Kor. J. Microbiol. Biotechnol.
Vol. 32, No. 4, 297–306 (2004)

Molecular Biological Studies on the Stress Protein HSP90β Gene from Flounder (*Paralichthys olivaceus*)

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Heat shock proteins (HSPs) were induced in cells in the thermal stress, and the HSP90 family is one of the major classes of HSPs. Gene encoding HSPs have been characterized from various mammals and piscine. We have cloned and sequenced the HSP90 cDNA from a brain cDNA library constructed from flounder (*Paralichthys olivaceus*). The result of sequence analysis shows it to be the HSP90β. The nucleotide sequence of the HSP90β was composed of 2791 long, encoding 726 amino acid residues. The flounder *hsp90β* gene showed very high sequence homology with *hsp90β* of European sea bass (96.6%), zebrafish (92.9%), Atlantic salmon (92.0%) and human (89.5%). We also constructed a phylogenetic tree based on HSP90 amino acid sequences from vertebrate species. Gene-specific primers were selected and used in RT-PCR reactions to measure the basal *hsp90β* mRNA. The *hsp90β* gene is constitutively expressed at a fairly high level in all examined tissues (brain, liver, kidney, muscle, and spleen). In order to express protein of flounder *hsp90β* in *E. coli*, we used the His-tagged pET-44(a)+ vector. Then, the expression of flounder HSP90β was confirmed by Western blot analysis.

Key words: Heat shock protein; cDNA library; flounder (*Paralichthys olivaceus*), expression, western blot

INTRODUTION

Heat shock proteins (HSPs) are ubiquitous, highly conserved proteins that are vital for cells from bacteria to mammals and plants [3]. The high expression of HSPs in cells is stress-inducible after exposure to a wide variety of stressors, such as heat shock, heavy metals, or almost sudden change in the cellular environment which induces protein damage [1, 22]. HSPs have known roles in chaperoning the correct folding of newly formed proteins, in maintaining the active conformation of existing proteins, and in preventing aggregation of improperly folded proteins. Once cells are exposed to heat and a variety of other stressful stimuli, the highly conserved HSPs are accumulated. However, non-stressful stimuli can regulate *hsp* gene expression [31].

The family of HSPs consists of HSP 100, 90, 70, 60, and 20 on the basis of their molecular weight. One of the major classes of HSPs induced in cells in response to thermal stress is the HSP90 family. HSP90 is constitutively abundant at 1-2% of cytosolic proteins in normal physiological conditions [28, 32]. The α and β paralogous forms of HSP90 have been descried in several vertebrates including zebrafish [19], Chinook salmon [27], carp [12], chicken [2, 23], mouse [14, 24], and human [13, 29]. It has been proposed that *hsp90α* and *hsp90β* evolved by duplication of a common ancestral gene more than about 500 million years ago, close to the time of emergence of vertebrates [9, 12, 23]. The *hsp90α* and *hsp90β* genes display different patterns of regulation during development and cell differentiation as well as in response to heat shock and other environmental stimuli. Both forms of HSP90α and HSP90β proteins exist mostly as homodimers. Slight differences in the C-terminal dimerization domain render the HSP90β dimers less stable than the α homodimers. This difference in stability also explains why the majority of HSP90 monomers come from the β isoform [9, 26]. HSP90 interacts with many cellular proteins, its specific functional domains are not well described, and organism-specific or isoform-specific functions remain uncharacterized. Considering the high cellular abundance and apparently high functional versatility of HSP90, it is likely that the biological relevance and/or method of a given HSP90 function differ between groups of organisms. Accumulating

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evidence suggests that HSP90 interacts with specific target proteins (e.g., kinases) and plays an essential role in steroid-receptor fidelity [7, 25, 28]. However, the detailed mechanisms of expressional regulation of HSP90 in a variety of organisms and tissues are not clearly understood.

The flounder (*Paralichthys olivaceus*), one of the most evolved teleosts, is commercially important marine aquaculture species in Korea and has been used for the molecular levels of the study on various functional genes [6, 15, 21].

In the present study, we initially focus on the isolation of cDNA encoding flounder HSP90β from the flounder (*P. olivaceus*) and characterize its expressions in adult tissues. Also, we have phylogenetically analyzed flounder HSP90β sequence with other vertebrate HSP90 sequences. Herein, we provide the molecular characteristics and tissue expressions of our newly identified the flounder HSP90β (GenBank accession number, AY214170) cDNA from adult flounder.

MATERIALS AND METHODS

RNA Isolation and cDNA Library Construction

Mature flounders (*P. olivaceus*) were purchased from a nearby fish market and ten brain glands from both sexes were collected. Total RNA was isolated with a TRIzol reagent (Invitrogen, Carlsbad, USA). The RNA pellet was washed with 70% ethanol, dried, and dissolved in DEPC-treated water. Poly(A) RNA was isolated with a Micro-FastTrack™ 2.0 Kit (Invitrogen, Carlsbad, USA). The quantity of RNA was determined by measuring the O.D. value at 260 nm. The construction of the brain cDNA library was performed using a ZAP-cDNA® Synthesis Kit (Stratagene, La Jolla, USA). The resulting library contained approximately 2×10^5 clones. The library was then amplified up to 4×10^9/ml.

Screening of HSP90β cDNA and DNA sequencing

Conserved nucleotide sequences of vertebrate HSP90β were searched using the NCBI (National Center for Biotechnology Information) nucleotide and protein sequence database and used for designing of oligonucleotide primers. Oligonucleotide degeneracy primers for probe preparation for screening HSP90 were synthesized at GenoTech (Taejeon, Korea). The probe used for screening of HSP90β was amplified by PCR using upstream (HSP90β-F; 5'-GCC/GGAG/AGACAAGGAG/CAACTAC-3') and downstream (HSP90β-R; 5'-GAAGAC/AACCAGT/CCTGTTG /TGA-3') primers and labeled with a DIG (digoxigenin) oligonucleotide 3' end labeling kit (Roche, Mannheim, Germany). The main PCR program consisted of 30 cycles at 94°C for 30 sec, 62°C for 30 sec, and 72°C for 30 sec. Approximately, 1×10^5 plaques from the cDNA library were screened with the above probe. Positive plaques recovered from the first screening were further confirmed by the second screening [5, 16, 17, 18, 21]. Positive plaques were recovered from the second screening and the phagemid containing the insert was excised according to the manufacturers instructions (Stratagene, La Jolla, USA). DNA sequencing of the excised phagemid was performed using the ABI PRISM™ DNA sequencing kit (Applied Biosystems, Foster, USA) and determined with ABI 377 Genetic Analyzer according to the manufacturers instructions (Applied Biosystems, Foster, USA).

Comparative Sequence Analysis of vertebrates HSP90

To define the molecular evolution of HSP90, several vertebrates HSP90 sequences were imported from the SwissPort data bank/GenBank as follows: *Dicentrarchus labrax* (European sea bass β; AAQ95586), *Danio rerio* (zebrafish β; AAH65359), *Salmo salar* (Atlantic salmon β; AAD30275), *Homo sapiens* (human β; NP-031381), *Mus musculus* (house mouse β; AAA37866), *Gallus gallus* (chicken β; Q04619), *Sus scrofa* (pig α; AAC48718), *H. sapiens* (human α; NP-031381), *Mus musculus* (house mouse α; AAA37865), *G. gallus* (chicken α; HHCH90), *D. rerio* (zebrafish α; Q90474), and *Oncorhynchus tshawytscha* (chinook salmon α; AAB49983) HSP90 sequences. The nucleotide sequences were analyzed using the program BLAST (http://www.ncbi.nlm.nih.gov/BLAST). A multiple sequence alignment was conducted using the program Clustal W (http://www.ebi.ac.uk/clustalw) and sequence identities were calculated using GeneDoc (http://www.psc.edu/biomed /genedoc). As an indication of confidence in the branching order, a bootstrap analysis (1000 replications) was completed for both distance and parsimony methods. A phylogenetic dendrogram presented by means of the Treeview program.

Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

In order to perform RT-PCR, total RNA was isolated

from brain, kidney, muscle, liver, and spleen from mature flounder (N=10; size: 45 cm ±10 cm, body weight: 900 g ±300 g; 3 years old). Titan™ one tube RT-PCR system (Roche, Mannheim, Germany) was used. Master mix 1 contained 0.2 mM dNTPs, 5 mM dithiothreitol, 50 pmol upstream (HSP-F2; 5'-ATGAGTACTGCGTCCAGCAGCTG-3') and downstream (HSP-R2; 5'-TTTGCATTTACTGCA-GTTTA-3') primers, template RNA, and 5U of Rnase inhibitor. Master mix 2 consisted of 5×RT-PCR buffer and enzyme mix. Mix 1 and mix 2 were added to a 0.2-ml thin-walled PCR tube on ice. Then the sample was placed in a thermocycler (Applied Biosystems, GeneAmp PCR system 2400) and incubated for 1 hour at 50°C for reverse transcription followed by thermocycling. The temperature profile of HSP90 was on prereaction at 94°C for 5 minutes; 30 cycling reactions at 94°C for 30 seconds, 60°C for 30 seconds, and 72°C for 40 seconds; and finally 15 min extension at 72°C.

Northern Blot Analysis

Flounders weighing 700-1000g were acclimatized under fasting conditions in well-aerated 300L water tanks over a 2-week period at 17°C. In heat shock treatments, fish were exposed to 25°C for up to 24h. At a given time, total RNA was isolated from liver tissue of heat shock treated flounder. Five μg of total RNA taken from a given time was separated by electrophoresis on a 1.5% formaldehyde gel. Denaturing gel loading mixture (RNA sample, 5X formaldehyde gel running buffer, 3.5 μl of 37% formaldehyde, 10 μl of formamide, and water to 20 μl) was made and incubated at 65°C for 15 min. After incubation, the mixture was rapidly cooled down on ice. 2 μl of formaldehyde gel-loading dye (50% glycerol, EDTA pH 8.0, 0.25% bromophenol blue and xylene cyanol) was mixed with it. Electrophoresis was performed using 1% formaldehyde gel in 1X MEA buffer (0.1 M MOPS [3-(N-morpholino)-propanesulfonic acid] pH 7.0, 40 mM sodium acetate, 5 mM EDTA pH 8.0). Then, the gel was transferred to 20X SSC (DEPC treated) and incubated twice for 15 min. RNA was transferred to a NC membrane using a capillary transfer method and cross-linked using a UV cross-linker with preset condition (1200 μJ/cm^2 at 254 nm). Hybridization and detection were performed as described on DIG labeling and detection kit manual (Boehringer Mannheim, Germany).

Overexpression of Flounder HSP90β Gene in *E. coli*

In order to express the HSP90β gene, the cloned HSP90β cDNAs was subcloned into pET-44a(+) expression vector (Novagen, USA) with His-tagged was modified by excised *Nde* I-*Xho* I fragment. The pET-44a(+) expression vector allows expression of a recombinant protein with a C-terminal fusion His-tag. A cDNA fragment encoding the entire HSP90β coding region was amplified by PCR with a pair of olignucleotides 5'-ACATATGCACCA-AGAAGAAG-3' and 5'-CTCGAGATCGACTTCCTC-3' on the cloned full-length flounder HSP90β cDNA. The resulting plasmid pET-44a(+)-HSP90β was used to transform the competent *E. coli* strain BL21(DE3). The cells harboring the HSP90β gene were cultured in LB medium (containing 50 μg/ml ampicillin) and induced by adding IPTG (isopropyl-β-D-thiogalactopyranoside) to a final concentration of 1 mM at a cell density corresponding to OD_{600} = 0.5. Expressed proteins were analyzed by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

Western Blot Analysis

The ten micrograms of the samples were used for 10% SDS-polyacrylamide gel electrophoresis and transferred onto a nitrocellulose membrane by a semi-dry method. The blot was incubated for 1h with 3% gelatin in TTBS (20 mM Tris-HCl, pH 7.4; 500 mM NaCl; 0.05% Tween 20) and then rinsed with TTBS. Subsequently, a polyclonal antibody against goat anti-6-Histidine (diluted 1:1000) was added and incubated for 1h at room temperature. After three washes with TTBS, the membrane was incubated with anti-goat antibody conjugated with alkaline phosphatase (Sigma; diluted 1:2000 in TTBS containing 1% gelatin) at room temperature for 30 min. The membrane was then rinsed three times with TTBS and developed at room temperature in a developing buffer (15 mg of Nitro Blue Tetrazolium; 0.7% N, N-dimethylformamide; 30 mg of 5-bromo-4-chloro-3-indolyl phosphate per 100 ml; 1 mM $MgCl_2$ and 100 mM $NaHCO_3$, pH 9.8).

RESULTS AND DISCUSSION

Fish are the largest and most diverse group of vertebrates. Their evolutionary position relative to other vertebrates and their ability to adapt to a wide variety of environments make them ideal for studying both organism

and molecular evolution. The major HSPs induced in response to thermal stress are the HSP90.

We report here the complete nucleotide and deduced amino acid sequence of HSP90β from flounder (*P. olivaceus*). We constructed a brain cDNA library using in ZAP-cDNA® Synthesis Kit with oligo-dT-purified mRNA from flounder brain tissues. Screening of approximately 10^4-10^5 independent plaques with a 500-nt PCR probe made from a flounder cDNA and primers based on the vertebrate *hsp90β* DNA conserved sequences, several plaques were isolated. Positive plaques were recovered from the second screening and the longest phagemid containing the insert was excised and its sequence was determined in both directions.

The cDNA encoding flounder HSP90β was cloned and its nucleotide sequence was determined as shown in Fig. 1. The cDNA sequenced was a 2791 bp fragment carrying a single open reading frame (ORF) of 2166 nucleotides, starting with an ATG-start codon and ending in an in-frame TAA-stop codon. The cDNA included 5-noncoding region, 197 nucleotides upstream of the ORF, and 3-noncoding region, 428 nucleotides beyond the termination signal, with an ATTAAA polyadenylation signal. The GenBank accession number for the HSP90β nucleotide sequence data in this paper is AY214170.

The ORF encoded a 722 amino acid protein with a predicted molecular weight of 82.9 kDa. The deduced amino acid sequence shown in Fig. 1 had significant homology to known HSP90 molecules, with EEVD at the carboxy-terminus, characteristics of known HSP90 sequences, and also HSP70 sequences, and several highly conserved domains, which have been recognized as useful for identifying the HSP90 family of proteins [11]. The flounder HSP90β contains a conserved consensus leucine zipper (LZ), identified originally as a protein joining motif [20, 27], from Met393 to Leu414, and consisting of M393-X_6-L400-X_6-L407-X_6-L414 (X is any amino acid) (Fig. 1). Little is known of the function of the LZ except that it is unlikely to be involved in HSP90 dimer formation [27].

The α and β paralogs of HSP90, highly homologous one another but encoded by separate genes have described in vertebrates [9, 12, 23]. The paralogs share about 85% amino acid identity within species in humans, mice, and chickens, but even greater identities are found comparing orthologous forms between species. For example, human HSP90α shares 95-99% identity with α forms from pig, chicken, and house mouse; and human HSP90β is 98% and 99% identical to its orthologous β forms in chicken and house mouse, respectively. The existence of two forms of HSP90 in flounder has not yet been established.

Comparison of the flounder HSP90β with other vertebrates is shown in Fig. 2. For investigating the similarity of flounder HSP90β with other vertebrates, the BLAST program was run. By this analysis flounder HSP90β gene showed high homology to HSP90β of European sea bass (96.6%), zebrafish (92.9%), Atlantic salmon (92.0%), human (89.5%), house mouse (89.8%), and chicken (88.5%). Also, flounder HSP90β gene showed high homology to HSP90α of pig (86.0%), human (86.0%), house mouse (85.6%), chicken (85.6%), zebrafish (81.6%), and Chinook salmon (80.5%). The conservation of the HSP90β amino acid sequence among different vertebrates is high and the identity. Regions A-E have been found to be ATP-binding sites [4, 8]. The high conservation of these regions highlights the importance of ATP-binding in the HSP90-involved processes of diverse function between them. The ATP-binding has been found to cause structural changes in HSP90 necessary for accommodating its interaction with target substrates [10, 27]. We found three highly divergent regions (I-III in Fig. 2) among vertebrates HSP90 amino acid sequences. Furthermore, we found 15 amino acids conserved among but differing between HSP90α and HSP90β sequences (Fig. 2). The flounder HSP90β protein had four N-linked glycosylation sites (N-X-S/T) (residues 45-47; 282-284; 388-390; 613-615) like other vertebrates, but had 5 different amino acids (F33, D36, D94, V477, M483) among vertebrates HSP90 proteins (Fig. 2).

The phylogenetic analysis with the entire sequences of the *hsp90β* gene was shown in Fig. 3. This data shows *hsp90α* and *hsp90β* genes duplication event shortly before the appearance of the teleosts, a relative vertebrate, from the rest of the vertebrate lineage. A consensus tree confirmed that the flounder HSP90 was a β form, and that it was most related to the European sea bass HSP90β (Fig. 3). Also, the flounder *hsp90β* gene shows a close relationship among the tetrapod vertebrates *hsp90α* genes rather than fish *hsp90α* genes. This phylogenic evidence on the *hsp90β* gene provides the clues that may be essential to understand the molecular evolution of this gene in vertebrates.

To characterize the tissue expressions of flounder *hsp90β*, the reverse transcription-polymerase chain reaction

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1    TCGAAATTAACCCTCACTAAAGGGAACAAAAGCTGGAGCTCCACCGCGGTGGCGGCCGCTCTAGAACTAGTGGATCCCCCGGGCTGCAGG
91   AATTCGGCACGAGCTCGAGTTGAATACGTGAGAGAGGAGGAGGGACGAAGCTTGGGTGGCATTTAATTCAAGATAAAGTCAACGAAAGTA
181  ACGAGATGCCTGAAGAAATGCACCAAGAAGAAGAGGCTGAGACCTTTGCCTTCCAAGCAGAGATCGCTCAGCTGATGTCCCTGATCATCA
         M  P  E  E  M  H  Q  E  E  E  A  E  T  F  A  F  Q  A  E  I  A  Q  L  M  S  L  I  I    28
271  ACACCTTCTATTTCAACAAAGATATCTTCCTCAGGGTGTTGATCTCCAATGCTTCTGATGCCTTGGACAAAATCCGTTACGAAAGCCTGA
     N  T  F  Y  F  N  K  D  I  F  L  R  V  L  I  S  N  A  S  D  A  L  D  K  I  R  Y  E  S  L    58
361  CTGAACCCACCAAGATGGACAGTGGCAAGGATTTGAAAATTGACATCATCCCCAACAAAGAGGACCGCACCCTGACCCTCATTGACACTG
     T  E  P  T  K  M  D  S  G  K  D  L  K  I  D  I  I  P  N  K  E  D  R  T  L  T  L  I  D  T    88
451  GAATCGGTATGACCAAAGCCGACCTGGTCAACAACCTGGGTACCATTGCCAAGTCTGGCACCAAGGCTTTCATGGAGGCTCTGCAGGCTG
     G  I  G  M  T  K  A  D  L  V  N  N  L  G  T  I  A  K  S  G  T  K  A  F  M  E  A  L  Q  A   118
541  GTGCTGACATCTCTATGATTGGTCAATTCGGTGTGGGTTTCTACTCCGCCTACCTTGTTGCCGAGAAGGTGGTTGTCATCACGAAGCACA
     G  A  D  I  S  M  I  G  Q  F  G  V  G  F  Y  S  A  Y  L  V  A  E  K  V  V  V  I  T  K  H   148
631  ATGACGATGAGCAGTATGCCTGGGAGTCCTCTGCCGGAGGTTCATTCACAGTCAAGGTCGACACCGGCGAGCCCATTGGCCGTGGTACAA
     N  D  D  E  Q  Y  A  W  E  S  S  A  G  G  S  F  T  V  K  V  D  T  G  E  P  I  G  R  G  T   178
721  AGATCGTCCTGCACCTGAAGGAGGACCAGACTGAGTACGTTGAGGATAAGAGGGTTAAGGAGATTGTGAAGAAGCACTCTCAGTTTATCG
     K  I  V  L  H  L  K  E  D  Q  T  E  Y  V  E  D  K  R  V  K  E  I  V  K  K  H  S  Q  F  I   208
811  GCTACCCCATCACCCTGTTTGTGGAGAAGGAGCGTGACAAGGAGATCAGTGACGACGAGGCAGAGGAGGAAAAGGCTGAGAAGGAGGAGA
     G  Y  P  I  T  L  F  V  E  K  E  R  D  K  E  I  S  D  D  E  A  E  E  E  K  A  E  K  E  E   238
901  AAGAGGATGAAGGTGAGGACAAGCCAAAGATTGAGGATGTGGGCTCAGATGATGAGGAAGACTCCAAAGACAAGGACAAGAAGAAGACAA
     K  E  D  E  G  E  D  K  P  K  I  E  D  V  G  S  D  D  E  E  D  S  K  D  K  D  K  K  K  T   268
991  AGAAGATCAAGGAGAAGTACATCGTCCAGGAGGAGCTGAACATGACCAAGCCCATCTGGACCAGAAACCCTGATGACATCACAAACGAGG
     K  K  I  K  E  K  Y  I  V  Q  E  E  L  N  M  T  K  P  I  W  T  R  N  P  D  D  I  T  N  E   298
1081 AGTATGGAGAGTTCTACAAGAGTCTGACCAATGACTGGGAGGATCATCTGGCTGTCAAGCACTTCTCAGTGGAAGGCCAGCTTGAATTCC
     E  Y  G  E  F  Y  K  S  L  T  N  D  W  E  D  H  L  A  V  K  H  F  S  V  E  G  Q  L  E  F   328
1171 GTGCCCTCCTCTTCATCCCCCGCCGTGCTCCTTTTGACCTCTTTGAGAACAAGAAGAAGAAGAATAACATCAAGCTGTACGTCAGGAGAG
     R  A  L  L  F  I  P  R  R  A  P  F  D  L  F  E  N  K  K  K  K  N  N  I  K  L  Y  V  R  R   358
1261 TCTTCATCATGGACAATTGTGAAGAGCTCATCCCAGAGTACCTGAACTTTGTCCGTGGTGTGGTGGACTCCGAGGATCTGCCCCTCAACA
     V  F  I  M  D  N  C  E  E  L  I  P  E  Y  L  N  F  V  R  G  V  V  D  S  E  D  L  P  L  N   388
1351 TCTCCAGAGAAATGCTGCAGCAGAGCAAAATCCTCAAGGTCATTCGCAAGAACATCGTCAAGAAGTGTCTGGAGCTGTTTGCAGAGCTGG
     I  S  R  E  M  L  Q  Q  S  K  I  L  K  V  I  R  K  N  I  V  K  K  C  L  E  L  F  A  E  L   418
1441 CCGAGGACAAGGAGAACTACAAGAAGTTCTATGAGGGTTTCTCAAAGAACATCAAGCTGGGAATCCACGAGGACTCACAAAACCGCAAGA
     A  E  D  K  E  N  Y  K  K  F  Y  E  G  F  S  K  N  I  K  L  G  I  H  E  D  S  Q  N  R  K   448
1531 AGCTTTCTGAGCTGCTGCGTTACCAAAGCTCTCAGTCTGGAGATGAGTCTACCTCCCTCACAGAGTACCTGTCCCGCATGAAGGAGAACC
     K  L  S  E  L  L  R  Y  Q  S  S  Q  S  G  D  E  S  T  S  L  T  E  Y  L  S  R  M  K  E  N   478
1621 AGAAGTCCATCTACTACATCACTGGTGAGAGCAAGGATCAGGTGGCCAACTCCGCCTTCGTCGAGCGCGTCCGCAAGCGTGGCTTCGAAG
     Q  K  S  I  Y  Y  I  T  G  E  S  K  D  Q  V  A  N  S  A  F  V  E  R  V  R  K  R  G  F  E   508
1711 TCCTGTACATGACAGAGCCAATCGATGAGTACTGCGTCCAGCAGCTGAAGGAGTTTGACGGTAAGACCCTGGTCTCTGTCACCAAGGAGG
     V  L  Y  M  T  E  P  I  D  E  Y  C  V  Q  Q  L  K  E  F  D  G  K  T  L  V  S  V  T  K  E   538
1801 GCCTGGAGCCGCCTGAGGATGAGGAGGAGAAGAAAAAGATGGAGGAGGACAAGGCCAAGTTTGAGAACCTCTGCAAACTCATGAAGGAGA
     G  L  E  P  P  E  D  E  E  E  K  K  K  M  E  E  D  K  A  K  F  E  N  L  C  K  L  M  K  E   568
1891 TCCTGGACAAGAAAGTGGAGAAGGTGACAGTGTCCAACAGACTGGTGTCTTCACCCTGCTGCATTGTGACAAGTACTTACGGATGGACAG
     I  L  D  K  K  V  E  K  V  T  V  S  N  R  L  V  S  S  P  C  C  I  V  T  S  T  Y  G  W  T   598
1981 CCAACATGGAGAGAATCATGAAGGCCCAGGCACTCAGGGACAACTCCACCATGGGCTACATGATGGCCAAGAAGCACCTTGAGATCAACC
     A  N  M  E  R  I  M  K  A  Q  A  L  R  D  N  S  T  M  G  Y  M  M  A  K  K  H  L  E  I  N   628
2071 CTGACCACCCCATCGTGGAAACTCTCAGGCAGAAGGCTGATGCTGACAAAAATGACAAGGCTGTGAAGGACCTTGTCATCCTGCTCTTTG
     P  D  H  P  I  V  E  T  L  R  Q  K  A  D  A  D  K  N  D  K  A  V  K  D  L  V  I  L  L  F   658
2161 AAACGGCCCTGCTGTCCTCAGGCTTCTCCCTGGACGACCCACAGACCCACTCCAACCGCATCTACAGAATGATCAAACTCGGCCTGGGTA
     E  T  A  L  L  S  S  G  F  S  L  D  D  P  Q  T  H  S  N  R  I  Y  R  M  I  K  L  G  L  G   688
2251 TCGATGACGACGATGTTCCGACAGAGGAAACCACTTCAGCAGCTGTCCCTGATGAGATTCCTCTCCTAGAAGGCGATGGCGAAGATGATG
     I  D  D  D  D  V  P  T  E  E  T  T  S  A  A  V  P  D  E  I  P  L  L  E  G  D  G  E  D  D   718
2341 CTTCACGCATGGAGGAAGTCGATTAAACCACCCCCCCCTCGTCCCAGATTTTTAACACTTTAGCCTCACTTTTCAATTCATCCCTTAAAC
     A  S  R  M  E  E  V  D  *                                                                  726
2431 TGCAGTAAATGCAAAACAAATAGTCATTCATGTTGTGTGGTGGACCAGTGTTGCTCTCGTGTCCAGAGCATTACTCTGCAACGCCCCCTG
2521 TTAAGAAAAGCAATTTTGGTTTTTGCTGTATAAGTTCATGGTGACAGCACATTTGTTTAACGAGTACCCTGTTGCACTGAGTTTTAAATG
2611 TCGGAGTGGTGAACATGGGAATGGTACATTCCATTGTCAGTCTGGAGGGTTAGGGGAGGTTCTGCTCATGTGCAACACTGCACGCTGCAT
2701 GGAGAGAGGACTGTATGATTCCTTTGCCTGAGTCCAGGCTTGTCTGTATTCCAAGTCTTTGTTTTGCAAAAAATTAAAGATGTAATACCT
2791 T
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Fig. 1. Nucleotide and deduced amino acid sequences of the flounder HSP90β cDNA. Numbers on the left and right margin correspond to the first nucleotide and last amino acid in the line, respectively. The postulated ATG start codon, TAA stop codon (*) are in bold and are underlined. The consensus polyadenylation signal (ATTAAA) is double-underlined. The consensus leucine zipper sequence is underlined. The conserved EEVD domain is double-underlined.

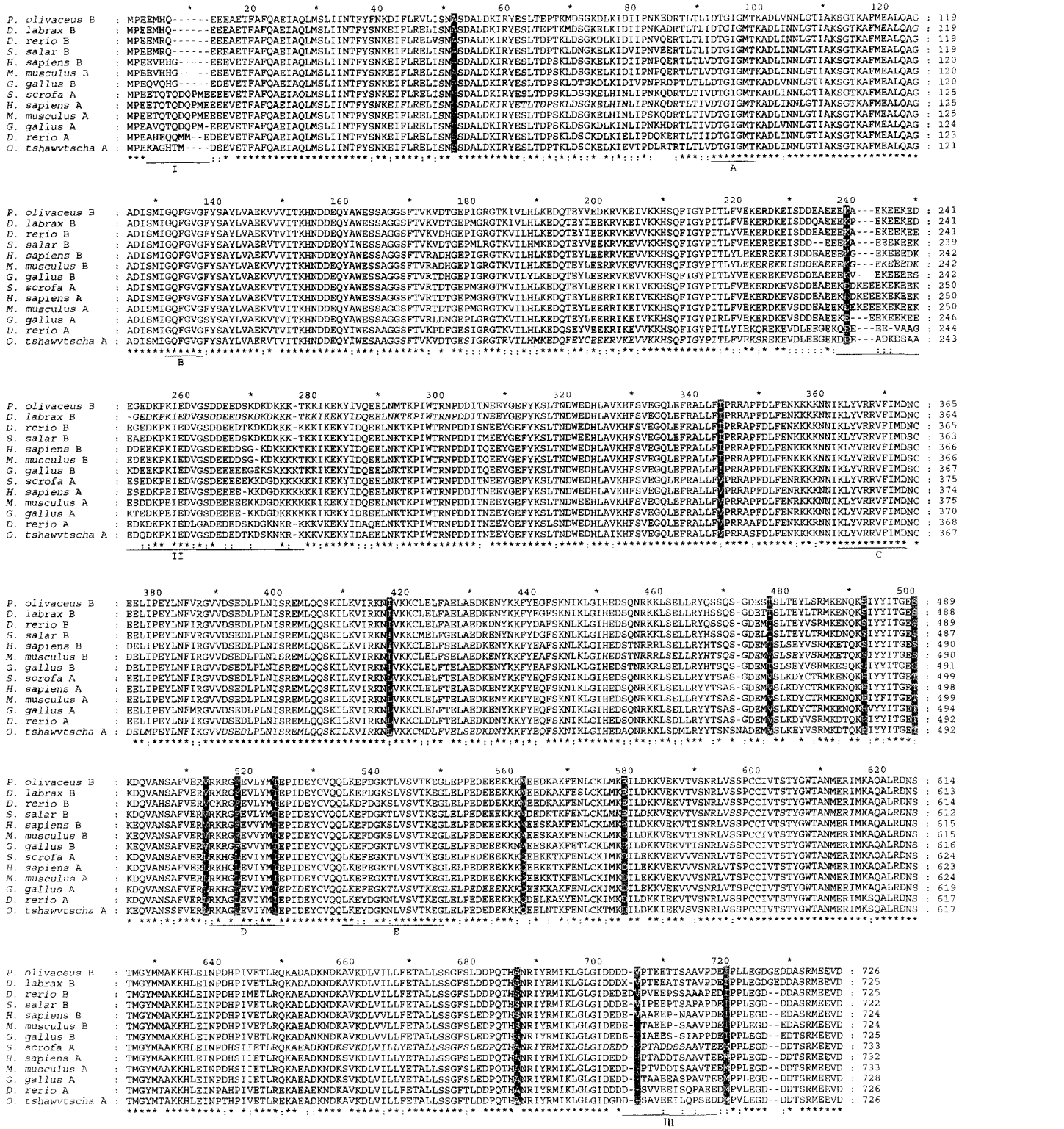

Fig. 2. Alignment of deduced amino acid sequence of the flounder HSP90β from other vertebrate HSP90. Identical amino acid residues among the HSP90 are marked as Asterisk (∗). The conservation of one of the following functional groups: non-polar, polar/uncharged, charged/acidic, or charged/basic are marked as colons. The postulated ATP-binding regions are underlined and alphabet (A-E). The highly divergent regions are underlined and roman numerals (I-III). Shaded amino acids indicate amino acid substitutions unique to and ubiquitous within α or β sequences but differ between them.

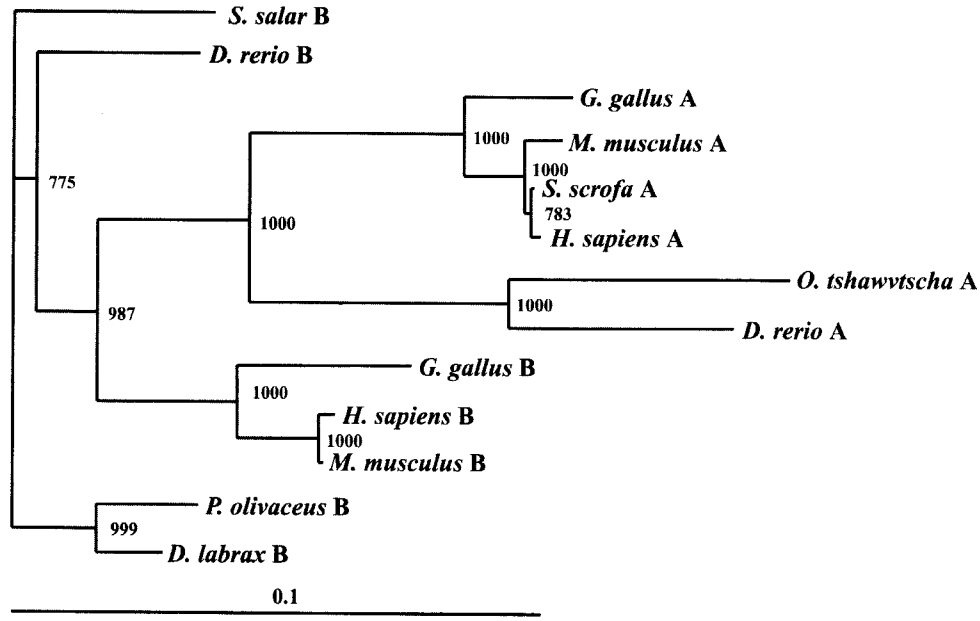

Fig. 3. Phylogenetic tree for HSP90 from vertebrates. The tree was drawn by the maximun-parsimony distance method on the amino acid sequence of HSP90 from *Dicentrarchus labrax* β, *Danio rerio* β, *Salmo salar* β, *Homo sapiens* β, *Mus musculus* β, *Gallus gallus* β, *Sus scrofa* α, *Homo sapiens* α, *Mus musculus* α, *Gallus gallus* α, *Danio rerio* α, *Oncorhynchus tshawytscha* α, *Paralichthys olivaceus* HSP90β amino acid sequences. The numbers at the nodes are the bootstrap values with 1000 trials.

(RT-PCR) was performed with primers specific for the *hsp90β* using total RNAs isolated from flounder tissues as a template. As shown in Fig. 4, the patterns of DNA fragments amplified from RT-PCR provided evidence for the expressions of the *hsp90β* gene. The expression of the *hsp90β* gene was detected in brain, kidney, muscle, liver, and spleen. Thus, flounder *hsp90β* mRNA has a wide tissue distribution. The *hsp90α* and *hsp90β* genes display different patterns of regulation during development and cell differentiation as well as in response to heat shock and other environmental stimuli. The response of the *hsp90α* expression to heat shock, at a transcriptional level, was previously investigated during embryo genesis in zebrafish [12, 19]. At normal temperature, *hsp90α* mRNA was not detectable in the developing brain and spinal cord but *hsp90β* mRNA was detectable various tissues include brain and spinal cord [12].

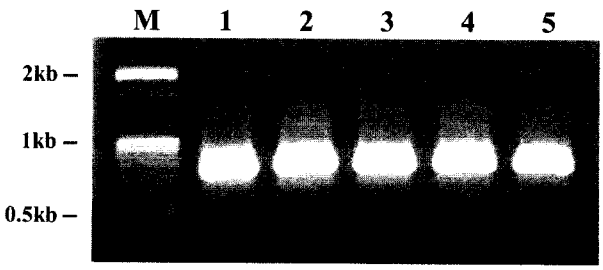

Fig. 4. Patterns of the expression of flounder HSP90β detected by RT-PCR. Lane M indicates a molecular marker. Lane 1 indicates brain; 2, kidney; 3, muscle; 4, liver; and 5, spleen.

To investigate the effects of hyperthermia, flounder were exposed to an 8°C increase of temperature. The expression pattern of the *hsp90β* gene was measured immediately after heat shock and also after a 2hr recovery period at the acclimatization temperature. Total RNA was prepared and hybridized with probe specific for the *hsp90β* and β-actin gene. There were 2.9, 3.3, 5.2, 5.6 fold increase of *hsp90β* after 2, 4, 6 and 8 hrs treatment, respectively. However, the expression level decreased to 4.7 fold after 24 hrs treatment (Fig. 5).

In order to express of flounder *hsp90β* in *E. coli*, we used the His-tagged pET-44(a)+ vector, which produces the recombinant protein of flounder HSP90β containing a short histidine tag at the carboxyl-terminus was overexpressed in *E. coli* BL21 (DE3) using an inducible T7 expression system. The HSP90β-histidine fusion protein was overexpressed under the control of the promoter and the expressed proteins were separated on 10% SDS-PAGE. The results show that the molecular weight of His-tagged HSP90β fusion protein is about 83.7 kDa (Fig. 6). Predicted actual protein size of HSP90β was about 82.9 kDa. The pET-44(a)+ vector has 0.8 kDa of C-terminal

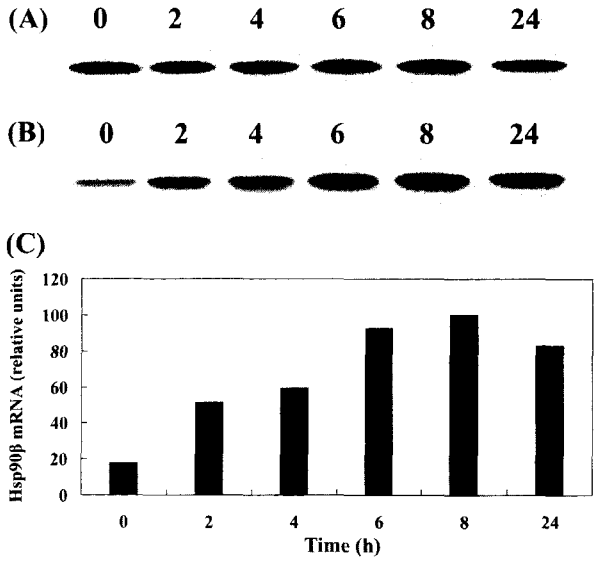

Fig. 5. Effect of heat treatment on the expression of β-actin and *hsp90β* in the flounder liver. The fish were exposed to an 8°C jump in temperature for up to 24 h. The β-actin mRNA levels were used as control (A). Messenger RNA of *hsp90β* was isolated from the tissue immediately after heat treatment and used Northern blot analysis for analysis (B). The Northern blot of *hsp90β* was quantified and plotted over time (C).

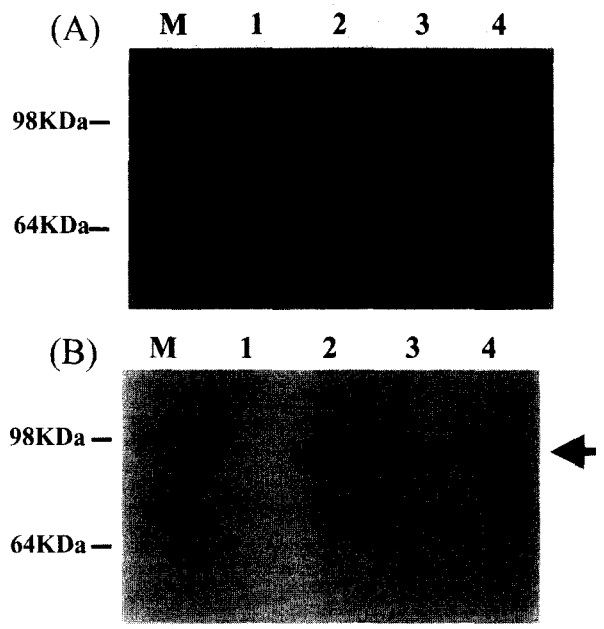

Fig. 6. Analysis of the expressed HSP90β in *E. coli* (A) and Western blot analysis (B). M indicates protein molecular weight marker; C, proteins from uninduced cell extracts of BL 21 (DE3) pLys. Lane 1-4, proteins from induced cell extracts 0, 1, 2, and 3 hr after IPTG induction, respectively.

fusion tag.

Fish are the most primitive vertebrates, and genetic information obtained from fish can reveal the origin and diversion of genes with a similar function in other organisms. Observations and genetic manipulations of the flounder HSP90 make this species a very useful model for studying the mechanism of HSP participation in chaperoning the correct folding of newly formed proteins, in maintaining the functionally active conformation of existing proteins, and in preventing aggregation of improperly folded proteins.

In conclusion, a comparison of the amino acid sequences of vertebrate HSP90β indicated that the flounder HSP90β is highly conserved with those of other species. The expression of the *hsp90β* gene was detected in brain, kidney, muscle, liver, and spleen. Thus, flounder *hsp90β* mRNA has a wide tissue distribution. These results will provide a wider base of knowledge on the primary structure of HSP90β at the molecular level and the functional diversity. Furthermore, it will contribute to explore the utility of heat shock proteins as biomarkers for monitoring a variety of environmental signals due to pollution, habitat destruction, and increased water temperatures.

Acknowledgment

This work was supported by Pukyong National University Research Fund in 2002.

국문초록

넙치(*Paralichthys olivaceus*) HSP90β 유전자의 분자생물학적 연구

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열 충격단백질(Heat shock protein: HSP)은 온도 스트레스에 대하여 세포 내에서 발현되는 단백질이다. HSP의 중요 분류군중의 하나가 HSP90 family이다. 여러 종류의 포유동물과 조류에서 HSP 유전자 특성에 대한 연구가 많이 진행되었다. 본 연구에서는 넙치(*Paralichthys olivaceus*)로부터 제조한 넙치 뇌 cDNA 유전자 은행을 이용하여 넙치

HSP90 cDNA 유전자를 분리하여 구성 염기서열의 특성을 밝혀 내었다. 염기서열의 분석결과 넙치의 *hsp90β* 유전자는 2,791개의 뉴클레오타이드로 구성되어 있고, 726개의 아미노산 잔기가 암호화되어 있었다. 넙치 *hsp90β* 유전자는 European sea bass와 96.6%, zebrafish와 92.9%, Atlantic salmon와 92.0%, 그리고 사람과는 89.5%의 염기서열 상동성을 지니고 있었다. 또한 HSP90 아미노산 서열을 바탕으로 척추동물 종들과의 진화계통수를 구축하였다. 넙치 *hsp90β* 유전자의 mRNA의 분포 정도를 RT-PCR를 이용하여 조사하였다. *hsp90β* 유전자는 조사한 모든 조직(뇌, 간, 신장, 근육, 비장)에서 높은 수준으로 발현이 되고 있었다. 또한, 넙치 HSP90β 단백질을 대량발현하기 위하여 대장균에서 발현을 유도하였다.

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(Received Nov. 25, 2004/Accepted Dec. 13, 2004)