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Effects of High Temperature Stress on mRNA Levels of Ubiquitin-activating Enzyme in Ovarian Tissue of Goldfish, *Carassius auratus*

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ABSTRACT

The stress effect of two different temperatures on mRNA level of ubiquitin-activating enzyme (E1) in ovarian tissue of goldfish was compared. For this purpose, PCR product of 600 bp of E1 was amplified in fish was kept at two different stress temperatures (2 h at+7°C and 2 h at+14°C). At the end of study, there was no any difference between the extent of RT-PCR bands of E1 at 2 h+7°C heat stress and the control group, there was not RT-PCR band of E1 at 2 h+14°C heat stress.

Key words: E1 enzyme, heat stress, RT-PCR, *Carassius auratus*

INTRODUCTION

The selective degradation of many proteins in eukaryotic cells is carried out by the ubiquitin-mediated pathway (Hershko and Ciechanover, 1992). The ubiquitin-activating enzyme (E1) plays an important role in the ubiquitination process and catalyses the first step of the ubiquitin-dependent proteolytic pathway (Haas and Rose, 1982). The initial reaction of protein ubiquitination involves the activation of ubiquitin by E1 in the presence of ATP (Hershko and Ciechanover, 1998; Sutovsky *et al.*, 2002; Tokumoto *et al.*, 2000). This ATP-ubiquitin-dependent process is responsible for intracellular activities. It is also responsible for the removal of aged housekeeping proteins, cell cycle regulation by cycling degradation and cellular immune response by antigenic peptide processing. A single E1 appears to be responsible for the activation of ubiquitin required for all modifications (Myung *et al.*, 2001).

Genes encoding E1 from mammals, plants and fish have also been cloned and characterized (Hatfield and Vierstra, 1992; Sun *et al.*, 1997; Tokumoto *et al.*, 2000). Characterization of several mammalian temperature-sensitive mutants of E1 showed that cells defective in E1 may exhibit a variety of abnormalities including cell cycle arrest, inhibition in the degradation of short-lived and abnormal proteins and defects in a number of other cellular functions (Finley *et al.*, 1984; Kulka *et al.*, 1988; Ayusawa *et al.*, 1992; Mori *et al.*, 1993). According to some studies the E1 enzyme in mammalian cells is inactivated by high temperature (Hershko and Ciechanover, 1992; Mayer *et al.*, 1989; Deveraux *et al.*, 1990) but there is no information on to this topic in fish. The E1 gene from goldfish was cloned by Tokumoto *et al.* (2000). The amplified cDNA by

Tokumoto *et al.* (2000) is 4069 bp long and the clone encodes a protein of 1059 amino acid residues with a predicted molecular mass of 117909 Da.

This study was performed to clarify the effects of stress induced by two different high temperatures stress on mRNA levels of ubiquitin-activating enzyme (E1) in goldfish.

MATERIALS AND METHODS

Fish and experimental design: Sexually mature male goldfish (*Carassius auratus*) were obtained from a commercial supplier. Average weight of fish was 114.46±6.14 g. During the acclimation period, total 30 fish were held for two weeks in a 300 L water volume fiberglass tank. During this period, water temperature was maintained at 25°C by heaters, water was aerated with air pump and fish were fed with commercial feed. Following acclimation a group of 10 fish were transferred to 32°C (Experiment I) and another group of 10 fish were transferred to 39°C (Experiment II) and fish were kept at these temperatures for two hours. A third group of fish remained as a control group at 25°C. Afterwards, the three groups of fish were taken and anaesthetised by immersion in a 1 mg L^{-1} solution of MS 222 (tricane methanesulfonate) for about 20 sec. As a final step, the ovaries of fish were obtained and immediately frozen in liquid nitrogen and followed by transport to the laboratory within 5 min.

Design of primers: The genomic DNA (including intronic and exonic regions) sequences of goldfish E1 were described by Tokumoto *et al.* (2000). The number of primers was designed to amplify regions of the gene. These primers were manufactured by SIGMA and stored at -20°C until use (all chemicals used in study were provided by SIGMA). The nucleotide sequences of sense and antisense primers were given as: 5'-CAG TGG GCT CGT GAT GAG TTT GAG -3' and 5'-TTT GTG TCA TCG TCC TTT TCA AAC-3'.

Total RNA purification: Total RNAs from the ovaries of fish kept at three different temperatures were purified according to Chrigwin *et al.* (1979)'s method. RNA samples were stored at -80°C until reverse transcription-polymerase chain reaction (RT-PCR) was performed.

Reverse transcription-polymerase chain reaction (RT-PCR): Total RNAs were denatured at 65°C for 10 min and chilled on ice and RT-PCR was performed as follows: samples of 1 μg total RNA were reverse transcribed in a total volume of 50 μL, containing 0.5 M oligo-dT as a primer, 0.5 mM dNTPs, 10 mM dithiothreitol, 20 U Rnasin, 20 U AMV reverse transcriptase, 50 mM Tris-HCl (pH 8.4), 75 mM KCl and 3 mM $MgCl_2$. First strand cDNAs were obtained after 60 min at 42°C in a 50 μL reaction mixture (Polat and Nalbantoglu, 2000).

Following inactivation at 95°C for 10 min, 5 μL of first cDNAs mixture was used as a template and PCR was performed in a 100 μL reaction mixture containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$ and 0.001 mL gelatine in the presence of 200 mM of each dNTP mix, 0.5 μM each of sense and antisense primers and 1 U of *Taq* DNA polymerase. Amphification was performed in a Techne PHC-3 thermal cycler for 40 cycles (90 s of denaturation at 95°C, 90 s of annealing at 63°C, 150 s of extension at 72°C, followed by a final elongation step at 72°C for 10 min).

To visualise PCR products, 2% agarose gel was used. 0.60 g agarose was mixed with 30 mL 0.5 X TBE buffer and melted in microwave oven. Six microliter PCR markers, 30 μL PCR products were added to the agarose gel wells. Then, 5.0 μL ethidium bromide (10 mg mL^{-1}) were added into TBE, the gel was run for 2 h under 100 volts. PCR bands were visualised under UV illuminator and photographed by Polaroid camera (Fig. 1).

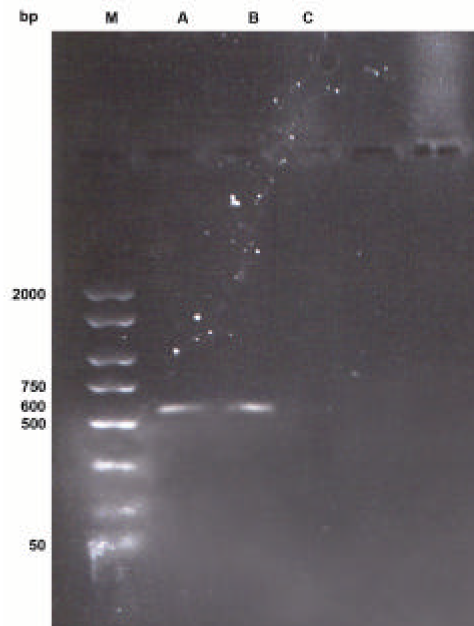

Fig. 1: RT-PCR products goldfish ovarian tissue, bp: Base pair, M: Marker, A: Control group (25°C), B: Heat stress experiment I (2 h at +7°C), C: Heat stress experiment II (2 h at +14°C)

RESULTS AND DISCUSSION

RT-PCR products synthesized from mRNA extracts of ovarian tissue of goldfish have been shown in Fig. 1. There are two clear bands, approximately 600 bp, as shown Line A (control group) and Line B (2 h at 7°C heat stress) but there is no Line C band (2 h at 14°C heat stress).

Extracellular ubiquitin-proteasome pathway is essential for ascidian fertilization, particularly in denaturation of proteinaceasus egg coat. It probably plays an important role in starfish oocyte maturation (Sakai *et al.*, 2004). In addition to functions of ubiquitin in reproduction system of vertebrates, there are some other vital functions such as control of events as diverse as cell cycle progression, a multitude of processes including cell differentiation, cell cycle, response to stress, chromatin modification in context of DNA repair, gene silencing (Baarends *et al.*, 1999; Hershko and Ciechanover, 1998; Varshavsky, 1997; Glotzer *et al.*, 1991), protein degradation and recycling (Ciechanover *et al.*, 1984) membrane receptor endocytosis (Strous and Govers, 1999) and even retroviral infection (Ott *et al.*, 1998).

The optimal temperature for goldfish is 23-25°C. The goldfish maintains vital functions such as reproduction and feeding up to 30°C. The goldfish is under high temperature stress at over 35°C and it's some vital activities interrupt at this level.

Stress factors appear to stimulate ubiquitin expression, including heat shock (Fornace *et al.*, 1989; Cheng *et al.*, 1994), respiratory stress (Cheng *et al.*, 1994), incubation with DNA damage agents (Cheng *et al.*, 1994; Fornace *et al.*, 1989; Treger *et al.*, 1988), starvation (Cheng *et al.*, 1994) and virus infection (Latchman *et al.*, 1987; Kemp and Latchman, 1988). For instance, Deveraux *et al.* (1990) showed that less than 10% of the E1 molecules present in ts85 cell at 31°C remain after heat stress (2 h at 39.5°C). But we have not found any on similar research in fish.

In a previous study, northern blot analysis of goldfish ovary tissue for E1 enzyme showed as a major band when it was compared with other tissues such as brain, eye, heart and kidney (Tokumoto *et al*., 2000). That is why ovarian tissues were used in our study. As seen from Fig. 1, RT-PCR amplification product of E1 in goldfish ovary tissue was seen at 2 h at 7°C heat stress (immediately change from 25 to 32°C). In other words, Line B was shown as a clear band. However, RT-PCR amplification product of E1 at 2 h at 4°C heat stress (Line C) was not clear (immediately change from 25 to 39°C).

In conclusion, a great deal of E1 mRNA level is interrupted under heat stress 2 h at 14°C and oocyte maturation may not continue but E1 mRNA level under heat stress 2 h at 7°C was the same with mRNA level of control group (25°C). Therefore, goldfish oocyte maturation continues under this heat stress.

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