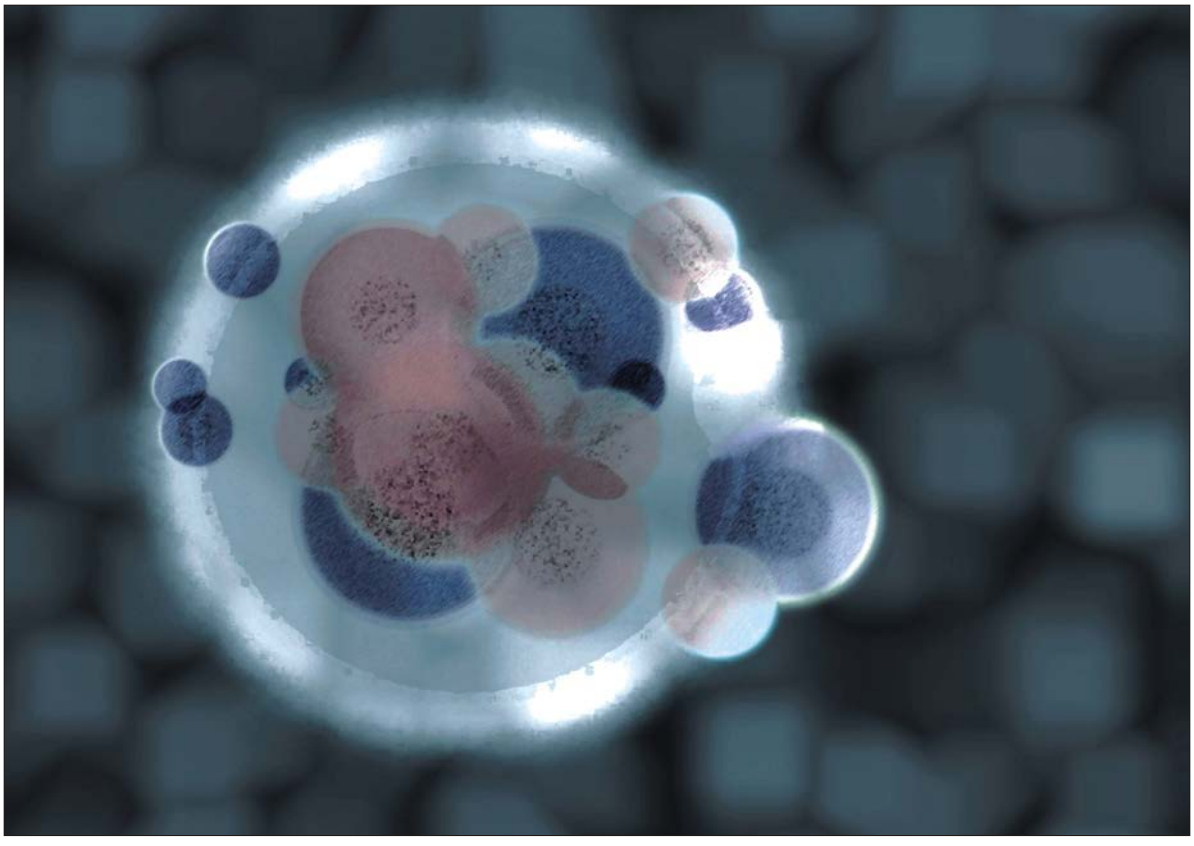

Journal of Biological Sciences

ISSN 1727-3048

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Journal of Biological Sciences 7 (5): 791-795, 2007
ISSN 1727-3048

Cloning and Expression of HSP70 and 90 mRNA from Bluegill *Lepomis macrochirus*

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Abstract: cDNA partial sequence of HSP70 and HSP90 were isolated from bluegill *Lepomis macrochirus*, with highly homologous to other teleost HSP genes. RT-PCR revealed that both HSP70 and 90 mRNA of bluegill showed large distribution with minor tissue-specific variation, but regardless of sex or seasonality. This result suggests that steady expression of HSPs would be advantageous of the acclimatization or survival in the environmental changes especially for poikilothermal animals that habit in wide ranges of different water temperature.

Key words: HSP70, HSP90, bluegill, teleost, mRNA, ecology

INTRODUCTION

Heat Shock Proteins (HSPs) are synthesized in response to various environmental stresses including heat shock. The major families of HSPs, HSP70 and HSP90 are most abundant and express in a negligible level in the absence of stress (Somero, 1995; Lai *et al*., 1984). HSP70 is known to assist the folding of nascent polypeptide chains, act as a molecular chaperone and mediate the repair and degradation of altered or denatured proteins (Kregel, 2002), whereas HSP90 supports various components of the cytoskeleton and steroid hormone receptors (Young *et al*., 2001). HSPs and heat shock response are highly conserved among phylogenetically divergent organisms (Basu *et al*., 2002), which attests to significant roles these molecules play in cellular function and adaptation.

For poikilothermal animals, temperature regulation of gene expression is certain to be critical for the survival and success in variable thermal environments. HSPs may play a role in physiological adaptation to wide ranges of temperature change, such as seasonal or diurnal shifts. The most common freshwater sunfish specie, bluegill *Lepomis macrochirus* Rafinesque is native to the middle and eastern regions of North America. It has been introduced to all over North America and other countries and its distribution is now confirmed in 20 countries (Froese and Pauly, 2006; Welcomme, 1992). Their successful invasion is thought to result from their omnivorous feeding habit and wide tolerance to different environments.

In the present study, partial sequences of HSP70 and 90 from bluegills were isolated and sex- and seasonal- specific expression; May (breeding season of bluegill), September and December, was assessed in the tissues of whole organisms to evaluate the role of HSPs in the tolerance to the seasonal environmental changes.

MATERIALS AND METHODS

Collection of experimental fish: Samplings of *L. macrochirus* were done by fishing on May, September and December 2006 at wetlands of Iwaki city, Fukushima prefecture, Japan. Immediately after captured, internal organs were extracted at collection site and soaked in 500 μL RNA stabilization solution RNAlater (Applied Biosystems, California, USA) in a 1.5 mL microfuge tube.

RNA extraction and cloning of the partial HSP genes: Total RNA was purified from brain, liver, reproductive organs (testis or ovary), heart, kidney, gill and muscle by RNeasy Mini Kit (Qiagen, Hilden, Germany). Reverse transcription of the RNA was performed by ThermoScript RT-PCR System (Invitrogen, California, USA). HSP70 cDNA and HSP90 cDNA were amplified by PCR with Takara Ex Taq Reaction Kit (Takara Bio, Shiga, Japan). Total PCR reaction volume of 30 μL was composed of 3.0 μL 10X Ex Taq Buffer, 3.0 μL dNTP mixture, 2.1 pmol of each primer, 0.8 units Ex Taq and 1.0 μL DNA solution containing 0.15 μg cDNA. Degenerate oligonucleotide primers used in PCR (HSP70-F; 5'-CARGAYTTYTTYAAYGGAAARGA-3', HSP70-R; 5'-CCCCCAGCACTYTGRTANAGKTT-3', HSP90-F; 5'-ATGCGCCAAGARGARGAGG-3', HSP90-R; 5'-CWGARAAGTGCTTGACAGCC-3') were based on previous reports (Palmisano *et al*., 1999; Deane *et al*.,

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2000). The profile of PCR conditions was as follows: initial denaturation at 95°C for 5 min; 35 or 40 cycles of denaturation at 95°C for 30 sec, annealing at 54°C for 30 sec, extension at 72°C for 1 min and a final extension at 72°C for 7 min. The PCR products of HSP genes were ligated into the pCR 2.1-TOPO vector by the use of TOPO TA Cloning (Invitrogen). The sequences of HSP genes were confirmed by DNA sequencing with ABI PRISM™ 3730xl DNA Analyzer (Applied Biosystems). The HSP gene sequences were compared with all other known gene sequences through a BLAST search. Similar DNA sequences were downloaded from the DNA Data Bank of Japan (DDBJ) and aligned with our sequences.

Evaluation of HSP expression: Detection of HSP70 and HSP90 transcripts were carried out using RT-PCR. At the same time, amplification of β-actin was performed and the product was used as an internal standard of RT-PCR. HSP specific oligonucleotide primers used in RT-PCR (RT-HSP70-F; 5'-GCTCAACAAGAGCATCAATCCAGAT -3', RT-HSP70-R; 5'- CTCCACAGTCGACTTCATGTTGAAA -3', RT-HSP90-F; 5'- CACCTTCTATTCCAACAAAGAGATC -3', RT-HSP90-R; 5'- TGAACTGGGAGTGCTTCTTGACAAT -3') were designed by sequence data of *L. macrochirus* HSPgenes reported in the present study. β-actin detective primers (Actin-F; 5'-CAATGGATCCGGTATGTGC-3', Actin-R; 5'-CGTTGTAGAAGGTGTGATGCC-3') were based on previous report (Naito *et al*., 1998). RNA extraction, cDNA synthesis and PCR amplification methods were as previously described.

RESULTS

cDNA partial sequence of HSP70 and HSP90: Two newly reported cDNA sequences were compared with the DDBJ database. *Lepomis* HSP70 partial sequence (751 bp, Fig. 1) showed 93.6% HSP70 sequence similarity to *Dicentrarchus labrax* Linnaeus (accession No. AY423555), 93.2% to *Rhabdosargus sarba* Forsskål (AY436786), 92.5% to *Paralichthys olivace* Temminck et Schlegel (AB006814), 88.0% to *Danio rerio* Hamilton (L77146), respectively. *Lepomis* HSP90 partial sequence (910 bp, Fig. 2) showed 94.8% HSP90 sequence similarity to *Dicentrarchus labrax* (AY395632), 91.4% to *Paralichthys olivace* (AY214170), 87.6% to *Oncorhynchus mykiss* Walbaum (AB196457), 87.0% to *Salmo salar* Linnaeus (AF135117), respectively. The cDNA sequence data reported in this study submitted to the DDBJ/EMBL/Gen-Bank database and were assigned the accession numbers AB296301 to HSP70 and AB296302 to HSP90.

Evaluation of HSP mRNA expression: RT-PCR revealed that *Lepomis* HSP70 and 90 mRNA were widely distributed in different organs (Fig. 3). HSP70 and 90 mRNA were found in brain, liver and gill regardless of

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GCTCAACAAGAGCATCAATCCAGATGAAGCTGTGGCCTATGGAGCTGCTGTCCAGGCTGCCATCCTGTCTGGTGACAAGTCTGAGAATGTCCAG
 L  N  K  S  I  N  P  D  E  A  V  A  Y  G  A  A  V  Q  A  A  I  L  S  G  D  K  S  E  N  V  Q

GACTTGCTGCTTCTGGACGTCACCCCTCTCTCCCTGGGAATTGAGACCGCTGGAGGTGTCATGACTGTCCTGATCAAACGTAACACCACTATT
 D  L  L  L  L  D  V  T  P  L  S  L  G  I  E  T  A  G  G  V  M  T  V  L  I  K  R  N  T  T  I

CCTACCAAGCAGACCCAGACCTTCACCACCTACTCTGACAACCAGCCTGGTGTGCTCATCCAGGTTTATGAGGGTGAACGTGCAATGACCAGG
 P  T  K  Q  T  Q  T  F  T  T  Y  S  D  N  Q  P  G  V  L  I  Q  V  Y  E  G  E  R  A  M  T  R

GACAACAACCTGCTGGGCAAGTTTGAGCTGACAGGCATCCCCCCTGCTCCTCGTGGTGTTCCACAGATCGAGGTGACGTTTGATATTGATGCC
 D  N  N  L  L  G  K  F  E  L  T  G  I  P  P  A  P  R  G  V  P  Q  I  E  V  T  F  D  I  D  A

AATGGCATCATGAATGTCTCTGCTGTAGACAAGAGCACTGGCAAGGAGAACAAGATCACCATCACCAATGACAAGGGTCGTCTCAGCAAGGAG
 N  G  I  M  N  V  S  A  V  D  K  S  T  G  K  E  N  K  I  T  I  T  N  D  K  G  R  L  S  K  E

GACATTGAACGCATGGTCCAAGAAGCTGAGAAGTACAAGGCTGAAGACGACGTCCAGCGTGACAAGGTGTCAGCCAAGAACGGCCTGGAGTCG
 D  I  E  R  M  V  Q  E  A  E  K  Y  K  A  E  D  D  V  Q  R  D  K  V  S  A  K  N  G  L  E  S

TATGCTTTCAACATGAAGTCGACTGTGGAGGATGAAAAGCTTGCTGGCAAGATCAGTGATGATGACAAGCAGAAGATTTTGGACAAGTGCAAC
 Y  A  F  N  M  K  S  T  V  E  D  E  K  L  A  G  K  I  S  D  D  D  K  Q  K  I  L  D  K  C  N

GAGGTCATTGGCTGGCTGGACAAGAACCAGACTGCTGAGAGGGATGAATATGAACATCAACAGAAGGAGCTGGAGAAGGTGTGCAACCCCATC
 E  V  I  G  W  L  D  K  N  Q  T  A  E  R  D  E  Y  E  H  Q  Q  K  E  L  E  K  V  C  N  P  I

ATCACC
 I  T
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Fig. 1: *Lepomis macrochirus* HSP70 cDNA partial sequence together with its deduced amino acid translation. GenBank DDBJ/EMBL/Gen-Bank database Accession No. AB296301

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CTGAGACCTTTGCCTTCCAGGCAGAGATCGCTCAGCTGATGTCCCTGATTATCAACACCTTCTATTCCAACAAAGAGATCTTCCTTAGGGAGTTG
  E  T  F  A  F  Q  A  E  I  A  Q  L  M  S  L  I  I  N  T  F  Y  S  N  K  E  I  F  L  R  E  L

ATCTCCAACGCCTCTGATGCTTTGGACAAAATTCGCTATGAAAGCCTGACTGACCCTACCAAGCTGGATAGTGGCAAGGATCTGAAAATTGAT
  I  S  N  A  S  D  A  L  D  K  I  R  Y  E  S  L  T  D  P  T  K  L  D  S  G  K  D  L  K  I  D

ATCATCCCCAACAAAGCAGACCGCACCCTGACCCTCATCGACACTGGAATTGGCATGACCAAAGCTGATCTGATAAACAACCTGGGTACCATC
  I  I  P  N  K  A  D  R  T  L  T  L  I  D  T  G  I  G  M  T  K  A  D  L  I  N  N  L  G  T  I

GCCAAGTCTGGCACCAAGGCCTTCATGGAGGCCCTGCAGGCTGGAGCTGACATTTCCATGATTGGTCAGTTCGGTGTGGGTTTCTACTCTGCT
  A  K  S  G  T  K  A  F  M  E  A  L  Q  A  G  A  D  I  S  M  I  G  Q  F  G  V  G  F  Y  S  A

TACCTTGTTGCCGAGAAGGTGGTTGTCATCACCAAACACAACGATGATGAGCAGTACGCCTGGGAGTCCTCTGCTGGAGGTTCCTTCACAGTA
  Y  L  V  A  E  K  V  V  V  I  T  K  H  N  D  D  E  Q  Y  A  W  E  S  S  A  G  G  S  F  T  V

AAGGTTGACAACGGTGAGCCCATGGGCCGTGGAACAAAGATTGTGCTGCACCTGAAGGAGGACCAGACGGAGTACGTTGAGGAGAAGAGAGTC
  K  V  D  N  G  E  P  M  G  R  G  T  K  I  V  L  H  L  K  E  D  Q  T  E  Y  V  E  E  K  R  V

AAGGAAATTGTCAAGAAGCACTCCCAGTTCATCGGCTACCCCATCACCCTGTTTGTGGAGAAGGAGCGTGACAAGGAGATCAGTGACGACGAG
  K  E  I  V  K  K  H  S  Q  F  I  G  Y  P  I  T  L  F  V  E  K  E  R  D  K  E  I  S  D  D  E

GCAGAGGAGGAGAAGGCAGAGAAGGAGGAGAAAGAGGAAGGTGAGGACAAGCCAAAGATTGAGGATGTGGGCTCAGACGATGAGGAGGACTCA
  A  E  E  E  K  A  E  K  E  E  K  E  E  G  E  D  K  P  K  I  E  D  V  G  S  D  D  E  E  D  S

AAGGATAAGGACAAGAAGAAGACTAAGAAAATCAAGGAGAAGTACATCGACCAGGAGGAGCTCAACAAGACCAAGCCCATCTGGACCAGGAAC
  K  D  K  D  K  K  K  T  K  K  I  K  E  K  Y  I  D  Q  E  E  L  N  K  T  K  P  I  W  T  R  N

CCTGACGACATCACAAACGAGGAGTATGGCGAGTTCTACAAGAGTCTGACCAACGACTGGGAGGATCACCT
  P  D  D  I  T  N  E  E  Y  G  E  F  Y  K  S  L  T  N  D  W  E  D  H
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Fig. 2: *Lepomis macrochirus* HSP90 cDNA partial sequence together with its deduced amino acid translation. GenBank DDBJ/EMBL/Gen-Bank database Accession No. AB296302

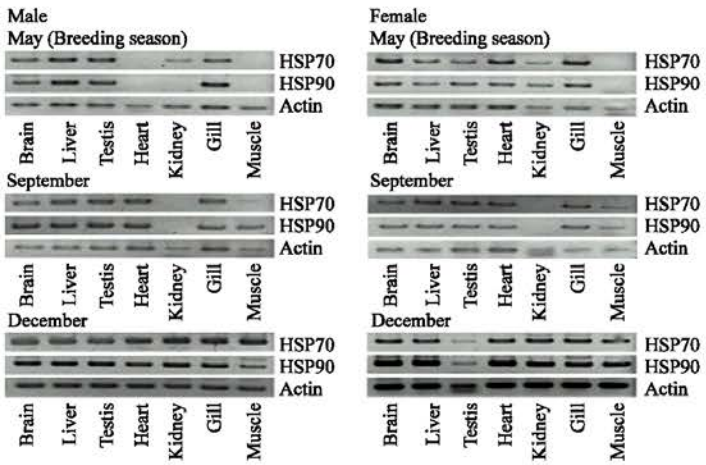

Fig. 3: Expression analysis in different organs at different season. RT-PCR amplification products for HSP70 and HSP90 fragments and the constitutive control of β-actin fragment

sex or seasonality. Although there were slight variations, they were also detected in gonad, heart, kidney and muscle and it seemed that there were neither sex- nor seasonal-specific expression. There was no apparent difference of tissue distribution between HSP70 and 90.

DISCUSSION

The present study reports the isolation and sequencing of cDNA partial clone corresponding to the *Lepomis* HSP70 and HSP90 that have highly sequence homology with other teleost species. And the comparison of the distribution of mRNA revealed that bluegill HSP70 and 90 mRNA were widely abundant in the tissues regardless of sex or seasonality including breeding season.

Numerous studies have been documented about HSPs mRNA expression induced by heat shock (Arai *et al*., 1995; Molina *et al*., 2000; Basu *et al*., 2002; Ojima *et al*., 2005). However, many of these were carried out using *in vitro* cell culture. Few studies were reported about the induction of HSP in the tissues of whole organisms in response to elevated temperature by the effect of naturally occurring variations in environmental

temperature. For example, the threshold induction temperature of HSP90 protein had a seasonal variation in the gobies *Gillichthys mirabilis* Cooper and *G. seta* Ginsburg (Dietz and Somero, 1992). Four marine teleost that habited in 10°C water showed tissue-specific variation in a specie and the wide variation in induction temperature among the species in the level of both HSP70 and HSP90 proteins (Dietz and Somero, 1993). As for mRNA expression, tissue-specific expression induced by heat shock was observed in embryonic zebrafish HSP70 and 90 mRNA (Krone *et al*., 1997) and semi-quantitative PCR revealed that HSP70 mRNA was detected in several organs of female tilapia *Oreochromis mossambicus* Peters by heat stress, while no signal was observed in the control fish (Molina *et al*., 2000). Furthermore, it was demonstrated that HSP expression was absent in an antarctic fish *Trematomus bernacchii* Boulenger (Hofmann *et al*., 2000). These results indicated that the expression manner of HSPs might be extremely variable among teleost species.

Steady expression of *Lepomis* HSP70 and 90 mRNA regardless of sex and seasonality as seen in the present study suggests that certain level of HSP expression might be advantageous to survive in labile environment, namely, bluegill could rapidly and well adapt the change of the environment. In fact, bluegill is tolerant to wide range of different water temperature; it generally prefers temperate water, but could adapt higher temperature (O'Hara, 1968) and sustain water temperature at 2.5°C (Petrosky and Magnuson, 1973). However, only qualitative evaluation, i.e., RT-PCR, was conducted in the present study, further quantitative investigation was necessary to reveal detailed HSPs functions in the thermotolerance of bluegill.

The result in the present study suggested that HSPs play some roles in environmental adaptation in poikilothermal animals and the heat shock response may have a significant variation among teleost species according to their capacity of adaptation.

ACKNOWLEDGMENT

The authors are thankful to the Iwaki Public Office of the Fukushima Prefecture and the Agricultural Land Division of Iwaki City for permission to use the agricultural water reservoir in Fukushima Prefectural Iwaki Park as the sampling site for bluegill.

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