and protein synthesis

The electrophoretic protein profile of A.parthenogenetica exposed to cold shock (17°C) at 80ppt and 120 ppt for 1h and also exposed to heat shock (42°C) at 40 and 80 ppt for 1 h along with control group are provided in plate 3.3.2. The patterns of polypeptide resolved in experimental samples stressed at high temperature provided the evidence of heat shock response. Further the pattern of protein expression in heat shocked A.parthenogenetica was distinctly differed from that noticed in control group. The densitometry analysis also revealed the temperature induced variation in expression of polypeptides in A.parthenogenetica (Fig. 3.3.2).

Data on relative front (RF), Molecular weight (MW) and Raw volume of poly peptides in A.parthenogenetica shocked at low (17°C) and high (42) temperatures at the tested salinities are shown in table 3.3.2. In control group nine poly peptides with the molecular weight range of 11.48 to 133.67 kDa were noticed. The corresponding RF and RV range recorded were; 1.0 to 0.048 and 88.86 to 10.04 respectively. In low temperature stressed
individuals 15 number of polypeptides were noticed both at 80 and 120 ppt salinities. The molecular weight recorded ranged from 11.53 to 158.85 kDa and from 11.48 to 163.32 kDa respectively at 80 and 120 ppt salinities. Here the RF and RV range recorded varied from 0.99 to 0.012 and from 173.75 and 44.14 in 80 ppt respectively. In individuals shocked at 42°C in 40 and 80 ppt 24 and 20 polypeptides were noticed with the molecular weight range of 11.75 to 156.90 and 11.48 to 152.14 respectively. At this high temperature shock, the RF and RV values registered ranged from 0.99 to 0.014 and 96.021 to 41.52 at 40 ppt and from 1.0 to 0.021 and 100.31 to 68.64 at 80 ppt respectively.

3.3.3. Formalin stress induced protein synthesis

SDS-PAGE analysis of protein profile of *A. parthenogenetica* stressed at 2% formalin at 20 ppt and 80 ppt for 6h. and also at 3% formalin in 20 ppt and 40 ppt for 1h. are shown in plate 3.3.3. The results indicated the occurrence of formalin and salinity dependent polypeptides in adult *A. parthenogenetica*. The results on densitometry scanning also revealed the expression of several polypeptides with varied molecular weight (Fig. 3.3.3).

Table 3.3.3 provides the data on relative front (RF), Molecular weight (MW) and Raw volume (RV) of protein profile of *A. parthenogenetica* stressed in 2% formalin at 20 ppt and 80 ppt and also in 3% formalin at 20 ppt and 40 ppt. The results clearly indicated the variation in the pattern of polypeptides between control and formalin stressed individuals. For instance, in control group 11 polypeptides were expressed with the molecular weight range of 11.13 kDa to 147.79 kDa. The corresponding RF and RV recorded were 0.99 to 0.028 and 196.21 to 22.25. In 2% formalin stressed individuals 21 and 25 polypeptides were recorded respectively at 20 ppt and 80 ppt with the molecular weight range of 11.04 kDa to 137.82 kDa and 11.08 kDa to 161.59 kDa. The RF and RV ranges registered in 2% formalin stressed
individuals were 1.0 to 0.042 and 104.38 to 30.09 in 20 ppt and 0.999 to 0.009 and 199.30 to 63.07 in 80 ppt salinity respectively. But in those individuals stressed in 3% formalin at 20 ppt and 40 ppt, 19 and 21 polypeptides were registered. Here the molecular weight range registered was 11.22kDa to 148.79 kDa and 11.41 kDa to 151.67 kDa. Likewise the RF and RV range registered were respectively ranged from 0.99 to 0.026 and from 152.89 to 58.68 in 20 ppt salinity; from 0.98 to 0.022 and from 162.07 to 62.51 in 40 ppt salinity.

3.3.4. Herbal product induced protein synthesis

The variation in protein profile of *A. parthenogenetica* fed herbal product, *Aswagandhathi churana* at 80 ppt and 120 ppt including control are provided in plate 3.3.4. At the tested concentration of 3.0% *A. parthenogenetica* showed salinity dependent change in the protein profile. Densitometry analysis also showed the occurrence of polypeptides with varied trend (Fig. 3.3.4.)

Table 3.3.4 provides the data on relative front (RF), Raw volume (RV) and Molecular weight (MW) of polypeptides expressed in *A. parthenogenetica* fed with 3% ayurvedic product in 80 and 120 ppt including control groups. The results indicated the occurrence of 18 polypeptides with the molecular weight range of 12.09 kDa to 123.13 kDa in control group. The corresponding RF and RV values ranged from 1.0 to 0.053 and from 55.13 to 3.07 respectively. In 80 ppt reared experimental group, 18 poly peptides with the molecular weight range of 12.15 kDa to 58.27 kDa were registered. Here the RF and RV values ranged from 0.99 to 0.253 and from 140.62 to 17.93 respectively. But in 120 ppt reared experimental group, 20 polypeptides with the molecular range of 12.40 kDa to 142.94 kDa were noticed. The corresponding RF and RV values recorded for this group were; 0.992 to 0.018 and 147.04 to 12.12 respectively.
3.4. Western blot result

In the present study, monoclonal antibody raised against Hsp70 purified from freshwater prawn *Machrobrachium rosenbergii* was used as a reference standard in order to identify Hsp70 band amongst many other stress protein bands found in *A.parthenogenetica* samples. The SDS-PAGE profile of heat shock and formalin treated is shown in Plate 3.4.1.

Lanes 2 – 5 show the protein profile of heat shock (T₃ and T₄) and formalin (F₃ and F₄) treated samples of *A.parthenogenetica*; wherein many protein bands were detected. Identification of Hsp70 by referring to its molecular weight was also not possible as the carbohydrate content of the samples may alter its mobility in SDS-PAGE. Hence, western blotting experiment was carried out to identify Hsp70 band by means of antibody-antigen interaction as explained in materials and methods section. The expression of Hsp70 is detectable in western blotting and the result is shown in Plate 3.4.2.

In western blotting, denatured Hsp70 was immobilized on a membrane surface, the immobilization of Hsp70 probably enhance the binding of antibody to Hsp70 antigenic region. Therefore as compared to the immunoassay technique (non-immobilized), western blotting may serve as a better tool for detecting denatured proteins.

The quantified Hsp70 expression is summarized in Table 3.4.1. wherein the heat shock conditions T₃ and T₄ registered the values of 24923.68 and 24332.17 ng/100µg samples respectively. The chemical shocks (different concentrations of formalin) induced the expression of Hsp70 as 25171.44 ng/100 µg for condition F₃ and 27309.1 ng/100 µg for condition F₄. The control sample did not display any detectable quantity of Hsp70 expression.
Table 3.4.1. Quantified Hsp70 expression

<table>
<thead>
<tr>
<th>Stress</th>
<th>Hsp70 quantity (ng)</th>
</tr>
</thead>
<tbody>
<tr>
<td>T₃</td>
<td>24923.68</td>
</tr>
<tr>
<td>T₄</td>
<td>24332.17</td>
</tr>
<tr>
<td>F₃</td>
<td>25171.44</td>
</tr>
<tr>
<td>F₄</td>
<td>27309.10</td>
</tr>
</tbody>
</table>

3.5. Query Protein sequence

Amino acid sequence of Hsp70 fragment containing 81 amino acid was obtained by Edman degradation technique and is shown below.

MSKAPAIGIL GTTSCVGVF QHGKVEIIN DQGNRTTPSY TDRLIGDAAK NQVAMNPNT

IFKRLIGRRF EDATVQSDMK H
3.5. Discussion

The Hsp70 family of stress proteins is well known for its major function in coping with damage caused by high temperatures and other stressors in a wide variety of cells and organism (Bukau and Horwich, 1998; Karlin and Broechleri, 1998; Kiang and Tsokos, 1998; Krebs, 1999; Nollen et al., 1999). Most research on this Hsp – family has focused chiefly on cellular and molecular aspects (Morimoto et al., 1994; Fiege et al., 1996; Beissinger and Bucher, 1998; Fink and Golo, 1998; Lorimer and Baldwin, 1998; Richardson et al., 1998; Ellis, 1999, 2000). As pointed out in the review by Feder and Hofmann (1999) the focus is changing as more attention is given to the evolutionary and ecological aspects of this important response. Krebs (1999), Tomanek and Somero (1999) respectively reported on Hsp70 in larvae and adults of three species of Drosophila from different thermal environment and on marine snails from different locations in the intertidal. These studies provide special opportunities to find connection between levels of stress proteins thermotolerance and ecological setting. The comparative approach has several advantages, one of which involves the choice of organisms. Because of their particular ecological setting, Artemia, a primitive crustacean known as the brine shrimp is chosen for the present study. Of major importance in Artemia’s life history is the production of encysted dormant embryos (cyst) that can survive dessication, repeated bouts of dessication / rehydration and also the prolonged anoxia, when fully hydrated, temperature extremes and exposure to ultraviolet and other forms of radiation (Clegg, 1997; Clegg et al., 1999).

In the present study, heat shock has been shown to induce synthesis of Hsp70 family in A.parthenogenetica. The patterns of polypeptides resolved in experimental samples stressed at low temperature (17°C, 80 ppt and 120 ppt) and at high temperature (42°C, 40 ppt and 80 ppt) revealed the heat shock response. It showed the presence of high, intermediate and low molecular
weight heat shock proteins. At 17°C in 80 ppt, the intermediate Hsps recorded were 62kDa and 73 kDa and were related to Hsp70 family. Likewise at the same temperature in 120 ppt the intermediate Hsps recorded were 62kDa and 73kDa. Low molecular weight proteins expressed at 17°C were 11, 13, 17, 27 and 30kDa. At 42°C both in 40 ppt and 80 ppt more or less similar intermediate Hsps were recorded (61, 63, 65 and 77 kDa) and the low molecular weight proteins expressed were 11, 14, 16, 20, 27, 28 and 30 kDa. Further confirmation through Western blot indicated the expression of Hsp70kDa in adult A.parthenogenetica stressed at 42°C both in 40 ppt and 80 ppt (T₃ & T₄). Earlier laboratory acclimation experiments showed another approach in studying the relationship between temperature and Hsp expression. Acclimation to warmer water has been shown to change Hsp expression in some organisms. Sanders et al. (1992) found that the synthesis of Hsp in Mytilus edulis mussels was rapid and that endogenous levels Hsp70 remained elevated for several months in individuals maintained in the laboratory at warm temperatures. However, other acclimation attempts have not resulted in increase of Hsp expression. The lack of an acclimation response suggests that in some instances Hsp expression may be heavily influenced by factors other than temperature and hence the results of acclimation experiments must be carefully interpreted (Roberts et al., 1997). The observed increase in levels of 70 KDa Hsps in specimens of one Antartic species Trematomus bernacchii is a direct effect of increasing temperatures on Hsp expression in this species (Carpenter and Hofmann, 2000).

In a study on stress protein in Indian major carp Cirrhinus mrigala (Ham.) acclimatized at 25 ± 2°C. Das et al. (2005) indicated the presence of biochemically significant levels of stress by recording the high levels of Hsp70 viz. 1.2 – 1.3 ng/µg total proteins in kidney and gill and 4.2 ± 5.3 ng/µg total protein in liver and brain tissues. They also further inferred that, a heat shock at 37°C for 48 hr resulted in only two or three fold increase in
Hsp70 levels in these organs. In correlation with the above study adult *A. parthenogenetica* stressed at 42°C both in 40 ppt and 80 ppt resulted high levels of Hsp70 *viz.* 24 – 25 µg/100µg.

Sheller *et al.* (1998) reported that a decrease in protein synthesis in both the axoplasm and the sheath of medical giant axon (MGA) when heat shocked either by *in vitro* and *in vivo*. They also reported that when compared to the control axons, newly synthesized proteins of 72, 84 and 87 kDa appeared in both the axoplasm and the sheath.

The relationship between environmental temperature and Hsp levels has been observed in a number of other systems, eurythermal marine goby (Place and Hofmann, 2001) and marine intertidal invertebrates (Hofmann and Somero, 1995; Tomanek and Somero, 1999). In their study correlation was made between 70 KDa Hsp level and low environmental temperature.

Studying the expression of Hsp70 in *Ostrea edulis* after exposure to heat shock and heavy metals, Piano *et al.* (2004) reported that thermal stress (exposed to 38°C) caused the expression of a 60 kDa inducible isoform in gills and those exposed to Cd showed a significant enhancement of Hsp70 although there was no clear appearance of Hsp69.

Roberts *et al.* (1997) reported a strong induction of new Hsp70 in *Mytilus californianus* at body temperatures within the range measured in the field specimens and also inferred that there were significant differences in endogeneous levels of Hsp70 as a function of season and collection tidal height. Tomanek and Somero (2000) reported the synthesis of putative Hsps of size classes 90, 77, 70 and 38 kDa in congeneric marine snails (Genus *Tegula*) from different tidal heights. Buckley *et al.* (2001) reported that the induction threshold temperature for Hsp production in *Mytilus trossulus* was found to be strongly influenced by the organisms thermal history. The
induction threshold temperatures for Hsps70 and low molecular mass class was 23\(^0\)C in the mussels collected in winter and 28\(^0\)C in the mussels collected in summer.

Hsp can be induced by a number of physical and chemical factors that have the ability to denature the proteins in common. In the present study adult *A.parthenogenetica* exposed to 3% formalin in 20ppt and 40ppt indicated the presence of significant levels of stress by recording the high levels of Hsp70 viz. 25 – 27µg/100µg. Yamuna *et al.* (2000) reported that Hg and Cu induced synthesis of Hsp70 in the fresh water prawn *Macrobrachium malcolmsonii*. Earlier studies indicated that the exposure to trace metals have been found to induce the synthesis of Hsp70 in aquatic organisms such as *Salmo gairdnerii* exposed to arsenite (Kothary and Candido, 1982); *Oncorhynchus tshawtscha*, exposed to zinc and cadmium (Heikkila *et al.*, 1982); *Pimphales promelas* due to chromium (Edington *et al.*, 1989) and arsenite (Dyer *et al.*, 1993 a and b), and *Mytilus edulis* due to copper (Sanders *et al.*, 1994). Tissue specific differences in accumulation of stress protein have also been reported in *Mytilus edulis* exposed to a range of copper concentrations (Sanders *et al.*, 1994).

Induction of Hsp70 in liver and gonads of Zebra fish (*Danio rerio*) upon copper exposure was also reported by Hofmann and Valenica (2004). They also inferred that the regulation of Hsp70 expression was found to arise by heat (28 – 37\(^0\)C) and cold (28 – 20\(^0\)C) shocks in the same fish. Kohler *et al.* (1992) also reported the expression of Hsp70 after heat shock or exposure to heavy metal (lead) through fluorography in *O. ascellus*.

An essential step in the induction of synthesis of Hsps is the interaction between a transcription factor, the heat-shock factor (HSF) and highly conserved regulating DNA sequences termed heat-shock elements (HSE) found in the promotor regions of heat – shock genes (Morimoto, 1998). In the
present study, the effects of chemical stress (formalin) were tested to induce
Hsps on *A. parthenogenetica* and the result was as in the case of heat stress.
SDS-PAGE protein profile studies indicated the occurrence of intermediate
and low molecular weight proteins in adult *A. parthenogenesis* stressed at 2%
formalin in 20ppt and 80ppt. The intermediate Hsps recorded were 63, 69 kDa
respectively and low molecular weight proteins were 11, 14, 17, 18, 22, 24,
27, 28,29 and 30 kDa respectively. Similarly at 3% formalin in 20 ppt and
40 ppt (F₃ & F₄) the intermediate Hsps were 68 and 67 kDa. The other Hsp70
family related proteins registered were 71, 73, 74, 75 kDa along with low
molecular weight proteins (11, 13, 14, 17, 19, 24, 27, 28, 29 and 30kDa).
Among the above tested concentrations (2% and 3%) Western Blot confirmed
the presence of Hsp70 protein only at 3% concentration both in 20 ppt and
40ppt (F₃ & F₄).

The variation in Hsp expression in formalin induced
*A. parthenogenetica* is the result of both an increase in Hsp production of
existing forms and the induction of new forms of Hsp70 gene family.
Additionally it was also noticed that both the level of Hsp expression and the
number of isoforms in *A. parthenogenetica* increase during laboratory
acclimation to warm temperature. This study conclusively demonstrates the
constitutive expression of some forms of Hsps in *A. parthenogenetica* and
provides indepth information for understanding the importance of heat shock
proteins in *A. parthenogenetica*.

Clegg *et al.* (2001) reported that *A. franciscana* (f), *A. sinica* (s) and
*A. tibetiana* (t) contained detectable amounts of two isoforms of 70 kDa Hsp
in cyst and larvae. There is no apparent difference in number or pattern of
isoforms of Hsp70 in gill tissue among *A. tibetiana* and *A.franciscana*. The
above results on cyst Hsp70 are similar to those obtained by Miller and
Mc Lennan (1987) who carried out a detailed study of cyst and larval stress
proteins induced by heat shock containing several isoforms of Hsp70 and Hsp90 families (Hsp68 and Hsp89).

*Artemin* appears to be a stress protein (De Herdt *et al.*, 1979; De Graff *et al.*, 1990) since its synthesis is strongly regulated following prolonged anoxia, which suppresses protein synthesis in general (Clegg and Jackson 1998), and its primary structure bears some resemblance to another stress protein, ferritin (De Graff *et al.*, 1990). The small heat shock/α-crystalline protein p26 in the stress resistance of cysts undergo extensive stress induced translocations from the soluble phase of cell extracts to nuclei and other cell compartment (Clegg *et al.*, 1994; 1995; 1999; Jackson and Clegg 1996). Liang *et al.* (1997a, b) and Liang and Macrae (1999) reported that P26 exhibits molecular chaperon’s activity *in vitro* and probably functions that way *in vivo.*
3.6. Conclusions

Stress induced protein synthesis in adult *A. parthenogenetica* as a function of hypo (20 and 40 ppt) and hyper (120, 160 and 200 ppt) salinity stress, low (17°C) and high (42°C) temperature stress respectively at 80 ppt and 120 ppt and also at 40 ppt and 80 ppt for 1 h; 2 % and 3 % formalin stress respectively at 20 and 80 ppt for 6 h. and at 20 and 40 ppt for 1 h and also 3% supplementary feeding of *Aswagandhathi churanam* at 80 and 120 ppt for 1 h indicated the expression of high, intermediate and low molecular weight heat shock protein. These environmental (salinity and temperature) and chemical (formalin and ayurvedic product) stress have been shown to induce the synthesis of Hsp70 family protein in *A. parthenogenetica*. Western blot analysis using anti Hsp70 primary antibody and goat anti-rat IgG, the secondary antibody inferred that, hyper temperature shock 42°C at 40 and 80 ppt (T3 and T4) registered 24.92 µg and 24.33 µg Hsp70 per 100 µg sample; Likewise 3% formalin at 20 ppt (F3) and 40 ppt (F4) sample induced the expression of Hsp70 in the levels of 25.17 µg and 27.31 µg per 100 µg sample. The sequence analysis of Hsp70 through Edman degradation technique indicated the presence N – terminal fragment sequence with 81 amino acids.