Four Members of Heat Shock Protein 70 Family in Korean Rose Bitterling (Rhodeus uyekii)

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ABSTRACT: Heat shock protein (HSP) 70, the highly conserved stress protein families, plays important roles in protecting cells against heat and other stresses in most animal species. In the present study, we identified and characterized four Hsp70 (RuHSP4, RuHSC70, RuHSP12A, RuGRP78) family proteins based on the expressed sequence tag (EST) analysis of the Korean rose bitterling R. uyekii cDNA library. The deduced RuHSP70 family has high amino acid identities of 72-99% with those of other species. Phylogenetic analysis revealed that RuHsp70 family clustered with fish groups (HSP4, HSC70, HSP12A, GRP78) proteins. Quantitative RT-PCR analysis showed the specific expression patterns of RuHsp70 family members in the early developmental stages and several tissues in Korean rose bitterling. The expression of 4 groups of Hsp70 family was detected in all tested tissue. Particularly, Hsp70 family of Korean rose bitterling is highly expressed in hepatopancreas and sexual gonad (testis and ovary). The expression of Hsp70 family was differentially regulated in accordance with early development stage of Rhodesus uyekii.

Key words: Development, Expression, Korean rose bitterling, HSP 70 family, Rhodesus uyekii

INTRODUCTION

The heat shock or stress response refers to the reaction of cellular organisms to adverse environmental stresses such as heat stress and heavy metals, which are highly conserved proteins (Welch et al., 1991). It composed of the rapid and coordinated induction of a group of proteins referred to as the stress proteins and the concomitant reduction of normal cellular proteins. The stress proteins can be divided into two groups of families: the heat shock proteins (HSPs) and the glucose-regulated proteins (GRPs). HSPs are categorized into several families and named according to their function, sequence homology and molecular mass in kilo-Daltons (kDa): HSP100, HSP90, HSP70, HSP60, HSP40 and several smaller HSP groups (Lindquist, 1992).

HSP70 is the largest and most highly conserved of the stress protein families (Sanders, 1993). HSP70 family contains four major members: HSP70, heat shock cognate 70 (Hsc70), HSP75 and GRP78 (Polanowska-Grabowska et al., 1997; Bausero et al., 2005). At least 121 proteins have been isolated in the HSP70 family and cross-hybridization occurs across mammals, fish and molluscs; indeed, humans and molluscs share the same antigenic and ATP binding domains (Margulis et al., 1989; Roberts et al., 2010). Hsp70 family was isolated from a variety of fishes such as rainbow trout (Kothary et al., 1984), Oryzias latipes (Arai et al., 1995), Zebrafish...
Hsp70 family also has been reported to be associated with differences in environmental temperatures (Feder & Hofmann, 1999). Most of the HSPs are also constitutively synthesized in considerable amounts even in the unstressed normal cells (Welch et al., 1991; Roberts et al., 2010; Hunt & Morimoto, 1985), which play a fundamental role in the regulation of normal protein synthesis within the cell. HSP families such as HSP90 and HSP70 are critical to the folding and assembly of other cellular proteins (Geising & Sambrook, 1992). These also have a wider role in relation to the immune, apoptotic and inflammatory processes (Moseley, 2000; Srivastava, 2002; Pockley, 2003). Depletion of either HSP70 or HSP90 in a transgenic zebrafish model caused defects in blood vessel formation through the modulation of VEGF-A-stimulated intracellular signaling, endothelial cell migration, blood vessel development and repair (Bruns et al., 2012).

In this study, we report the identification and molecular characterization of the Korean rose bitterling (Rhodeus uyekii) HSP70 family members. We analyzed multiple alignments and phylogenetic tree of the deduced RuHSP70 family sequences and other homologs. We investigated the expression of RuHSP70 transcript during early development and in several tissues of Korean rose bitterling. This is the first report of molecular and functional analyses of the Korean rose bitterling HSP70 gene.

MATERIALS AND METHODS

1. Fish maintenance and sample preparation

Rhodeus uyekii were collected from the Yangchun River, Uiryung-gun, Gyungnam, Republic of Korea. The fish were maintained at the National Fisheries Research and Development Institute (NFRDI) in Busan, Republic of Korea (Kim et al., 2014). The adults were maintained in 40 L glass aquaria at a density of approximately 20 fish per aquarium. The water was renewed weekly and the temperature in the rearing tanks was maintained at 20 ± 1°C. The room was maintained on a 12:12-h light:dark cycle. Adults were fed TetraBits (Tetra) and frozen bloodworms (Advanced Hatchery Technology) twice a day. For RNA extraction, the sample of 10 randomly selected embryo or fish were collected and immediately frozen in liquid nitrogen, and stored at −80°C before use.

2. Identification of Korean rose bitterling R. uyekii RuHSP70 family

The RuHSP70 family cDNA sequences were isolated from the expressed sequence tag (EST) analysis of the Korean rose bitterling R. uyekii cDNA library. EST clones were isolated using a Plasmid Miniprep Kit (Qiagen), and sequenced using T3 reverse primers (Promega) and an ABI3730xl automatic sequencer (Applied Biosystems). The nucleotide sequence was analyzed and compared using the BLASTX search program (http://www.ncbi.nlm.nih.gov/BLAST/).

3. Multiple sequence alignment and phylogenetic analysis

The relevant sequences were compared using the BLASTX search program (http://www.ncbi.nlm.nih.gov/BLAST/) and retrieved from GenBank for multiple sequence alignments using CLUSTALW (http://www.genome.jp/tools-bin/clustalw). MEGA (ver. 4) was used to assess homologies among the aligned sequences. A phylogenetic tree based on the deduced amino acid sequences was constructed using a neighbor-joining algorithm, and the reliability of the branching was tested using bootstrap resampling with 1,000 pseudo-replicates.

4. Quantitative real-time PCR

Total RNA was prepared from tissues using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions, treated with DNase I (New England BioLabs, Beverly, MA, USA) and quantitatively...
determined; 500 ng samples were used for reverse transctiption (RT). First-strand cDNA was synthesized using Transcriptor First Strand cDNA Synthesis Kit (Roche). Quantitative real-time PCR was performed using Fast SYBR Green Master Mix (Applied Biosystems, Inc.). The PCR primers used for real-time PCR are listed in Table 1. Following an initial 10-min Taq activation step at 95°C, real-time PCR was performed using the following cycling conditions: 40 cycles of 95°C for 10 s, 60°C for 15 s, and fluorescence reading in an SDS 7500 system (Applied Biosystems, Inc.). Transcript levels were quantified as expression relative to the β-actin transcript level.

**RESULTS AND DISCUSSION**

1. Identification of four Hsp70 family cDNA sequences in the Korean rose bittering

The partial sequences of Korean rose bittering Hsp70 family were identified from the expressed sequence tag (EST) analysis of the *R. uyekii* cDNA library. A search using the BLASTX program (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi) and pairwise alignment revealed that the deduced amino acids of EST clones RU-1-2a_I02, RU-1-3a_F01, RU-2-1a_111 and RU-2-3a_F17 showed the high homology with heat shock protein 70kDa 4-like (HSP4), heat shock cognate 70 (HSC70), heat shock protein 70kDa 12A-like (HSP12A) and glucose regulated protein 78 (GRP78) of other species, respectively. Accession no. XM008284359.1, AY538777.1, NM001045435.1 and N595368.1.

2. Comparison of RuHSP70 family with other homologs

The deduced amino acids of RuHSP70 family were aligned with Hsp70 proteins from other species including Tongue sole, biocolor damselfish, Zebrafish, Amazon molly, Korean rose bittering, Cichlidae, Asiatice ricefish, Prussian carp, Minnows, Grass carp, Channel catfish, Mexican tetra, Zebra mbuna, Cichlid, guppy, Southern platyfish, Alpaca, Mouse, Human, Olive baboon, Cow, Hamster, Sheep, Falcon, Burmese python, Hill pigeon, African elephant (Fig. 1). Pairwise alignment revealed RuHSP70 family showed high amino acid identities of 72-99% with those of other species. In Table 2, RuHSP4 showed high homology with Stegastes parties and Poecilia formosa. RuHSC70 showed high homology with Carassius gibelio, Hypophthalmichthys molitrix and Pimephales promelas. RuHSP12A showed high homology with Danio rerio and Astyanax mexicanus. RuGRP78 showed high homology with Ctenopharyngodon idell and Danio rerio.

3. Phylogenetic analysis of RuHSP70 family with other homologs

A phylogenetic analysis, based on the deduced amino acid sequence of RuHSP70 family and related sequences,
Fig. 1. Multiple alignment of the amino acid sequences of the Korean rose bitterling heat shock protein 70 family (RuHSP70 family) and related sequences. A multiple alignment of amino acid sequences of Hsp70 family was produced using ClustalW 1.81. GenBank accession numbers for the analyzed sequences are the following: (a) Haplochromis burtoni (XP-005921284), Orezias latipes (XP-004073389), Cynoglossus semilaevis (XP-008331072), Stegastes partitus (XP-008296412), Poecilia formosa (XP-007562090), Danio rerio (NP_999881), Homo sapiens (AAH02526), Papio anubis (XP-003900145), Mus musculus (EDL36302), Vicugna pacos (XP-006212876). (b) Anio rerio (NP-00103873), Hypoehalithys molpiteix (ACJ03595), Carassius gibelio (AAO43731), Pimephales promelas (AAS46619), Ctenopharyngodon idella (ACJ03596), Ictalurus punctatus (ABD77547), Bos taurus (AAI54390), Homo sapiens (AAH08907), Cricetulus griseus (EGW02963), Mus musculus (BAE29904). (c) Danio rerio (NP_001038900), Astyanax mexicanus (XP-007244701), Maylandia zebra (XP-004572562), Pundamilia nyererei (XP-005747498), Stegastes partitus (XP-008305029), Bos taurus (AAI54390), Ovis aries (XP-004020391), Homo sapiens (XP-005269729), Falco peregrinus (XP-005239629), Python bivittatus (XP-007434939). (d) Poecilia reticulata (XP-008422585), Xiphophorus maculatus (XP-005803813), Neocamprologus brichardi (XP-006789208), Ctenopharyngodon idella (ACJ65009), Danio rerio (AAH63946), Homo sapiens (EAW87621), Papio anubis (XP-003919999), Mus musculus (AA37315), Loxodonta africana (XP-003407784), Columba livia (XP-005531063). Identical residues are indicated by asterisks (*); conservative substitutions are indicated by dots (.).
Table 2. Pairwise ClustalW analysis of the deduced amino acid sequences of RuHSP70 family with those of other species

| Species                          | GenBank no.         | Identity(%) |
|----------------------------------|---------------------|-------------|
| (A) RuHSP4                       |                     |             |
| *Haplochromis burtoni* Heat shock 70 kDa protein 4-like | XP_005921284       | 80          |
| *Oryzias latipes* Heat shock 70 kDa protein 4-like | XP_004073389       | 81          |
| *Cynoglossus semilaevis* Heat shock protein 105 kDa isoform X1 | XP_008331072       | 84          |
| *Stegastes partitus* Heat shock 70 kDa protein 4-like | XP_008296412       | 87          |
| *Poecilia formosa* Heat shock 70 kDa protein 4-like | XP_007562090       | 87          |
| *Danio rerio* Heat shock protein 4a | NP_999881    | 84          |
| *Homo sapiens* Heat shock 70kDa protein 4 | AAH02526 | 72          |
| *Papio anubis* Heat shock 70 kDa protein 4 | XP_003900145 | 73          |
| *Mus musculus* Heat shock protein 4, isoform CRA_b | EDL33602 | 74          |
| *Vicugna pacos* Heat shock 70 kDa protein 4 isoform X2 | XP_006212876 | 74          |
| (B) RuHSC70                      |                     |             |
| *Danio rerio* Heat shock cognate 71 kDa protein | NP_001103873     | 97          |
| *Hypophthalmichthys molitrix* Heat shock protein 70 | ACJ03595 | 98          |
| *Carassius gibelio* Heat shock cognate 70 kDa protein | AAO43731 | 99          |
| *Pimephales promelas* Heat shock cognate 70 kDa protein | AAS46619 | 98          |
| *Ctenopharyngodon idella* Heat shock protein 70 | ACJ03596 | 97          |
| *Ictalurus punctatus* Heat shock cognate 70 kDa protein | ABD77547 | 95          |
| *Bos taurus* HSPA8 protein | AA154390 | 92          |
| *Homo sapiens* HSPA8 protein | AAH08907 | 92          |
| *Cricetulus griseus* Heat shock cognate 71 kDa protein | EGW02963 | 91          |
| *Mus musculus* Unnamed protein product | BAE29904 | 95          |
| (C) RuHSP12A                     |                     |             |
| *Danio rerio* Heat shock protein 12A | NP_001038900 | 92          |
| *Astyanax mexicanus* Heat shock 70 kDa protein 12A isoform X1 | XP_007244701 | 91          |
| *Maylandia zebra* Heat shock 70 kDa protein 12A-like isoform X4 | XP_004572562 | 85          |
| *Pandamilia nyererei* Heat shock 70 kDa protein 12A-like isoform X5 | XP_005747498 | 84          |
| *Stegastes partitus* Heat shock 70 kDa protein 12A isoform X3 | XP_008305029 | 85          |
| *Bos taurus* Heat shock 70 kDa protein 12A isoform X1 | XP_002698580 | 76          |
| *Ovis aries* Heat shock 70 kDa protein 12A | XP_004020391 | 75          |
| *Homo sapiens* Heat shock 70 kDa protein 12A isoform X1 | XP_005269729 | 75          |
| *Falco peregrinus* Heat shock 70 kDa protein 12A | XP_005237627 | 74          |
| *Python bivittatus* Heat shock 70 kDa protein 12A-like isoform X1 | XP_007433493 | 74          |
was performed. RuHSP70 family was divided into two distinct groups, one as fisheries and the other one as mammals. The tree indicated clear clustering of RuHSP4 sequences into two groups: Amazon molly; Bicolor damselfish; Tongue sole; Zebrasfish; Cichlidae; Asiatic ricefish VS alpaca; mouse; olive baboon and human. The tree indicated clear clustering of RuHSC70 sequences into two groups: Prussian carp; Minnows; Zebrafish; Cuvier et valenciennes; Grass carp; Channel catfish VS Cow; Human; Hamster and Mouse. The tree indicated clear clustering of RuHSP12A family sequences into two groups: Zebrasfish; Mexican tetra; Bicolor damselfish; Zebra mbuna; Cichlid VS Falcon; Burmese python; Human; Cow and Sheep. The tree indicated clear clustering of RuGRP78 sequences into two groups: Grass carp; Zebrasfish; Guppy; Southern platyfish VS Hill pigeon; African elephant; mouse; olive baboon and human (Fig. 2).

4. Tissue distribution of Hsp70 family mRNA in Korean rose bitterling

Tissue distribution of Hsp70 family mRNA in Korean rose bitterling were investigated by quantitative real-time PCR. The expression levels of Hsp70 family mRNA were quantified after normalization to β-actin as an internal reference gene. The expression of RuHSP4 mRNA was detected in all tissue examined; highly in the ovary, testis and hepatopancreas. Levels of the RuHSP4 mRNA in the ovary, testis and hepatopancreas were 14.6, 11.3 and 9.8 folds that in gill where expression was the lowest, respectively. The expression of RuHSC70 mRNA was detected in all tissue examined; highly in the hepatopancreas, testis and spleen. Levels of the RuHSC70 mRNA in the ovary, testis and hepatopancreas were 2.7, 1.8 and 1.1 folds that in muscle where expression was the lowest, respectively. The expression of RuHSP12A mRNA was detected in all tissue examined; highly in the hepatopancreas, testis and spleen. Levels of the RuHSP12A mRNA in the ovary, testis and brain were 2.8, 1.1 and 1 folds that in gill where expression was the lowest, respectively. The expression of RuGRP78 mRNA was detected in all tissue examined; highly in the hepatopancreas, spleen and ovary. Levels of the RuGRP78 mRNA in the hepatopancreas, spleen and ovary were 42.1, 28.2 and 12.7 folds that in fin where expression was the lowest, respectively (Fig. 3). Overall,

| Species                              | GenBank no.     | Identity(%) |
|--------------------------------------|-----------------|-------------|
| (D) RuGRP78                          |                 |             |
| *Poecilia reticulata* 78 kDa glucose-regulated protein | XP_008422585  | 89          |
| *Xiphophorus maculatus* 78 kDa glucose-regulated protein-like | XP_005803813  | 90          |
| *Neolamprologus brichardi* 78 kDa glucose-regulated protein-like | XP_006789208  | 88          |
| *Ctenopharyngodon idella* GRP78      | ACJ65009        | 95          |
| *Danio rerio* Heat shock protein 5    | AAH63946        | 93          |
| *Homo sapiens* Heat shock 70kDa protein 5 | EAW87621      | 84          |
| *Papio anubis* 78 kDa glucose-regulated protein-like | XP_003911999  | 84          |
| *Mus musculus* Immunoglobulin heavy chain binding protein | AAA37315      | 82          |
| *Loxodonta africana* 78 kDa glucose-regulated protein | XP_003407784  | 86          |
| *Columba livia* 78 kDa glucose-regulated protein | XP_005513063  | 86          |
we found that Hsp70 family of Korean rose bitterling is highly expressed in hepatopancreas and sexual gonad (testis, ovary). This expression pattern is consistent with high previous results. The results showed that Hap 70 family was highly expressed in hepatopancreas and sexual gonad (testis, ovary) in Rhynchocypris kumgangensis (Im et al., 2013) and Paphia undiata (Wu et al., 2014).

5. Expression analysis of the HSP70 family mRNA during early development

The expression of Hsp70 family mRNA during early development of Korean rose bitterling was determined by quantitative real-time PCR at 1, 3, 6, 15 and 21 days post-fertilization (dpf). After the first dpf, the relative expression of RuHSP4 sharply decreased and was maintained at similar
Fig. 3. Tissue distribution of The Korean rose bitterling RuHSP70 family. Quantitative real-time RT-PCR was performed on equal amounts of total RNA isolated from tissues of normal conditioned fish. Korean rose bitterling β-actin was used as an internal control. Expression levels of (A) RuHSP4, (B) RuHSC70, (C) RuHSP12A, (D) RuGRP78 transcript were quantified by expression relative to the β-actin transcript level. B, brain; E, eye; G, gill; F, fin; K, kidney; Hp, hepatopancreas; St, stomach; Sp, spleen; I, intestine; M, muscle; T, testis; O, ovary. The values represent the mean±SD (n=3).

level, until the last dpf. The expression of RuHSC70 mRNA was detected from 1 dpf and moderately increased until 21 dpf during the early development. The relative expression of RuHSP12A and RuGRP78 increased significantly at the 15 dpf and decreased slightly at 21 dpf (Fig. 4).

The results of previous research are similar to our study. It is consistent that the expression of Hsp70 family gene was increased after fertilization in Oncorhynchus tshawytscha (Kong et al., 1996), Oncorhynchus mykiss (Currie & Tufts., 1997; Currie et al., 2000; Ojima et al., 2005a, b) and Salmo salar (Lund et al., 2002). We expected that differences in the expression are due to differences on the role of Hsp70 family mRNA at various stage of development. Further investigations are required to determine the function of Hsp70 family in development stage of Korean rose bitterling under in vivo and vitro conditions.

In this study, the partial sequences of Korean rose bittering Hsp70 family were identified from the expressed sequence tag (EST) analysis of the R. uyekii cDNA library. We found 4 members (RuHSP4, RuHSC70, RuHSP12A, RuGRP78) of Hsp70 family in R. uyekii. Pairwise alignment and phylogenetic analysis revealed RuHSP70 family showed RuHsp70 family has been conserved during evolution. The expression patterns of RuHsp70 family suggest that they play a unique or specific role during early development. Further investigations are required to elucidate the functional role of HSP70 families in R. uyekii.

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