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Full Length Research Paper

Real-time quantitative (PCR) applications to quantify and the expression profiles of heat shock protein (HSP70) genes in Nile tilapia, *Oreochromis niloticus* (L.) and *Oreochromis mossambicus* (P.)

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Quantitative real-time PCR (qRT-PCR) has already been used to study the expression profiles of heat shock protein (Hsp) genes in Nile tilapia, *Oreochromis niloticus* (Lineaus, 1752) and *Oreochromis mossambicu* (Peter, 1983). Young fish were exposed to heat stress for 5.5 h followed by qRT-PCR of Hsp70 mRNA, using tubulin (*tub*) as a reference gene and flourogenic dyes. Expression of Hsp70 mRNA peaked at 1 h of heat stress and decreased at 5.5 h. This method proved to be a very sensitive technique in quantifying Hsp70 transcripts in 6.70 ng of total DNA from the two tilapia species. A standard curve was prepared, for both tilapia fishes showed almost uniform results throughout the experiment. The threshold PCR cycle (Ct), at which RT -PCR products from DNA standards accumulated to a critical level, was determined for samples in the range of 2×10^4 to 2×10^{10} molecules/ l. From conclusion of this study, it is shown that when the tilapia fish muscles are subjected to stress, with the help of abiotic factors- temperatures, whether above or below optimum condition within a stipulated period of time in a peculiar way, will lead to the production of the Hsp70 protein.

Keywords: Hsp70, quantitative RT-PCR, *Oreochromis mossambicus*, *Oreochromis niloticus.*

INTRODUCTION

Like all heat shock proteins (HSPs) family, HSP70 has been recognized as a molecular chaperone, playing a central role in cell biology and biochemistry (Bukau and Horwich, 1998; Mayer and Bakau, 1998; Iwama et al., 1998). HSP70 and its co-chaperones, encoded by multi-gene family members, are developmentally regulated and differentially expressed in response to temperature stress and also, to other conditions that interrupt normal protein folding or favour protein denaturation (Schlesinger, 1990; Sanders, 1993) . Many Hsp 70 genes are strongly and rapidly induced at 37 - 45°C within from a period of 30 min to 2 h (Liu and Saint, 2002).

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Under adverse environmental conditions, the new synthesis of stress HSPs -70 increases, and it takes on new but related role to protect the cell from other adverse effect. Sambrook (1992), Basu et al. (2002) described the functions of HSPs70 in various aspects of fish physiology, including development and aging, stress physiology, endocrinology, immunology, environmental physiology, acclimation then with stress tolerance. Molina et al. (2001) reported that a tilapia heat shock protein in (Hsp70) promoter in tilapia, *Oreochromis mossambicus* promoter is able to confer the heat shock response on a reporter gene after transient expression, both in cell culture and in microinjection process.

The most widely studied member of the Hsp families, especially in response to proteotoxic is the 70 kDa (Hsp70) family of proteins (Feng et al., 2003; Luciana et al., 2006). Basu et al. (2003) reported that a physiological

response as well as an inducible cellular stress response occurs in Hsp70 family. It is only this specie that is subjected to these stressful conditions, for this has not been observed in the cellular and tissues-responses of other teleosts species.

In real time, reverse transcription polymerase chain reaction PCR (RT -PCR) is a variant of polymerase chain reaction (PCR), a laboratory technique commonly used in molecular biology to generate many copies of a DNA sequence and a flourogenic dye, which is used to continuously monitor product accumulation. With this method, the initial concentration of template DNA is assessed by using the number of PCR amplification that enters the exponential phase. Other methods such as northern hybridization, RNase protection assay and *in situ* hybridi-zation have been used for quantification of gene expression. However, RT-PCR has many advantages over these methods, including analysis time and sensitivity, specificity, case of use and reproducibility (Freeman et al., 1999; Liu and Saint, 2002; Radonic et al., 2004; Klein, 2001).

MATERIALS AND METHODS

Primers for RT-PCR

Primers were designed with the help of primer 3 program (http: frodo.wi.mit.edu/cgi-bin/primer3/primer3-www.cgi). Specific PCR primers for the HSP70 protein corresponding gene were designed on a muscle part of both fish Hsp70 gene (data not published) - A sense strand primer - ß - Actin (ACTIN) primer sequence of Forward: 5'-ATTGCTGGAGCTCTTCTGGA-3' and anti sense 5'-CAGCGGTGTACTGATGGATG-3'. Then second degenerate primers for the tub gene were designed on known tub sequences, ESTs found on the Internet databases: EBI (http://www.sbi.ac.uk/Databases/) and NCBI (http://www.ncbi.nlm.nih.gov/) – A sense strand primer was Tub A1f 5'-CAAGGAGGAAGGATCTGGAA- 3' and antisense primer TubA1r 5'-TGCATGCAGACCTGTAAAGC-3'

Animal

The present study was undertaken, using tilapia, *Oreochromis niloticus* (Linaeus, 1972) and common tilapia, *O. mossambicus* (Peters, 1983). They were used because of their wide availability and suitability as a model for analysis of Hsp70 proteins. The fish size 10 ± 2.11 to 13 ± 1.80 g was acclimated to the laboratory tank. Fish were fed daily once with tilapia grown diet.

Heat shock exposure

Ten fishes were acclimatized for 72 h at the normal temperature of 28°C. These experimental fish were moved to a similar tank where the water previously heated up to 34°C, and then the fish were left for 2 h. Then the fish were moved back to the tank of normal temperature and again left for two to five hours. After the fish were anaesthetized by putting on the dry ice, then the flesh tissues were obtained from the fish directly. The tissues obtained directly with dry ice were kept under - 70°C for further use.

Heat stress

Muscle tissues (flesh) were collected from experimentally subjected fish. After taking five gram of such muscle tissues, they were analysed at 1 and 5.5 h stress initiation. After this, tissues were stored at - 40°C for further experiment.

DNA extraction

Grind muscles were grinded with lysis buffer and then 2 ml of RNA extraction buffer and also 2 ml of phenol with cold condition were added for 5 min. This crude extract was allowed to vortex for 15 s and centrifuged at 5000 rpm (2500 g) for 10 min. After that transfer, the upper layer was transferred to a fresh tube and mixed with an equal volume of phenol, and then extracted again with an equal volume of phenol. Again these contents were mixed thoroughly and centrifuged at 7000 rpm (2500 g) for 10 min. The upper layer after centrifugation was immediately transferred to a fresh tube, and to this extract an equal volume of mixed phenol-chloroform-isoamyl alcohol was added (25:24:1). It was then extracted after centrifuged at 8000 rpm (3000 g) for 10 min.

Again the upper layer was transferred to a fresh tube and then mixed with an equal volume of chloroform - isoamylalcohol (24:1). Then this cleared solution was centrifuged at 5000 rpm for 10 min. The upper layer solution was transferred to a new eppendrof (Genei, Bangalore), and then to this, 2.5 volume of ethanol and 0.1 volume of 3M Sodium acetate were added for purification purpose. These contents were inverted several times for thoroughly mixing of both upper and lower region. Then the sample immediately was incubated at -20^{O}C for 12 h, centrifuged at 15,000 rpm (19,000 g) for 30 min at 4^{O}C.

The supernatant was discarded after allowing the pellet to dry. Again this pellet was washed with double distilled water. Finally, the RNA concentration was determined.

Then to 20 ng of the above RNA solution, 5 µl of RNase free DNase I (1 U/ µl) (Qiagen), 10 µl of DNase buffer and 65 µl of distilled water were added after incubating at 37^{O}C for 45 min. To this 200 µl of distilled water was added, and also with an equal volume of phenol to the above solutions. The solutions of these contents were allowed to vortex for 15 s and centrifuged at 13,000 rpm (17,000 g) for 5 min.

Again, the upper layer was transferred to a fresh tube and extracted with an equal volume of chloroform-isoamyl alcohol (24:1). The solutions were mixed, and then the contents were centrifuged at 13,000 rpm (17,000 g) for 5 min. Finally, the upper layer was transferred to a fresh tube, and 2.5 volume of ethanol and 0.1 volume of .3M sodium acetate (pH 4.8) were added. The sample kept at -20^{O}C for 12 h were incubated. Then, these contents were centrifuged at 13,000 rpm (17,000 g) for 35 min at 5^{O}C. The supernatant was discarded, and then the pellet was allowed to dry, and to dissolve in 100 µl TE buffer. After that the RNA concentration was determined, using the UV spectrophotometer (IFS: SL171 Minispec).

Reverse transcription

5 µl of 10X Buffer RT (GENI, Bangalore), 2 µl of dNTPs (5 mM each), 0.5 µl Oligo-dt (10 µM), 0.25 µl RNAse inhibitor (10U/ µl), 1 µl sensiscript Reverse Transcriptase and RNAse – free water up to 20 µL were added to 50 ng of RNA. The mixed solution was allowed to vortex for 5 min after incubating the contents at 37°C for 60 min.

PCR

PCR analysis was carried out by using a PCC 200 PCR cycler. 5 µl of cDNA sample was mixed with 10 µl 5X Buffer (1X), containing $MgCl_2$ (1.5 mM) 2 µl dNTPsl dNTPs (0.2 mM each), 2 µl of 0.3 mM

Table 1. Results of gene expression in four samples (OMx, ON, ONx) of genetic material A (statistical analysis; Wilcoxon, *tub* p = 0.715, Hsp-P = 0.068).

Name of the sample	T	H	T/ H
OM	-	-	-
OM1	0.78	4.03	6.03
OM2	0.83	1.16	1.21
OMx	-	-	-
OMx1	1.05	7.93	5.63
OMx2	0.85	1.44	1.19
ONy	-	-	-
ONy1	0.90	5.31	6.02
ONy2	0.85	0.30	0.28
ONz	-	-	-
ONz1	0.60	8.93	13.14
ONz2	0.85	5.25	5.26

1, x1,y1,z1 indicates 1h of heat stress; x2,y2,z2 for 5.5 h of heat stress. T-fold of change of *tub* (reference gene) H-fold of change of HSP70, T/ H - fold of change of HSP70 corrected by the reference gene. OM-*O. mossambicus.* ON – *O. niloticus.*

of each primer, 0.25 µl Taq DNA polymerase (1.25U), water up to 50 µl. Thermal profile is as follows: 95°C for 2 min; then 30 cycles of 95°C for 30 s; 55°C for 40 s; t 72°C for 10 min; and 4°C, forever.

RT-PCR

Quantitative RT-PCR analysis was carried out with a Berkin-Elmer PCR cyler. Then 2.5 µl of cDNA sample (5.50 ng/ µl) was mixed with 2.5 µL 10X buffer (1X), 0.75 µl of Mgcl2 (1.5mM), 1.5 µl dNTPs (0.15mM each), 0.6 µl of 0.20 mM of each primer , 0.30 µl Platinum Taq DNA Polymerase (1.25U). The above ingredients were made of 25 µl doubled distilled water (sterile).

Thermal profile

Thermal profile was as follows: 90°C for 2 min; then 35 cycles of 90°C for 30 s; 55°C for 40 s; 72°C for 1 min; melting curve 60 - 90°C; hold 1 s; 72°C for 5 min extension; and 4°C, for ever.

RESULTS AND DISCUSSION

Initially, several primers were designed for *hsp*70 and tested using RT-PCR. The reaction was optimized by choosing the best primers and concentration of the remaining components of the master mix and by optimised the thermal profile. During optimization, it was necessary to determine the fraction of the RT-PCR signal that originated from primer- dimer or by-product production. This was established by plotting the melting curve. Finally, with the use of melting peak, the desired products were amplified, and then the segment was determined.

Before RT-PCR, RNase-free DNase was used to digest the all DNA contamination in the RNA samples. In all cases, a control experiment (that is assay in the absence of reverse transcriptase) was used to determine the fraction of the RT-PCR experiments, depending upon the choice of PCR primers. Other researches have used tub as a reference gene in quantification studies (Giulietti et al., 2001; Fronhoffs et al., 2002) However, tub expression had to be initially tested. This was accomplished by monitoring its expression in several RT-PCR reactions on many different samples (data was not given) (Schlesinger, 1990; Sanders, 1993; Bukau and Horwich, 1998). The stable expression proved that tub was suitable to be used as reference gene in the following experiments.

RT-PCR gene quantification is based upon the use of specific standards, which are serial dilutions of plasmid DNA including the gene under study and the reference gene, with known amount of number of molecules and on the construction of standard curves for both genes in the various fish species (Feder et al., 1999; Stordeur et al., 2002; Frohoffs et al., 2002) . The presence of the desired sequence was confirmed by digesting the plasmid *Escherichia coli* bacterium with the restriction enzyme *Eco*RI. And DNA was quantified. A standard curve was prepared for each gene by using serial dilutions of the *E. coli* plasmid DNA template. The threshold PCR cycle (Ct), at which RT- PCR products from DNA standards accumulated to a critical level, was determined for samples in the range of 2×10^6 molecules/µl to 2×10^{15} molecules/µl. Molina et al. (2000) find out first transcriptional analysis of a Hsp70 promoter from fish, revealing a powerful tool to direct controlled tissue-independent gene expression in fish. The standard curves for HSP70 and tub were linear throughout their range (Figures 1 and 2), indicating that the developed method is a reliable and sensitive tool for Hsp70 DNA quantitation in *O. mossambicus*.

RT-PCR reactions were performed on the experimental tilapia fish samples. To interpret our RT -PCR results on gene expression, the fold of change (T) of the reference gene - tub was calculated by dividing the gene copy number at 1 and at 5.5 h of stress to the gene copy number just before the heat stress. The fold change of (H) of HSP70 was calculated as above and the ratio T/ H gave the fold of change of HSP70 corrected by the Reference gene. According to our present results calculations, the expression on *tub* did not change at all during the 5.5 h of heat stress (Popgene > 0.05 for gene). In contrast, according to RT-PCR, the expression of *O. mossambicus* fish HSP70 was followed by expression pattern during the heat stress. Expression increased during the first 1 h and decreased at 5.5 h (Table 1).

Conclusion

In conclusion, this study demonstrates the use of RT-PCR to study Hsp70 expression in tilapia fish (*O. mossambicus and O. niloticus*) during heat stress and

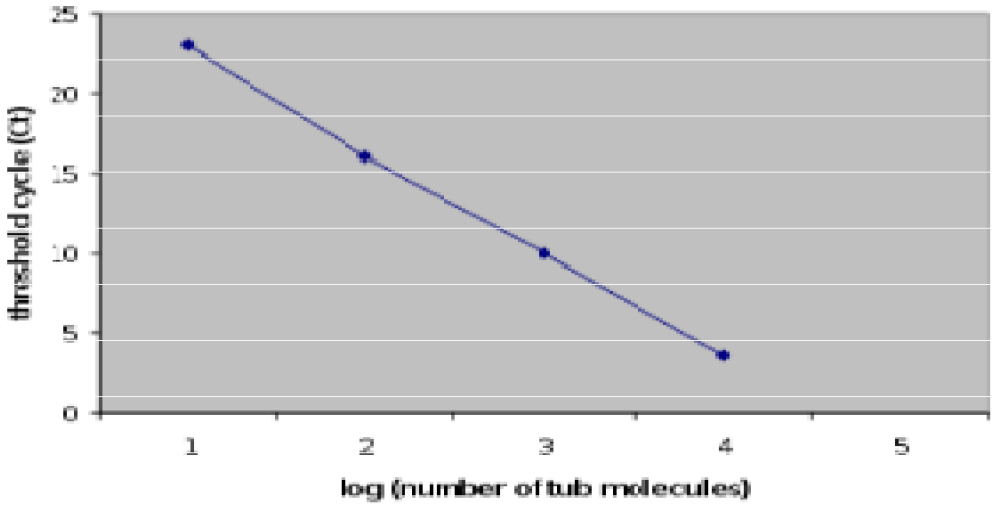

Figure 1. Standard curves for tubulin (*tub*) reference gene. Standard curve is generated by plotting Ct values against the logarithm of the number of DNA molecules indicating a precise linear relationship with a correlation coefficient of r^2 ~ 0.986 and ard curve was generated by threshold cycle values against the (based upon the quantitative estimation) number DNA molecules.

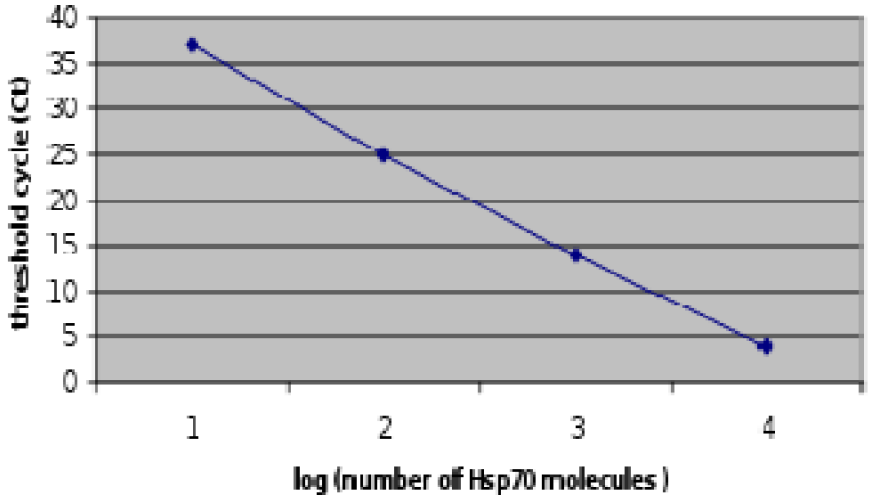

Figure 2. Standard curve for Hsp 70 gene Standard curve is generated by plotting Ct values against the logarithm of the number of DNA molecules indicating a precise linear relationship with a correlation coefficient of r^2 = 0.9743.

indicates that tub is an applicable reference gene for such study. Apart from the sensitivity, specificity of RT-PCR makes this method of quantification easy to perform and more economical than other approaches. This could be powerful molecular tool for future quantification studies between among HSP families or even among members of the same family in tilapia fishes. Results indicated that the two different species in tilapia fish did not show much more remarkable variations throughout the present experiment. Hence, we concluded that this RT-PCR was performed uniformly on both species. The standard curves for HSP70 and tub were linear throughout their range, indicating that the developed method is a reliable and sensitive tool for HSP70 quantitation in tilapia fishes.

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