Expression of heat shock protein 70 in Cirrhinus mrigala (Ham.) exposed to thermal variations

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ABSTRACT
The present investigation reveals the results of heat and cold shock expression of HSP-70 in different organs of Cirrhinus mrigala (Ham.). Fishes were exposed to heat and cold shock treatments and thermosensitive organs viz. liver, brain, kidney and gills were dissected out and tissues were analyzed for HSP70 quantification. At acclimatised temperature (26°C) the HSP – 70 level was highest in liver (10.35 ± 0.8704) and lowest in gills (1.96 ± 0.29). The highest HSP-70 levels were obtained in brain and liver, respectively during heat shock treatment at 37°C for 1 hr, 2hrs, 4 hrs, 22 hrs, 27 hrs, 44 hrs and 48 hrs whereas the lowest HSP-70 levels were obtained in kidney and gills, respectively. In cold shock treatment at 17°C for 18 hrs, 27 hrs and 48 hrs the highest HSP-70 levels were obtained in liver and brain, whereas the lowest levels were obtained in gills.

Introduction
Organisms respond at the cellular level to abrupt changes in their environmental conditions, such as temperature, by the coordinated increase in expression of small number of specific genes i.e, heat shock genes. The product of these genes, heat shock proteins, are involved in protecting the cells from the adverse effect of stress, allowing the cells to survive and recover. However, it has become clear that heat shock proteins are also present in stressed cells and are essential for normal function. Among the four major heat shock proteins in the HSP family, HSP-70 is the most prominent product of protein synthesis in temperature-stressed cells. Under adverse environmental conditions, synthesis of this protein (HSP-70) increases and they act to repair and protect cellular proteins from stress/or induced damage and to minimize protein aggregation. Meyer (1978) studied some physiological aspects of sublethal heat stress in the juvenile steelhead trout and cohosalmon. The present paper reports the expression of heat shock protein 70 in different organs of Cirrhinus mrigala(Ham.) exposed to thermal variations.
Materials and methods

Apparently healthy, disease free Cirrhinus mrigala (Ham.) ranging from 14-17 cm in length and 40-46 gm in weight were collected from nearby local market at Barrackpore, North 24-Parganas, West Bengal and acclimatized in laboratory condition in flow through system where the water temperature was approximately 26°C, for 2 weeks. Fishes were fed with tubifex larvae. Feeding was stopped prior to experiment.

Heat and cold shock treatment

The heat shock treatment was carried out at 37°C temperature for 1 hr, 2 hrs, 4 hrs, 22 hrs, 27 hrs, 44 hrs and 48 hrs of exposure while cold shock treatment was carried out at 17°C temperature for 18 hrs, 27 hrs and 48 hrs of exposure. In each experiment, (heat as well as cold treatment) 5 fishes were used. They were kept in thermostatic aquarium to maintain uniform temperature. Water was exchanged at every 2 hr interval. After a specified time duration (i.e. 24 hrs for acclimatized temperature, 1, 2, 4, 22, 27, 44 and 48 hrs for heat shock treatment and 18, 27 and 48 hrs for cold shock treatment), fishes were dissected and liver, brain, kidney and gills were carefully collected in ice-chilled PBS containing ATP. Then tissues were sonicated for 20 seconds at 75 watt. and 20 khz output. To prevent the protein denaturation, test tubes were kept in ice. Then the solution was centrifuged at -6°C to -2°C at 10000 x g for 10 minutes and the supernatant was preserved at -20°C before quantification.

Protein quantification

The protein quantification was done as per Lowry's method (1951).

HSP 70 quantification

HSP 70 (Sigma H9776, from borine brain) was serially diluted with ice-chilled PBS to a final concentration of 5 ng/ml. Each dilution was coated in triplicate wells in ELISA plate (Maxisorp, Nunc). Each well received 100 µl of diluted HSP 70 (lowest concentration for coating was 0.5 ng/well). Plates were covered and incubated overnight at 4°C. On the 2nd day, wells were washed thrice for 3 minutes each with 200 µl PBS. Then wells were blocked with 200ml PBS containing 3% BSA (Blocking step) and incubated for 2 hr. at 37°C. After another washing step (3 times PBS) the plate was incubated with anti-HSP 70 antibody (100 ml) at a dilution of 1:500 for 2 hr at 37°C. Subsequently, plate was washed thrice with 200 µl PBS containing 0.1% tween 20 and again incubated with anti-mouse HRPO conjugate (100 µl/well) at 1:1000 dilution for 1½ hr at 37°C. The plate was again washed 3 times with PBS containing 0.1% Tween 20. Then 100 µl freshly prepared substrate solution was added to each well and plate was incubated at 37°C for 18-20 mins. The plate was read in a 96 well multiplate reader at 450 nm wavelength. Background activity was determined in wells containing PBS instead of HSP / sample. A concentration vs. absorbance curve was prepared in mm graph paper.

Preparation of tissue homogenate

The tissues were kept in individual test tubes (containing PBS and ATP) and were sonicated for 20 seconds at 75 watt. and 20 khz output. To prevent the protein denaturation, test tubes were kept in ice. Then the solution was centrifuged at -6°C to -2°C at 10000 x g for 10 minutes and the supernatant was preserved at -20°C before quantification.

Estimation of HSP 70 in tissue samples

Same procedure was followed except that 100 µl of tissue supernatant was
coated in each well instead of HSP 70. The tissue homogenate was diluted with ice-chilled PBS without ATP to a final concentration of 20 µg protein / ml. The plate was read in a microplate reader at 450 nm wavelength. The HSP 70 concentration in tissue samples were extrapolated from absorbance values using the standard curve of HSP 70. The concentrations were expressed as ng of HSP 70/ µg of tissue protein.

Results and discussion

Effect of acclimatized temperature (at 26°C) on HSP 70

At acclimatized temperature (26°C) the highest HSP 70 level was observed in liver that was 10.38 ± 0.8704 ng/20 µg and the lowest HSP 70 level was observed in gills, that was 1.96 ± 0.2908 ng/20 µg. The values at acclimatized temperature were taken as control and the other two observations were made in comparison to this temperature. The HSP 70 level at acclimatized or room temperature is shown in Table 1.

Effect of heat shock (at 37°C) on HSP 70

The highest HSP 70 values were observed in brain for 1 hr, 2 hrs and 4 hrs of exposures and liver, for 22 hrs, 27 hrs, 44 hrs and 48 hrs of exposures, respectively. Whereas, the lowest HSP 70 levels were observed in kidney for 1hr, 22 hrs, 27 hrs, 44 hrs and gill for 2 hrs, 4 hrs and 48 hrs of exposure, respectively. The HSP 70 level at 37°C (heat stock treatment) is shown in Table 2.

The effect of heat shock treatment increases the HSP 70 level in liver during 22 hrs followed by a steady decrease upto 48 hrs. The sharp increase in HSP 70 depicts high metabolic activity at higher temperature. However, on chronic exposure HSP 70 level declined at 27 hrs, 44 hrs and 48 hrs of duration as shown in Fig.1.

The level of HSP 70 in brain rose from 10.96 (± 1.98) to 17.75 (± 0.9605) within 2 hrs of heat treatment.

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Table 1. HSP-70 level in different organs of Cirrhinus mrigala at acclimatized temperature (26°C)

<table>
<thead>
<tr>
<th>Duration (hr)</th>
<th>No. of fishes examined</th>
<th>Liver HSP-70 level (ng/20 µg)</th>
<th>Brain HSP-70 level (ng/20 µg)</th>
<th>Kidney HSP-70 level (ng/20 µg)</th>
<th>Gills HSP-70 level (ng/20 µg)</th>
<th>Remarks</th>
</tr>
</thead>
<tbody>
<tr>
<td>24</td>
<td>5</td>
<td>10.38 ± 0.8704</td>
<td>7.52 ± 0.3868</td>
<td>2.0 ± 0.3406</td>
<td>1.96 ± 0.2908</td>
<td>Fishes were healthy, kept in flow through system and fed by tubifex larvae</td>
</tr>
</tbody>
</table>

Values expressed in Mean ± S.D.
TABLE 2. HSP-70 level in different organs of *Cirrhinus mrigala* during heat shock treatment (37°C) at different durations.

<table>
<thead>
<tr>
<th>Duration (hr)</th>
<th>No. of fishes examined</th>
<th>Liver HSP-70 level (ng/20 µg)</th>
<th>Brain HSP-70 level (ng/20 µg)</th>
<th>Kidney HSP-70 level (ng/20 µg)</th>
<th>Gills HSP-70 level (ng/20 µg)</th>
<th>Remarks</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>5</td>
<td>4.78 ± 0.0829*</td>
<td>10.96 ± 0.198*</td>
<td>2.44 ± 0.277</td>
<td>3.88 ± 0.8288*</td>
<td>Fishes were healthy</td>
</tr>
<tr>
<td>2</td>
<td>5</td>
<td>10.94 ± 0.1293</td>
<td>17.75 ± 0.9605*</td>
<td>1.63 ± 0.1479</td>
<td>1.05 ± 0.118*</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>5</td>
<td>12.23 ± 0.2385*</td>
<td>16.45 ± 0.2693*</td>
<td>6.18 ± 0.0087*</td>
<td>1.95 ± 0.7228</td>
<td></td>
</tr>
<tr>
<td>22</td>
<td>5</td>
<td>15.23 ± 0.2947*</td>
<td>9.58 ± 0.3491*</td>
<td>3.10 ± 0.0707*</td>
<td>3.83 ± 0.0829*</td>
<td>Food intaking rate reduced</td>
</tr>
<tr>
<td>27</td>
<td>5</td>
<td>11.5 ± 0.0707*</td>
<td>8.1 ± 0.0707*</td>
<td>3.1 ± 0.0707*</td>
<td>4.08 ± 0.0829*</td>
<td></td>
</tr>
<tr>
<td>44</td>
<td>5</td>
<td>8.38 ± 0.0829*</td>
<td>7.83 ± 0.0829*</td>
<td>2.1 ± 0.1248*</td>
<td>3.2 ± 0.0707*</td>
<td>One of the fishes died</td>
</tr>
<tr>
<td>48</td>
<td>5</td>
<td>5.68 ± 0.0433*</td>
<td>5.08 ± 0.0829*</td>
<td>3.73 ± 0.0829*</td>
<td>2.98 ± 0.0829*</td>
<td>Two of the fishes died</td>
</tr>
</tbody>
</table>

Values expressed in Mean ± S. D.
*= The significant values.

Thereafter it maintained a more or less static level of expression. All these are in parity with role of brain in maintenance of thermal integrity. The decline in both liver and brain at 44 hrs and 48 hrs may be a response of acclimation at 37°C. In the present study it has been observed that, the chronic exposed fish failed to maintain the cell function integrity, for which, HSP 70 level dropped. At high temperature (37°C), when fishes were exposed for 48 hrs some of the fishes succumbed. Warm acclimation result in higher basal levels of HSP 70 and increases the threshold induction temperature of HSP in fish (Dietz, 1994).

In the present study, it has been observed that the concentration of HSP 70 in the tissues of kidney and gills were much lower than those of liver and brain. Thus, apparently, it seems that, the ability of heat tolerance and to control the changed metabolism with the physical stress are minimum in these two organs. That is why, they are affected (or, damaged) by heat as well as cold stress. However, it is known that 80 – 90% of circulatory heat is lost through gills. Being an exposed organ responsible for thermal homeostasis (atleast partially) it is unlikely that gills are more susceptible to heat and cold induced damage. Cellular functions, other than HSP, may be involved more in maintenance of protein structure and functional integrity. It is also noticeable that, HSP 70 expression increased in gills and kidney even at 48 hrs than those at acclimatized temperature (at 26°C). Currie et al. (2000) studied the effect of heat shock and acclimation temperature on HSP 70 in rainbow trout.

**Effect of cold shock (at 17°C) on HSP 70**

The cold shock treatment was carried out at 17°C for 18 hrs, 27hrs and
Expression of heat shock protein 70 in mrigal

**Table 3.** HSP-70 level in different organs of *Cirrhinus mrigala* during cold shock treatment (17°C) at different durations.

<table>
<thead>
<tr>
<th>Duration (hr)</th>
<th>No. of fishes examined</th>
<th>HSP-70 level (ng/20µl/0µg)</th>
<th>Remarks</th>
</tr>
</thead>
<tbody>
<tr>
<td>18</td>
<td>5</td>
<td>5.34 ± 0.0439*</td>
<td>Food intake reduced, fishes looked apparently healthy, water was changed at every 12hr</td>
</tr>
<tr>
<td>27</td>
<td>5</td>
<td>4.9 ± 0.1871*</td>
<td></td>
</tr>
<tr>
<td>48</td>
<td>5</td>
<td>3.45 ± 0.0476*</td>
<td>Food intake much reduced, very sluggish movement</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5.16 ± 0.1192*</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>5.73 ± 0.0829*</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>2.44 ± 0.0828*</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>2.8 ± 0.0024*</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.63 ± 0.1090</td>
<td></td>
</tr>
</tbody>
</table>

Values expressed in Mean ± Standard deviation.

*=* The significant values.

Significant at P < 0.05

48 hrs of exposures. The highest HSP 70 level were obtained in liver, for 18 hrs and 27 hrs and brain for 48 hrs of exposure, respectively. Whereas lowest HSP 70 level were obtained in gills for 18 hrs, 27 hrs and 48 hrs of exposures, respectively. The values of HSP 70 level during cold shock treatment is shown in Table 3.

As per Morcillo and Dietz (1996), heat shock puffs persists until about 7 hrs of recovery, indicating that the...
synthesis may be active after removal of the stressor. After denovo synthesis of HSPs has stopped, a gradual decrease in HSP levels occurs, as the proteins are broken down.

References


