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Cloning and expression analysis of a HSP70 gene from Korean rockfish (*Sebastes schlegeli*)

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ABSTRACT

The gene encoding HSP70 was isolated from Korean rockfish *Sebastes schlegeli* by homologous cloning and rapid amplification of cDNA ends (RACE). The full-length of HSP70 cDNA was composed of 2259 bp and encoded a polypeptide of 639 amino acids. BLAST analysis showed that HSP70 of *S. schlegeli* shared high identities with those of the *Lates calcarifer*, *Oreochromis niloticus*, *Seriola quinqueradiata* HSP70s (88–89%). Our current study also revealed that HSP70 of Korean rockfish was expressed in many tissues by RT-PCR under unstressed condition. Quantitative real-time PCR showed that the expression patterns of Korean rockfish HSP70 were developmental stage-dependency. The expression of HSP70 was measured by quantitative real-time PCR after different oxygen treatments. The results showed that expression of HSP70 increased significantly after exposure to hypoxia for 30 min in gill and ovary, and then decreased for 60 min, and the level in spleen and liver gradually increased and reached the highest at 60 min. In addition, in gill, spleen and liver, the HSP70 mRNA level reached the maximum in hypoxia group after one hour different oxygen concentration stress. Increased amounts of serum thyroxine (T_4), and triiodothyronine (T_3) were also found during 30 min hypoxia treatment and 60 min normoxia group in our study. All of the results provide information to further study the mechanism of physiology and immune function under stress conditions of ovoviviparous teleosts.

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1. Introduction

Heat shock proteins (HSPs), are the proteins known to help organisms to modulate stress response and protect organisms from environmentally induced cellular damage [1–4]. The HSP members are usually grouped according to their molecular weights, and divided into three main families: HSP90s, HSP70s and small HSPs [1,5]. Among the HSPs families, HSP70s were the most conserved and most extensively studied proteins which play a key role in the cell as molecular chaperones, such as membrane translocation, degradation of misfolded proteins and other regulatory processes [6–9]. Furthermore, many studies indicated that HSP70s could also function as potent activators of the innate immune system [10,11]. Except for heat shock, there is a great diversity of stressors, including hyperthermia, reduced oxygen levels, osmotic stress, heavy metal exposure and oxidative stress inducing the expression of HSP70 [12,13].

Several cDNAs encoding HSP70 of teleosts from rainbow trout [14], medaka [15] or zebrafish [16,17] have been described. Under non-stress conditions, HSP70 was mainly detected in hemocytes, muscle, mantle, gill, digestive gland and heart, in many aquatic animals [18,19]. It is well known that the organism habited in a naturally fluctuant dissolved oxygen environments and they evolved a variety of physiological and behavioral strategies to cope with the lack of oxygen [20]. In recent years, there are many hypoxia studies have been conducted in fish in order to understand the role of oxygen in physiology of aquatic organisms [21]. Many reports showed that the synthesis of HSP70 protein could be induced in mammals under hypoxia conditions, and the physiologically relevant levels of hypoxia induced a regulatory pathway different from that of heat shock and that a different set of genes is induced [22–24]. The expression of HSP70 gene, regulated by environmental factor, leads to protect cells against to harmful conditions by binding and refolding of damaged proteins [25]. Immune parameters including serum levels of thyroxine, triiodothyronine and Red blood cell (RBC) count may reflect the immunization situation after hypoxia. In addition, although our knowledge of detailed HSP70 expression profiles is about in hypoxia, heat shock or bacteria progress, their functions during gonadal development are not yet fully understood.

Korean rockfish, a typical ovoviviparous teleost belongs to the Scorpaenidae and inhabits rocky reefs in waters of Japan, Korea and

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China. Recent years, it is utilized as an important cultured species for artificial seeding production [26,27]. Ovoviviparous species, which the eggs hatch in the female body and do not obtain nutrition from female, but rely on yolk proteins accumulated in oocytes as an energy source during the vitellogenesis [28].

In the present study, cloning and characterization of HSP70 from Korean rockfish (*Sebastes schlegeli*) are described. The gene expression profiles for HSP70 during gonadal development stages are showed using fluorescent real-time quantitative RT-PCR. In addition, this is the first study to clearly define molecular regulation of HSP70 gene in ovoviviparous Korean rockfish acclimated to different oxygen exposure, to clearly define molecular regulation of HSP70 genes, serum levels of thyroxine and triiodothyronine and Red blood cell (RBC) count in fish acclimated to different water oxygen concentration and to demonstrate how the HSP70 response with aspects of growth and immune physiology. Data obtained in present study will supply information for further study in physiological status of fish HSP70 and of environmental stress, which will also help us for getting the basis for researches on reproductive physiology, blood physiology, genetic breeding and improvement of hypoxia-tolerance of Korean rockfish.

2. Materials and methods

2.1. Experimental fish and holding conditions

2.1.1. Fish

Adult Korean rockfish were obtained from a commercial fisheries farm in costal area of Shandong province, China. Fish were maintained for 3–4 days in culture tanks, the temperature and salinity fluctuated with ambient conditions. After excising the gonads, sexual maturity was determined by the presence of mature ova and sperm.

2.1.2. Developmental period

About twenty samples including adult male and female Korean rockfish (*S. schlegeli*) were obtained from costal area of Shandong province every two months for temporal and spatial expression. After maintained for 3–4 days in indoor, the fish were anaesthetized in tricaine methanesulfonate MS-222 (100 mg/L, Sigma, St. Louis, MO). Various tissues including heart, liver, spleen, head kidney, ceaca, ovary, testis, fat, kidney, gill, brain and intestine were collected, immediately frozen in liquid nitrogen and stored at −80 °C until analyses. In order to perform seasonal cycle studies, adult male fishes sampling ($n \geq 3$) were collected in mainly at stage II (spermatogonia stage), stage III (immature sperm stage), stage IV (mature testes stage), and stage V (spermiation stage); and female sampling were mainly at stage II (perinucleolus oocyte stage), stage III (primary yolk stage), stage IV (secondary yolk stage), stage V (tertiary yolk stage) and stage VI (gestational ovary stage) [72].

2.1.3. Acute hypoxia challenge

In addition, about forth female samples (weighing 635.9 ± 18.9 g, at perinucleolus oocyte stage) were also obtained from the Shandong costal area. Then, fish were divided into three groups ($n \geqq 10$) and one group ($n = 6$) with no treatment. And the tanks of treatments groups were all airtight, filling with ambient seawater (average temperature: 24 °C, average salinity 30‰). The mean measured initial concentrations ± S.D. in the airtight tanks were: 8.48 ± 0.11 mg O_2/L for the hyperoxia treatment; 7.23 ± 0.01 mg O_2/L for the normoxia treatment; 4.48 ± 0.25 mg O_2/L for the hypoxia treatment; 7.24 ± 0.01 mg O_2/L for the control group. The oxygen tension in each tank was regulated by the use of gas mixer combining air and nitrogen. Oxygen concentrations and temperatures were measured using COD reactor (Hach, USA). All of the samples were collected in non-treatment group, half of the samples were collected from each airtight tank group at 30 min and others were collected at 60 min. Tissues were collected as described previously. Blood samples were collected from caudal vein and clotted at 4 °C for 4–6 h. Then, serum was centrifuged at 16 000 r min^{-1} for 10 min and stored at −40 °C before steroid analysis.

2.2. Total RNA extraction and reverse transcription (RT)

Tissues were extracted with RNAiso reagent (Takara, Japan) following the manufacturer's protocols. Total RNA was quantified using a UV spectrophotometer (ChampGel 5000, China), integrity and purity of RNA samples was evaluated with an Nucleic acid analyzer (Biodropsis BD-1000, China) and 1.5% agarose gel. First-strand cDNA was synthesized from 1 μg of total RNA from each sample using random primers and Reverse Transcriptase M-MLV (Takara, Japan) in a 10 μL reaction.

2.3. Rapid amplification of 5′ and 3′ cDNA ends (RACE)

One pair of degenerate primers, HSP70F and HSP70R (Table 1), were designed according to conserved sequences of HSP70 genes from closely related species were used to amplify a HSP70 cDNA fragment of Korean rockfish. Touchdown polymerase chain reaction (PCR) was used for HSP70 amplification. The program was: 5 min at 94 °C, 10 cycles of 30 s at 94 °C, 30 s at a range of annealing temperature from 70 °C to 60 °C, decreasing 1 °C each cycle, and 35 s at 72 °C, then followed by additional 30 cycles of 30 s at 94 °C, 30 s at 62 °C and 35 s at 72 °C, finally ended with 10 min at 72 °C for extension. The PCR products were separated by a 1.5% agarose gel and purified using the TIAN gel midi Purification Kit (Tiangen,

Table 1
Primers used for KrHSP70 gene cDNA RACE, RT-PCR and real-time PCR.

Primers	Sequence (5′–3′)	Position	Usage
RT-PCR			
HSP70F	GAGGGMRTCGACTTCTAYACBTCCATCAC	969–998	Degenerate primer
HSP70R	CTTGTACTTCTCTGCNTCYTGBACCAT	1930–1957	Degenerate primer
3′,5′RACE			
HSP70-5-R1	TCAAAGCGAGCCCTAGTGATGGAAGTA	985–1012	5′-RACE primer
HSP70-5-R2	CCCTAGTGATGGAAGTATAGAAGTCG	975–1002	Nested 5′-RACE primer
HSP70-3-R1	GTAACACCACCATCCCCACTAAACAA	1351–1377	3′-RACE primer
HSP70-3-R2	AACACCACCATCCCCACTAAACAAAC	1353–1379	Nested 3′-RACE primer
RT-qPCR			
HSP70-eF	AGAGCCGGTGGAGAAAGC	1046–1064	RT-PCR and qPCR primer
HSP70-e-R	CCTCGTCTGGGTTGATGC	1189–1206	RT-PCR and qPCR primer
18S-e-F	CCTGAGAAACGGCTACCATC	–	Reference primer
18S-e-R	CCAATTACAGGGCCTCGAAAG	–	Reference primer

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China) and then cloned into the PGM-T vector (Tiangen, China), propagated in *Escherichia coli* (Trans5α, Transgen, China); clones were sequenced using the ABI3730XL sequencer (ABI, USA).

The 5′ and 3′ RACE reactions were used the SMART™ RACE cDNA amplification kit (Clontech, USA), primers and nested primers were shown in Table 1. PCR was performed using the following PCR cycling conditions: 5 min denaturing step, at 94 °C, 40 cycles of 30 s at 94 °C, 30 s at 59 °C for H1-5-1 and H1-5-2, or 30 s at 69 °C for H1-3-1 and H1-3-2, then followed by additional step at 72 °C for 1 min, finally ended with 10 min at 72 °C for extension. The PCR products were gel-purified and sequenced as above.

2.4. Phylogenetic analysis and sequence analysis

The searches for nucleotide and amino acid sequences similarities were carried out with BLAST program at the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/blast). The deduced amino acid sequence information was analyzed with the Expert Protein Analysis System (http://www.expasy.org/). Furthermore, multiple sequence alignment and phylogenic tree were constructed using the software of Clustal X 1.81 [29] and MEGA 4.0 [30] based on the amino acid sequences of KrHS70, and some other known deduced HSP70 sequences downloaded above. Bootstrap analysis was conducted with 1000 replicates to test the relative support for the branches produced by the neighbor-joining analysis [31].

2.5. Semi-quantitative RT-PCR analysis of HSP70 mRNA tissue expression

The expression profiles of HSP70 mRNA in different tissues were studies through RT-PCR, including heart, liver, spleen, head kidney, caecus, gonads, stomach, fat, kidney, gill, brain and intestine from both male and female adult black rockfish (sampled in November). Total RNA of those tissues was extracted using RNAiso reagent (Takara, Japan) and treated with RNase-free DNase I before proceeding for the first strand synthesis. The primers used for examination of tissue expression pattern are listed in Table 1. After comparing 18S rRNA, β-actin and GAPDH, we found that the expression of 18S rRNA was more stable and suitable for this study as a reference gene. PCR cycling conditions were as follows: 95 °C for 5 min; 40 cycles of 95 °C for 5 s, 58 °C for 30 s, and 72 °C for 30 s; and 72 °C for 10 min. The PCR products were checked by 1.5% agarose gel electrophoresis. The gel was pre-stained with ethidium bromide and visualized on a Gel system (ChampGel 5000, China).

2.6. qPCR analysis of the HSP70 gene

Three or four fish samples were chosen for studying the temporal expression of HSP70 in each ovarian developmental stage. The expressions of HSP70 gene on different oxygen stress were also measured by the qPCR. The cDNAs of those gonads were also prepared and PCR conditions were described as above. Real-time PCR assays (25 μL), each individual sample of which was run in triplicate wells, were carried out using iQ™ SYBR Green Supermix (Takara, Japan) performing on Multicolor Real-Time PCR Detection System (Roche Lightcycler480, German). As an internal control, 18S ribosomal RNA was amplified in an identical manner using primers specific for Korean rockfish and all data were normalized to the 18S calculated threshold-cycle (Ct) level. To confirm the primer specificity, melting curve analysis of amplification products was performed at the end of each PCR reaction that only one PCR product was amplified and detected. The samples from stage II and control group with serial dilutions of total cDNA were used as calibrators in temporal expression study and hypoxia treatment study respectively in our research. The result was analyzed according to the $2^{-\Delta\Delta Ct}$ method [32].

2.7. Red blood cell (RBC) count

Blood samples with different oxygen concentration treatments were collected from the caudal vein with use of ethylenediaminetetraacetic acid (EDTA) K2. After collection, the samples were immediately stored at a temperature of 4 °C. Within 10 min, they were centrifuged at 3000 *g* and 4 °C for 10 min. Aliquots of the plasma were stored at −80 °C. Red blood cells counts ($\times 10^{12}$ μL) (RBC), were determined from the venous blood samples by conventional methods using BS-1800 Auto Hematology Analyzer (ShenZhen Mindray Bio-Medical Electronics Co., Ltd., Guangzhou, China).

2.8. Steroid radioimmunoassay

Serum levels of triiodothyronine (T_3) and thyromine (T_4) were respectively measured using Iodine [^{125}I]. Radioimmunoassay Kits (Tianjin Nine Tripods Medical & Bioengineering Co., Ltd., Sino-US joint-venture enterprise) under different dissolved oxygen levels fish. The binding rate is highly specific with an extremely low cross reactivity to other naturally occurring steroids, which was less than 0.1% to most circulating steroids. The coefficients of intra-assay and inter-assay variation were 7.1–10.4% and 5.5–12.3%, respectively, for the T_3 and T_4 assay. Any samples with coefficient of variation higher than 10% were not included in the analyses. The assay sensitivity reached to 0.1 pg/mL and 0.08 pg/mL for T_3 and T_4 by the kit protocol, respectively.

2.9. Statistics

The relevant values in this study were analyzed through ANOVA followed by Duncan's multiple range tests. Statistical significance was considered as $P < 0.05$.

3. Results

3.1. Isolation and characterization of HSP70 cDNA

The PCR product amplified using the degenerated primers (HSPF1/HSPR1) was 988 bp, and its nucleotide sequence was homogeneous to other known HSP70 genes by BLAST search at NCBI (http://www.ncbi.nlm.nih.gov/). Then two fragments corresponding to the 5′ and 3′ end of the KrHSP70 cDNA were amplified by RACE and nested PCR approaches. The full-length cDNA of KrHSP70 (Genbank accession number: AGF90789) was 2259 bp, containing a 241 bp-3′ terminal UTR, 98 bp-5′ terminal UTR with a canonical polyadenylation signal sequence AATAAA, a poly (A) tail. And the open reading frame (ORF) of KrHSP70 cDNA was 1917 bp, encoding a protein of 639 amino acids (Fig. 1).

Amino acid sequence analysis indicated that KrHSP70 contained an ATP-GTP-Binding site motif A (P-loop) AEAYLGAA (residues 133–140), the bipartite Nuclear Targeting Sequence with KRKHKKDISPDREHWRS (residues 248–265), and the glycosylation domains NKSI (residues 362–365), NVSA (residues 521–524) (Fig. 1). Three highly conserved signatures including 1 (IDLGTTYS), 2 (IFDLGGGTFDVSIL), 3 (PTIEEVD) and the cytoplasmic characteristic motif EEVD (residues 636–639) were all identified in *S. Schlegeli*, *Lates calcarifer*, *Lutjanus sanguineus*, *Danio rerio*, *Oncorhynchus mykiss*, *Paralichthys olivaceus*, *Oreochromis niloticus* (Fig. 2). However, three GAP repeat and GGMP motif were all absent in *S. Schlegeli.*

BLAST program analysis showed that the amino acid sequences of KrHSC70 shared the high similarity with that of other known

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1     ACATGGGGACTGACGACACCACGAGGCAGTAATCTCTTCTCCTGCTCTTCTCTCCAGATTATCATCTTAACCACAGACACAAATTAAAAACAGCCAAGATGTCTGCAGCTAACAGTGTAG 120
1                                                                                                     M S A A N S V 7
121   CGATCGGCATTGACCTGGGCACCACCTACTCCTGTGTCGGGGTTTTCCAGCACGGAAAAGTAGAAATCATCGCCAACGACCAGGGCAACAGGACCACCCCCAGCTACGTGGCGTTCACTG 240
8     A I G I D L G T T Y S C V G V F Q H G K V E I I A N D Q G N R T T P S Y V A F T 47
241   ACACAGAGAGGCTCATCGGGGATGCAGCCAAGAACCAGGTGGCTTTGAACCCCAGCAACACCGTGTTTGATGCCAAGAGACTGATTGGAAGAAAGATTGATGATGCAGTGGTGCAGGCGG 360
80    D T E R L I G D A A K N Q V A L N P S N T V F D A K R L I G R K I D D A V V Q A 87
361   ACATGAAGCATTGGCCCTTCAAGGTGATCGGAGATGGAGGGAAGCCCAAAATTCAAGTGGAGCACAAAGGGGAGGAGAAAGCCTTCAACCCTGAGGAGATTTCCTCCATGGTCCTGGTGA 480
88    D M K H W P F K V I G D G G K P K I Q V E H K G E E K A F N P E E I S S M V L V 127
481   AGATGAAGGAGATTGCTGAAGCCTACCTGGGCCAAAAGGTGTCCAAAGCTGTCATCACAGTCCCGGCGTACTTCAACGACTCCCAGCGACAGGCGACTAAAGACGCAGGCGTCATCGCAG 600
128   K M K E I A E A Y L G Q K V S K A V I T V P A Y F N D S Q R Q A T K D A G V I A 167
601   GACTGCATGTCCTGAGGATCATCAACGAGCCGACGGCGGCCGCCATCGCGTACGGTCTGGACAGAAGCAAGACAGGAGAACGTAACGTCCTGATCTTTGACTTGGGCGGAGGAACCTTCG 720
168   G L H V L R I I N E P T A A A I A Y G L D R S K T G E R N V L I F D L G G G T F 207
721   ACGTGTCCATCCTGACCATTGAAGATGGTATCTTTGAGGTGAAAGCCACGGCTGGAGACACTCACCTGGGTGGAGAAGACTTTGACAACCGCATGGTCAGCCACTTTATGGAGGAGTTCA 840
208   D V S I L T I E D G I F E V K A T A G D T H L G G E D F D N R M V S H F M E E F 247
841   AGAGGAAACACAAGAAGGACATCAGCCCAGACAGAGAGCATTGGAGGAGCTGCGCACAGCTTGTGAGAGGCCAGAGGACCTGTCTCAGCTCCAGCAGCATCGAGATCGATTCTCTGTTTG 960
248   K R K H K K D I S P D R E H W R S C A Q L V R G Q R T C L S S S S I E I D S L F 287
961   AAGCAATTGAGGGCGTCGACTTCTATACTTCCATCACTAGGGCTCGCTTTGAGGAGCTGTGCTCCGACCTGTTCAGGGGAACCTTAGAGCCGGTGGAGAAAGCCATGAGGGATGCCAAAA 1080
288   E A I E G V D F Y T S I T R A R F E E L C S D L F R G T L E P V E K A M R D A K 327
1081  TGGACAAGGGGAAGATCCATGACATCGTCCTGGTCGGAGGCTCCACCCGCATCCCTAAAATCCAGAAACTCCTGGCCGATCTCTTCAACGGCAGGGAGCTGAACAAGAGCATCAACCCAG 1200
328   M D K G K I H D I V L V G G S T R I P K I Q K L L A D L F N G R E L N K S I N P 367
1201  ACGAGGCGGTGGCTTATGGCGCTGCCGTCCAGGCCGCCATTCTCACAGGCGATACCTCGGGCAACGTTCAGGACGTGCTGCTGCTGGACGTGGCGCCTCTGTCCCTGGGTATCGAGACCG 1320
368   D E A V A Y G A A V Q A A I L T G D T S G N V Q D V L L L D V A P L S L G I E T 407
1321  CTGGAGGGGTCATGACGTCTCTGATTAAGCGTAACACCACCATCCCCACTAAACAAACCCAGGTGTTCACCACCTACTCTGACAACCAGCCCGGGGTCCTCATCCAGGTCTATGAAGGGG 1440
408   A G G V M T S L I K R N T T I P T K Q T Q V F T T Y S D N Q P G V L I Q V Y E G 447
1441  AACGGGCCATGACCAAGGACAACAACCTGCTGGGCAAGTTTGGGCTGACGGGAATCCCGCCCGCTCCACGAGGGGTCCCGCAGATCGAGGTCACCTTCGACGTGGATGCAAACGGCATTT 1560
448   E R A M T K D N N L L G K F G L T G I P P A P R G V P Q I E V T F D V D A N G I 487
1561  TGAACGTGTCCGCGGTGGACAAAAGCACCGGCAAAGAGAAAAAGATCACTATCACCAACGATAAGGGCCGACTGAGCAAAGAGGATATCGAGAGGATGGTGCAGGACGCCGACAAATACA 1680
520   L N V S A V D K S T G K E K K I T I T N D K G R L S K E D I E R M V Q D A D K Y 527
1681  AAGCTGAGGACGACCTTCAGAGGGATAAAGTCTCTGCCAAGAACTCCCTGGAGTCCTACGCCTTCAACATGAAGAGCAGCATGCAGGACGAGAACATGAAGGGCAAAATCAGTGCCGAGG 1800
560   K A E D D L Q R D K V S A K N S L E S Y A F N M K S S M Q D E N M K G K I S A E 567
1801  ACGAGAAGACGGTGATTGAGAAGTGTGACGAGACCATCGCCTGGATGGAGAGCAACCAGCTGGCTGAAAAAGAGGAGTACCAACACCGGCAGAAAGAGCTGGAGAAAGTGTGTAACCCCA 1920
568   D E K T V I E K C D E T I A W M E S N Q L A E K E E Y Q H R Q K E L E K V C N P 607
1921  TCATCAGCAGCTTGTACCAGGGAGGAAAGCCTCCAGGCAGCTGTGGAGAGGAAGCACGCGGTGGCTCCCAGGAGCCCACCATTGAGGAGGTGGACTAAAATGGTCCCCATGGACTCTGAT 2040
608   I I S S L Y Q G G K P P G S C G E E A R G G S Q E P T I E E V D *             640
2041  GGCCATTATGGACTCTGATATCACTGGGACTGTTTGAAATGGAACTGTAACTTTATACCTCTTCTTCATATGTTTTTATCACCATCATATTTATGATAAAAAGTGTTAATATTCTAAACT
2161  TTTGGATAATAGGTGCCATCTTAATGTCATTTTCATTTCTGCGTTGCTGGACATTGGAATAAAGATGAACATAAATACAAAAAAAAAAAAAAAAAAAA
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Fig. 1. Nucleotide sequences and deduced amino acid sequences of Korean rockfish (*Sebastes schlegeli*) HSP70 cDNA. Asterisk (*) means the termination codon. Three classical HSP signature motifs are marked with gray box; ATP/GTP-Binding Site Motif A are marked with opened box; Bipartite Nuclear Targeting are shown with closed box; The glycosylation domains are marked with double underlines; Consensus sequence EEVD at the C-terminus is indicated in EEVD; Tailing signal was underlined; This nucleotide and deduced amino acid sequence data was registered in the GenBank (No. AGF90789).

HSP70, and the closest matches with *L. calcarifer* Sequence comparison revealed that the deduced amino acid sequence of KrHSP70 showed 89%, 88% and 88% similarity with the inducible HSP70s of *L. calcarifer*, *O. niloticus*, *Seriola quinqueradiata*, respectively (Fig. 3).

Based on the amino acid sequences of KrHSP70 along with other HSP70 from other species, a phylogenetic tree was constructed using the program of MEGA 4. The phylogenetic tree is composed of three major groups: reptile, mammals and fish. The relationship

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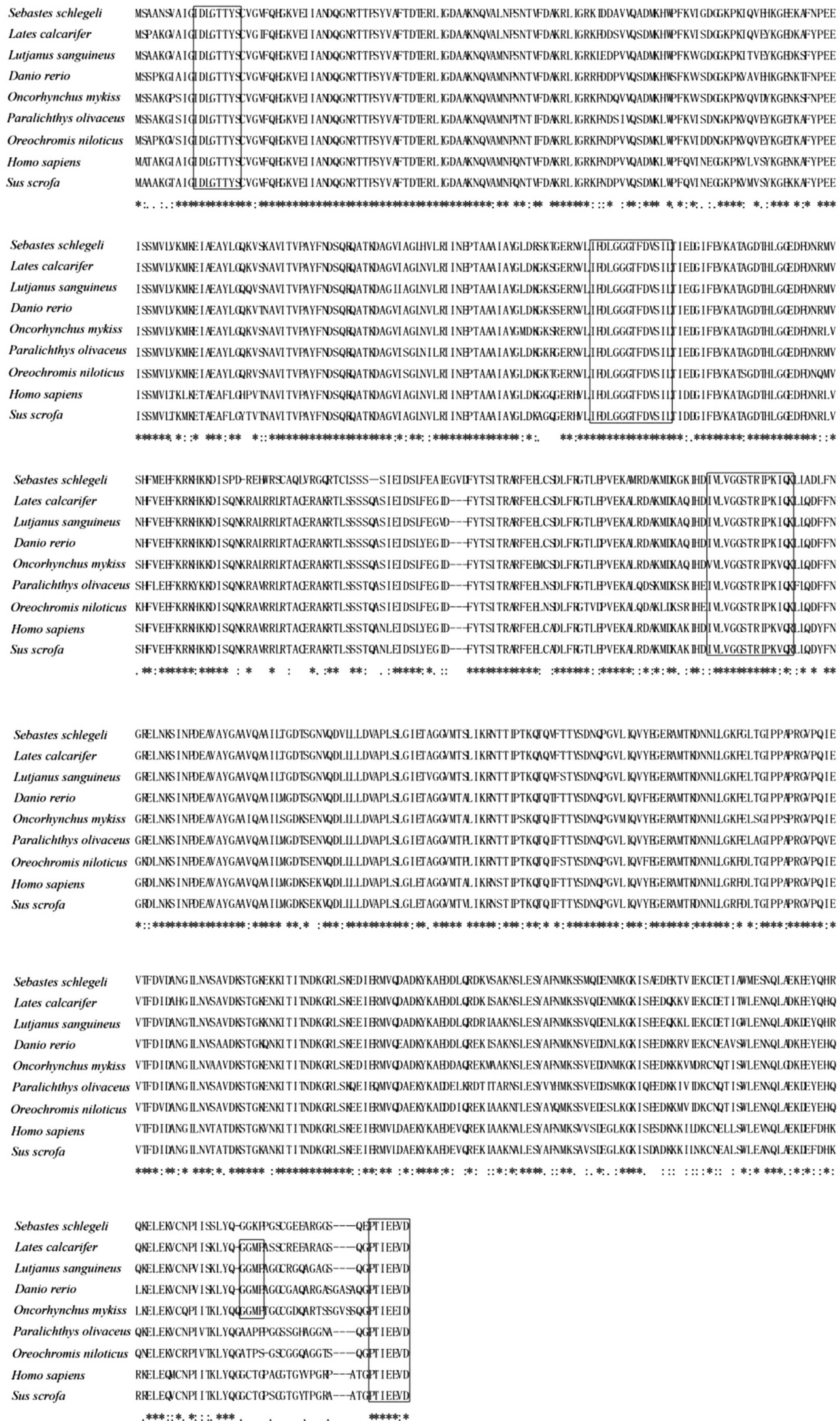

Fig. 2. Alignment of HSP70 amino acid sequences with other species HSP70s. Alignment of deduced amino acid sequences of HSP70 from *Sebastes schlegeli* (GenBank ID: AGF90789) with those from *Lates calcarifer* (GenBank ID: AEH27544), *Lutjanus sanguineus* (GenBank ID: ADO32584), *Oncorhynchus mykiss* (GenBank ID: NP_001117700), *Paralichthys olivaceus* (GenBank ID: BAA31697), *Oreochromis niloticus* (GenBank ID: XP_003444871), *Sus scrofa* (GenBank ID: ABX82832) and *Homo sapiens* (GenBank ID: NP_005518).

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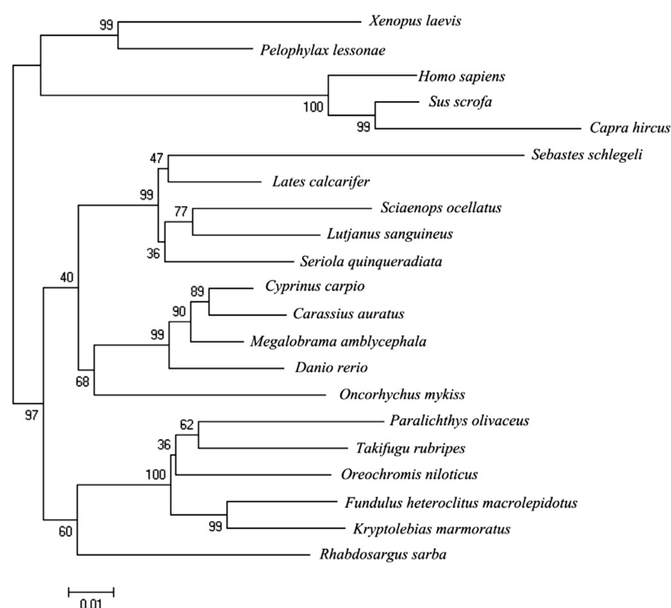

Fig. 3. Phylogenetic tree for HSP70 amino acid sequences of mammals and teleosts. The tree was generated by MEGA 4.0 software using the neighbor-joining method, following Clustal X. Scale bar indicates an evolutionary distance of 0.01 amino acid substitution per position in the sequence. Bootstrap values are indicated (1000 replicates). GenBank ID for reptiles: *Xenopus laevis*, NP_001721147; *Pelophylax lessonae*, ACY69994; for mammalians: *Sus scrofa*, ABX82832; *Homo sapiens*, NP_005518; for fishes: *Sebastes schlegeli*, AGF90789; *Lates calcarifer*, AEH27544; *Danio rerio*, AAH56709; *Oncorhynchus mykiss*, NP_001117700; *Paralichthys olivaceus*, BAA31697; *Oreochromis niloticus*, XP_003444871; *Sciaenops ocellatus*, ADL18372; *Seriola quinqueradiata*, BAG82850; *Cyprinus carpio*, AEQ44578; *Carassius auratus*, BAC67184; *Megalobrama amblycephala*, ACG63706; *Takifugu rubripes*, CAA69894; *Fundulus heteroclitus macrolepidotus*, ABB17042; *Kryptolebias marmoratus*, AEM65179; *Rhabdosargus sarba*, AAR97294.

displayed in the phylogenic tree was generally in agreement with the traditional taxonomy of these species (Fig. 4).

3.2. Expression of KrHSP70 in tissues under unstressed condition

Semi-quantitative RT-PCR results showed KrHSP70 mRNA was widely expressed in those of 13 tissues in both male and female. There was a little mRNA expression of KrHSP70 gene in stomach, fat and ceaca, whereas, the level of KrHSP70 mRNA was the highest in liver in female. In male, its expression was undetectable in the kidney, and was the highest in head kidney (Fig. 5).

Fig. 4. Expression pattern of HSP70 in different adult tissues detected by RT-PCR without treatments. The integrity of the RNA from the each tissue was ensured by uniform amplification of 18S transcripts (lower panel). Ma: marker; H: heart; L: liver; SP: spleen; HK: head kidney; C: caecus; M: muscle; HK: head kidney; C: caecus; T: testis; O: ovary; F: fat; K: kidney; G: gill; B: brain; I: intestine.

3.3. Expression of KrHSP70 in gonads and brain during gonadal development stages

Fluorescent real-time RT-PCR results showed that quantity of KrHSP70 mRNA was appeared to be tissue dependant. Levels of HSP70 measured increased in ovary from stage II (perinucleolus oocyte stage) to the stage III (primary yolk stage) and decreased from stage IV (secondary yolk stage) to stage VI (gestational ovary stage) (Fig. 6(A), $P < 0.05$). For males, the HSP70 levels decreased significantly from stage II (spermatogonia stage) until stage IV (mature testes stage) and increased gradually in stage V (spermiation stage) (Fig. 6(B), $P < 0.05$). A different pattern was observed in brain for HSP70 levels in both males and females (Fig. 6). The HSP70 levels from females were decreased from stage II to stage III, and increased gradually from stage IV to stage VI (Fig. 6(C), $P < 0.05$). In male, the levels of HSP70 were increased from stage II to the top in stage III (immature sperm stage) and decreased to the bottom in stage IV, then finally increased in stage V (Fig. 6(D), $P < 0.05$).

3.4. Expression of KrHSP70 under hypoxia stressed condition

In order to know whether hypoxia influences HSP70 expression in Korean rockfish, HSP70 mRNA levers in tissues including ovary, spleen, liver and gill had been treated with hypoxia were detected by qPCR. The mRNA expression of HSP70 at different time points after hypoxia treatments is shown in Fig. 6. The samples at 0 min group and normoxia group were used as calibrators for comparative relative qPCR. The result showed a similar change trend of KrHSP70 transcript was observed in the ovary and gill, the mRNA expression of KrHSP70 greatly increased and was very significantly higher than that of 0 min after hypoxia in 30 min, and then greatly dropped at 60 min (Fig. 6(A) and (B), $P < 0.05$). However, for spleen and liver, the expression level of KrHSP70 was increased gradually from 0 min, and reached the highest in 60 min (Fig. 6(C) and (D), $P < 0.05$). HSP70 expression was induced by different oxygen concentration at one hour and was shown in Fig. 7. In ovary, the expression level of HSP70 was highest under normoxia (Fig. 7(A)). The expression level of KrHSP70 was much higher than other two groups in spleen (Fig. 7(B), $P < 0.05$). And the transcript levels in hyperoxia and hypoxia groups were higher than normoxia group (Fig. 7(C) and (D), $P < 0.05$).

3.5. Serum T_3 and T_4 analysis

The changes of serum levels of T_3 and T_4 levels in female Korean rockfish under hypoxia treatments were shown in Fig. 8. And our result showed that the serum levels of T_3 and T_4 have the similar pattern of during hypoxia treatment. Under the hypoxia treatment, the average concentration of T_3 and T_4 in female Korean rockfish was increased at 0 min (1.37 ± 0.12 ng dL^{-1} and 1.47 ± 0.5 ng dL^{-1}), then peaked at 30 min (1.98 ± 0.17 ng dL^{-1} and 2.12 ± 0.13 ng dL^{-1}), finally decreased at 60 min (1.64 ± 0.13 ng dL^{-1} and 1.64 ± 0.13 ng dL^{-1}). Meanwhile, the amount of serum T_3 and T_4 levels in different hypoxia treatments after one hour was shown the highest level under the normoxia treatment (3.11 ± 0.76 ng dL^{-1} and 1.98 ± 0.17 ng dL^{-1}).

4. Discussion

In this study, full-length of HSP70 cDNA sequence, cloned from Korean rockfish (*S. schlegeli*) ovary, shared the highest degree of

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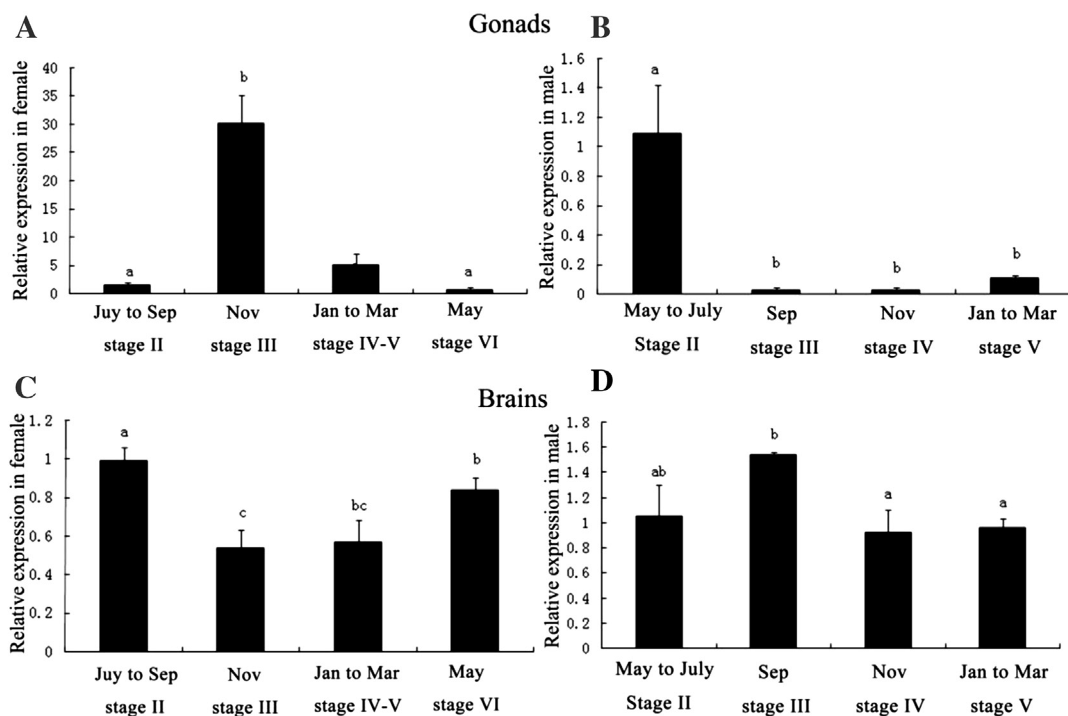

Fig. 5. mRNA expression of HSP70 in gonads (A and B) and brain (C and D) of Korean rockfish during the annual reproductive cycle by relative quantitative real-time PCR. Expression of 18S rRNA was used as an internal control for real-time PCR. Each histogram represents the mean of three determinations. Values are expressed as mean ± standard error of mean. Different letters indicate significant difference ($P < 0.05$, one-way ANOVA, followed by Duncan's test).

homology with known HSP70 genes of other species, as much as 89% similarity with barramundi perch HSP70. The amino acid sequence of ATPase domain of HSP70 from various species appears to be highly conserved whereas the C-terminal domain was more divergent [33], the situation which was also revealed in our study. Although the C-terminal end is less conserved, the last 7 amino acids, PTIEEVD, are identical in all the species, the feature above is previously shown to be characteristic of nuclear-cytosolic HSP70s

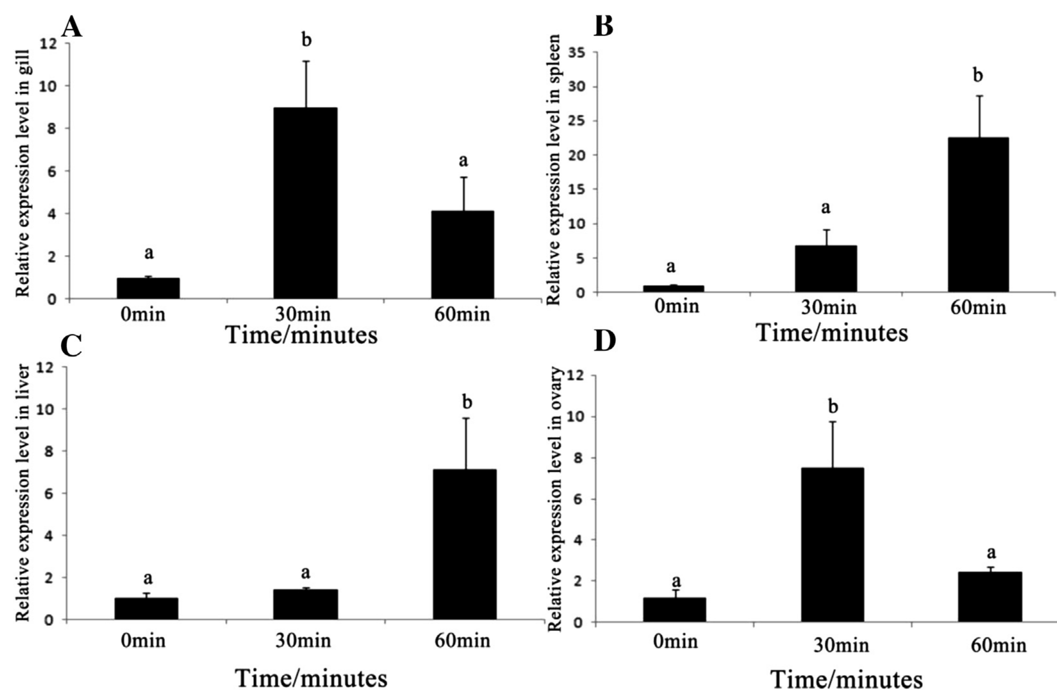

Fig. 6. Expression analysis of female Korean rockfish HSP70 by relative quantitative real-time PCR in gill (A), spleen (B), liver (C) and ovary (D), during 60 min of treatments with hypoxia. Expression of 18S rRNA was used as an internal control for real-time PCR. Each histogram represents the mean of three determinations. Values are expressed as mean ± standard error of mean. Different letters indicate significant difference ($P < 0.05$, one-way ANOVA, followed by Duncan's test).

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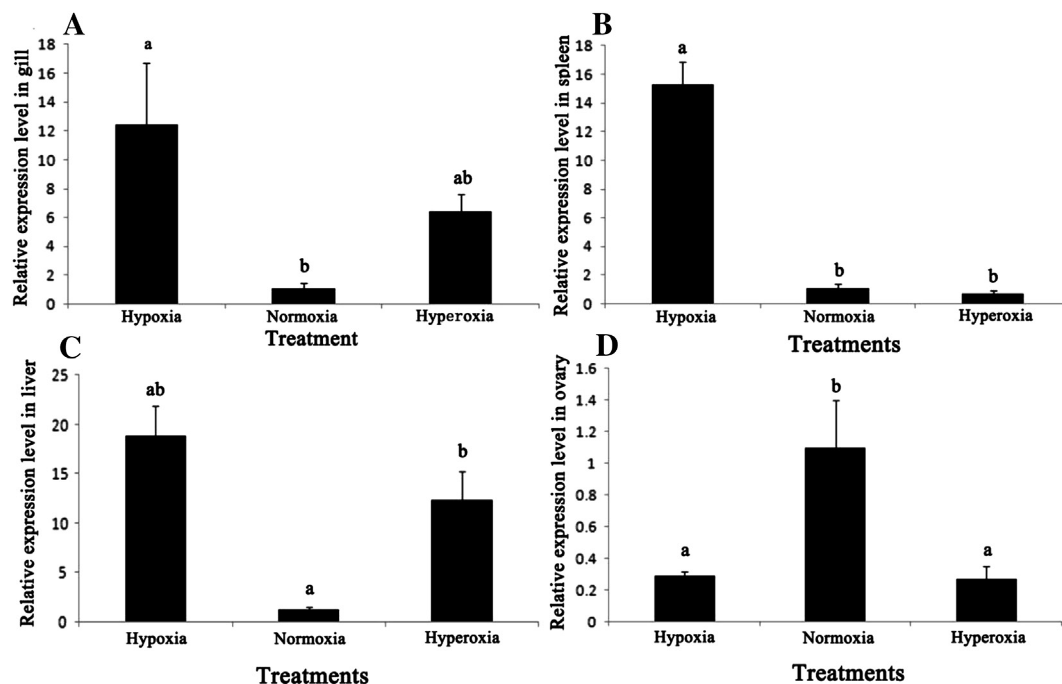

Fig. 7. Expression analysis of female Korean rockfish HSP70 by relative quantitative real-time PCR in gill (A), spleen (B), liver (C) and ovary (D), after 60 min different oxygen concentration treatments. Expression of 18S rRNA was used as an internal control for real-time PCR. Each histogram represents the mean of three determinations. Values are expressed as mean ± standard error of mean. Different letters indicate significant difference ($P < 0.05$, one-way ANOVA, followed by Duncan's test).

[6]. However, the deduced amino acid sequence of KrHSP70 lacks repeats of a conserved tetrapeptide GGMP which are located at the C-terminal portion of the protein in most of the species, this structure might mediate the association of HSP70 with HSP90 into a multichaperone complex and involve in the regulation of protein co-chaperone interaction [34–36]. The motif of EEVD, a cytosolic HSP70-specific motif, is highly conserved within most animals HSP70 family members [34,35,37]. Our deduced amino acids also have three highly conserved HSP70 family motifs [38], and consensus pattern of glycosylation positions NKSI, NVSA [39].

The tissue expression analysis of HSP70 showed that the HSP70 was ubiquitously expressed in several organs or tissues in both of male and female Korean rockfish, indicating that HSP70 was synthesized under unstressed conditions. Furthermore, the high expression pattern of KrHSP70 in gill, liver, kidney and head kidney indicated that HSP70 might be correlated with immune response [40,41]. However, the KrHSP70 was found in head kidney, but was not detected in kidney of male fish, indicating that the different functions of these two tissues. The head kidney is an important hematopoietic organ [42], and the kidney is a key lymphoid tissue [43]. In the study of gill of Pacific abalone and swimming crab, this gene appears to be highly expressed [18,44], which was consistent with our result. In addition, our result firstly detected there was a higher expression level in ovary than in testis. However, whether the result was the same or not in gene expression after hypoxia treatment or other environment changes needed a further investigation. Meanwhile, we found the KrHSP70 expressed in tissues including intestine, stomach, fat and caecus, indicating HSP70 also plays an important role in the digestive organs. Several reports proved the cytoprotective functions of HSPs against environmental

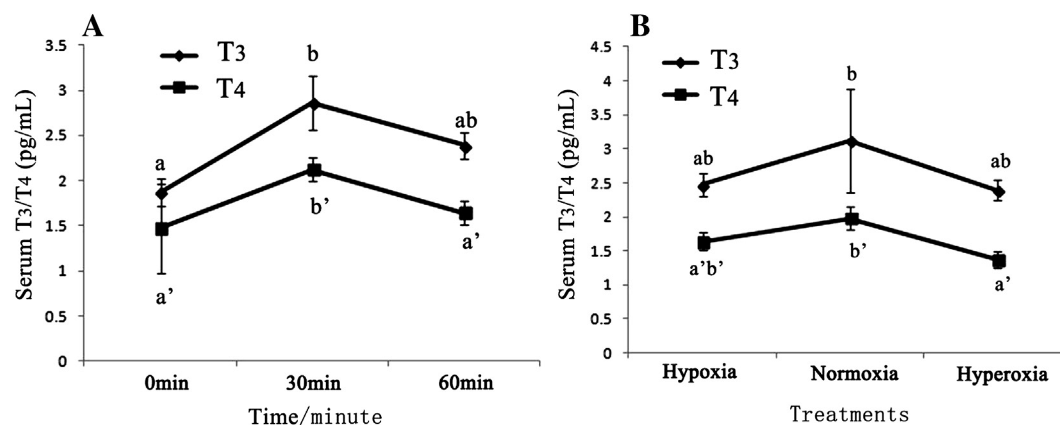

Fig. 8. Serum triiodothyronine (T_3) and thyromine (T_4) levels of female Korean rockfish, during 60 min of treatments with hypoxia (A) and after 60 min different oxygen concentration treatments (B). Values are expressed as mean ± standard error of mean. Different letters indicate significant difference ($P < 0.05$, one-way ANOVA, followed by Duncan's multiple).

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stresses also in digestive organs, those functions are known to be necessary for living cells to obtain tolerance to adapt to environmental changes [45,46].

The knowledge of detailed HSP70 expression profiles is constantly gaining by us, however, their functions in gonads during gonadal development are not yet fully understood [47], especially in teleost. It is significant of differences in the levels of KrHSP70 in gonads during gametogenesis, indicating an important role for this protein during reproduction. In male fish, the expression level of HSP70 increased with the development of testis, reaching peak before immature sperm stage. In female, the level continued to precipitously decrease from perinucleolus oocyte stage to secondary yolk stage, and increased slightly in gestational ovary stage. Many reviews suggested that HSPs expressed in spermatogenesis in order to provide for their specialized needs [48,49] and to serve a molecular chaperone function that allowed spermatogenic cell types to accommodate the unique set of proteins synthesized during the development and differentiation of these cells [50]. In female, the constitutive HSP70 molecular chaperone was also previously indicated for mitosis in early embryo cleavage of undifferentiated cells [51]. In addition, the high expression level of HSP70 at immature ovary stage was found in shrimp, reflecting its function in endocytosis and as molecular chaperone [52]. Lo et al. also pointed out that the expression of the vitellogenin gene may be suppressed by the binding of heat shock-related protein, revealing that promoter sequence of vitellogenin gene appeared to be associated with heat shock transcription factor [52]. Unlike expression in ovary, which was higher in early development stage, the KrHSP70 expression level was high in all development cycles in brain. Study in mammalians indicated that HSP70 expression appears closely linked in early mammalian development to critical differentiation and proliferation stages in early brain [53]. Our result demonstrated that the HSP70 might involve in teleost brain development. In numerous studies, heat shock proteins have been identified to induce by a variety of stressors including hypoxia [54]. Furthermore, little information is known at the level of the cell, tissue, organ and whole organism for stress proteins mitigating the impact of stress in individuals [1]. In present study, the mRNA expression of the HSP70 gene after hypoxia treatment was clearly time-dependent in tissues, with the highest maximum at 30 min for ovary and gill, at 60 min for spleen and liver (Fig. 6). This showed the HSP70 was up-regulated by hypoxia in various tissues, and hypoxia had effects on development and physiology of fish was mentioned in many reports on teleost [54–56]. We supposed that the up-regulated mRNA expression of KrHSP70 in the following hypoxia indicated that the KrHSP70 gene was inducible and involved in the immune response. Pockley suggested that heat shock proteins could be immunoregulatory agents, because these proteins could elicit cytokine production by, and adhesion molecule expression of, a range of cell types, and they could deliver maturation signals and peptides to antigen presenting cells through receptor-mediated interactions [41].

Many reports indicated the gene expression in response to hypoxia appear to be tissue specific [57–60], which was also found in our present study. Tissues of liver, spleen ovary and gill were used as our target tissues to investigate the expression profiles of KrHSP70 gene following different oxygen stress. Our result showed the expression level was highest in hypoxia groups in spleen, liver and gill, and the transcript levels of KrHSP70 were higher in hyperoxia groups than in normoxia groups in liver and gill (Fig. 7). Increased evidences indicated that HSP70 acts as a biochemical marker linked to hypoxia-caused proteo-toxicity, facing hypoxia treatment [61–63]. In the study of Nile Tilapia, there was a significant difference in gills and liver of the HSP70 protein levels between control and hypoxic fish, suggesting that they were primary organ to be affected by hypoxia [63]. The increase in the number of circulating erythrocytes during adaptation to hypoxia raises blood oxygen capacity and improves oxygen delivery to the tissues [64]. In this process, spleen is a tissue with important roles as regards the red blood cells in the immune system [65–67]. The red blood cells number was also accounted in our experiment (Table 2), it is showed that both of hypoxia and hyperoxia increased the red blood cells in Korean rockfish. All results revealed HSP70 also played a crucial role in the spleen during hypoxia treatment, confirming the immune function of HSP70. In the present experiment, we discovered alterations of the HSP70 mRNAs in the ovary, where the expression level was highest in normoxia group. HSPs were hypothesized to play a role in ovulation and maintenance of the postovulatory metabolic activity and oocyte survival [68]. Furthermore, pattern in ovary expression level was similar with the serum T_3 and T_4 level in our present experiment. The thyroid hormones, T_3 and T_4, were widely accepted to be acted as modulators of the immune response [69]. The level of T_3 and T_4 in Korean rockfish decreased at one hour hypoxia, and the level in oxygen-changed group was much lower than in normoxia group. Many reports suggested that the circulating levels of thyroid hormones are known to decline exposed to stress condition [70]. Coimbra and Reis-Henriques found that under stress factors, ovary and testicular alterations with decline in T_4 levels and normal levels of T_3 in Tilapia fish [71].

In conclusion, a novel HSP70 gene from the Korean rockfish (*S. schlegeli*) was identified and characterized for the first time. HSP70 gene expression was detected in male and female fish during reproductive development cycles in gonads and brains. We also analyzed the HSP70 expression pattern in female Korean rockfish under different oxygen stress in gill, spleen, liver and ovary, linking with the serum T_3 and T_4 levels. The cloning and expression analysis of HSP70 gene will provide more information of the characterization of HSP70 family members in the ovoviviparous fish. These data would be useful for further study of the expression characteristics under various stress conditions, and to reveal indepth knowledge in the immune function of HSP70 in teleost.

Table 2
Changes in RBCs under different oxygen concentration treatments. Different letters indicate significant difference ($P < 0.05$, one-way ANOVA, followed by Duncan's multiple).

Treatments (Hypoxia)	Number of RBCs division ($\times 10^{12}$) L^{-1}	Treatments 2(One hour)	Number of RBCs division ($\times 10^{12}$) L^{-1}
0 min	2.11 ± 0.11^a	Hypoxia	1.91 ± 0.03^a
30 min	2.00 ± 0.08^a	Normoxia	1.46 ± 0.09^b
60 min	1.91 ± 0.03^a	Hyperoxia	1.75 ± 0.03^a

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