Molecular studies on the Two Ornithine Decarboxylase Antizyme genes from Flounder (*Paralichthys olivaceus*)

넙치 Ornithine Decarboxylase Antizyme 유전자에 대한 분자생물학적 연구

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A Dissertation

by

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ABSTRACT

Ornithine decarboxylase (ODC) antizyme is a key regulatory protein in the control of cellular polyamines. The ODC antizyme is a protein that represses ornithine decarboxylase, which is a key enzyme for polyamine biosynthesis, through accelerating enzymatic degradation of ODC by 26S proteasome. The cDNA clones coding for two distinct ODC antizymes (*AZS* and *AZL*) were isolated from a flounder brain cDNA library. The nucleotide sequences of *AZS* and *AZL* genes revealed that both clones require translational frameshifting for expression. Taking account of +1 frameshifting, *AZS* and *AZL* gene products corresponded to a protein of 221 and 218 amino acid residues, respectively and shared 83.3% amino

acid sequence identity with each other. Comparison of the structure and nucleotide sequence of antizyme genes from flounder, zebrafish, mouse, and human showed that the genes were highly conserved. A phylogentic tree based on antizymes amino acid sequences were constructed from various species. The presence of the two antizymes mRNA species in brain, kidney, liver, and embryo were confirmed using the reverse transcription-polymerase chain reaction (RT-PCR) and Northern blot analysis. A recombinant protein of flounder ODC antizyme containing a short histidine tag at the amino-terminus was overexpressed in *Escherichia coli* BL21 (DE3) pLys using the pET-44a(+) expression vector. Antizyme genes were efficiently expressed in *E. coli* as a His-*AZL* and His-*AZS* fusion protein.

Key words: ornithine decarboxylase, antizyme, polyamine, 26S proteasome, flounder, frameshifting

INTRODUCTION

Ornithine decarboxylase (ODC) antizyme plays an important role in the control of intracellular levels of polyamines, such as putrescine, spermidine, and spermine, which are essential for cell growth, proliferation, differentiation, transformation, and apoptosis (Tabor and Tabor, 1984; Cohen, 1998; Coffino, 2001). Antizyme binds to ODC and targets to rapid ubiquitin-independent degradation catalysed by the 26S proteasome (Murakami *et al.*, 1992; Gandre *et al.*, 2002). Antizyme is the only known non-ubiquitin signal for a substrate of the proteasome (Li and Coffino, 1993). Another function of antizyme is to suppress cellular uptake of polyamines (Mitchell *et al.*, 1994; Suzuki *et al.*, 1994). These dual functions of antizyme effectively prevent from an excessive accumulation of cellular polyamines.

Antizyme expression is regulated translationally by a polyamine stimulated ribosomal frame-shifiting (Coffino, 2001; Matsufuji *et al.*, 1995). Synthesis of antizyme requires translational frameshifting, resulting in by passing a stop codon located shortly downstream of the intiation codon of open reading frame 1 (ORF1) (Matsufuji *et al.*, 1995). The amino-terminal portion is encoded by open reading frame1 (ORF1), and the remainder is encoded by the overlapping ORF2 in the +1 reading frame. High

concentration of polyamines converts the ribosome from its original reading frame to the +1 frame to encode a second ORF and synthesize complete functional antizyme protein. Translation experiments *in vitro* showed that the frameshifting is stimulated by polyamines (Ivanov *et al.*, 1988).

Antizyme binds to ODC subunit to form enzymatically inactive heterodimers (Mamroud-Kidron *et al.*, 1994). The affinity of antizyme to ODC subunits is higher that of ODC subunits each other. Therefore, interaction between antizyme and ODC subunits results in the inactivation of ODC enzyme and the degradation of ODC subunits (Murakami *et al.*, 1992; Li and Coffino, 1993).

Antizyme cDNA species and genes have been cloned from various sources including *Homo sapiens* (Tewari *et al.*, 1994; Ivanov *et al.*, 1998), *Mus musculus* (Kajiwara *et al.*, 1996), *Drosophila melanogaster* (Matsufuji *et al.*, 1995), *Danio rerio* (Saito *et al.*, 2000), *Xenopus laevis* (Ichiba *et al.*, 1995), *Gallus gallus* (Drozdowski *et al.*, 1998), and *Rattus norvegicus* (Miyazaki *et al.*, 1992). All these antizymes require translational frameshifting for their synthesis.

In fish, zebrafish has two different types of antizyme: antizyme small form (*AZS*) and antizyme large from (*AZL*). Two zebrafish antizymes have different expression and activities (Saito *et al.*, 2000). However, the knowledge of molecular structure of

antizyme in the marine fishes is extremely limited. Also, the nature of two antizymes in these fish and their roles in the control of polyamine pathway is still unclear. The flounder (*Paralichthys olivaceus*) is a commercially important marine aquaculture species in Korea, one of the most evolved teleosts and has been used for the molecular levels of the study on various functional genes (Cho *et al.*, 2001; Lee *et al.*, 2001; Kim and Kim, 1999; Lee *et al.*, 2003). Recently, a cDNA coding for flounder ODC has been discovered (Lee *et al.*, 2004). Its genetic characteristics and tissue expression were also reported (Lee *et al.*, 2004). In the present study, we initially focus on the isolation of cDNAs encoding antizyme cDNA from flounder (*Paralichtyhys olivaceus*) and characterizes its expressions in adult tissues. Herein, we provide the molecular characteristics and tissue expressions of our newly identified the flounder *AZS* (GenBank accession number, AY257551) and *AZL* (GenBank accession number, AY257552) cDNAs from adult flounder. These data will provide a wider base of knowledge on the primary structure of antizyme at the molecular level and the functional diversity.

EXPERIMENTAL PROCEDURES

MATERIALS

Bacterial strains

The *E. coli* strain XL1-Blue (F'::Tn10 $proA^{+}B^{+}$ $lacI^{q}$ △(lacZ) M15/*recA1 endA1 gyrA96* (Nal^{r}) *thi hsdR17* (r_k^{-} m_k^{+}) *supE44 relA1 lac*) was used for transformation and color selection. The *E. coli* strain BL21 (DE3) pLys [F, *ompT hsd*S_B(r_B -r_B) *dcm, gal,* (DE3) Cm^{r}] was used for the overexpression of flounder ODC antizyme gene.

Fish tissues

The brain, liver, kidney, and embryo tissues were obtained from mature flounder (n=10; size: 45±10㎝, body weight; 900 ±300 g; 3 years old) and stored at -70℃ until use.

Enzymes

Restriction enzymes were purchased from Promega (U.S.A.). *Taq* polymerase and reverse transcriptase were obtained from Bioneer (Taejeon, Korea). Sequencing ready reaction kit (ABI PRISM™ dye terminator) was purchased from Perkin Eelmer (U.S.A.).

Oligonucleotide primers

The primers for ODC antizyme cloning were designed on the basis of the conserved sequences from known ODC antizyme sequences and synthesized from GenoTech (Tajeon, Korea). The oligonucleotide primers used for this study were summarized in Table 1.

Other Materials

DIG labeling and detection kit were purchased from Boehringer Mannheim (Germany). Nitro blue tetrazolium chloride (NBT) and 5-bromo-4-chloro-3-indolyl (BCIP) solutions were purchased from Bio-Rad (U.S.A.). Protein low and prestained molecular weight markers and were obtained from Fermentas (U.S.A.). TRIzol reagent for total RNA isolation was purchased from Invitrogen (U.S.A.). Other chemicals were purchased from Fluka (U.S.A.) and Sigma (U.S.A.)

Table 1. Primers used in this study

Name	Sequence	Remark
AZ-F	5'-GGGCCTC(T/G)GTGGTGCTCCTGATG-3'	Probe for antizyme, Forward
LT7	5'-TTGTAATACGACTCACTATAGGGC-3'	T7 modified primer
AZS-R	5'-ACTCTGCCGTTGGCAGGG-3'	Probe for *AZS*, Reverse
AZL-R	5'-ATCCCACTGTATCGTCTTGGTAA-3'	Probe for *AZL*, Reverse
AZS-F1	5'-ATGGTTAAATCTAACCTTCAG-3'	*AZS* RT-PCR, Forward
AZL-F1	5'-GATGGCGAATTTGAGTAGCG-3'	*AZL* RT-PCR, Forward
AZS-R1	5'-GGATACCCGGTCTCAC-3'	*AZS* RT-PCR, Reverse
AZL-R1	5'-CTCGAGGGGCTGTCCAGAG-3'	*AZL* RT-PCR, Reverse
AZS-F2	5'-GGATCCATGGTTAAATCTAACCTTC-3'	Forward primer for *AZS* expression (*BamH* I)
AZL-F2	5'-GTCGACATATGGTAAAATCCACC-3'	Forward primer for *AZL* expression (*Sal* I)
AZS-R2	5'-CTCGAGGTCGTCATCAGAGGG-3'	Reverse primer for *AZS* expression (*Xho I*)
AZL-R2	5'-CTCGAGCTCTTCGTCCGAAGAG-3'	Reverse primer for *AZL* expression (*Xho I*)

METHODS

Isolation of RNA

Total RNA from flounder brain, liver and kidney tissues were isolated using total RNA isolation kit (Promega). Each of fresh flounder tissue (100mg) was homogenized in 1 ㎖ of denaturation reagent using a power driven homogenizer (Polytron™) for 30 sec at 25,000 rpm. The sample was kept on ice for 5 min and 200 ㎕ of chloroform was added and vigorous shaked for 15 sec. Then, the sample was kept on ice for 15 min and centrifuged for 15 min at 13,000 rpm. The aqueous phase was carefully transferred to a new tube, and 0.5 volume of isopropanol was added to it and mixed by inverting several times. After that, RNA Tack™ resin (0.05 V/V) was added, vortexed for 30 sec, and spun for 1 min. Pellet was washed twice with 1 ㎖ of 75% ethanol, dried briefly, resuspended in 0.1 volume of diethyl pyrocarbonate (DEPC) treated water, and centrifuged for 2 min at 13,000 rpm. Supernatant was transferred to a new tube. Poly(A) RNA was isolated using a Micro-Fast Track™ 2.0 Kit (Invitrogen). Ten ㎕ (500㎍) of purified poly(A) RNA was aliquoted to a new

microcentrifuge tube and 1 ㎖ of Micro-Fast Track™ 2.0 lysis buffer was added. Then, the mixture was heated up to 65℃ for 5 min and placed immediately on ice for 1 min. Sixty-three ㎕ of 5 M NaCl was added, and mixed well with the oligo-T cellulose for 2 min. The poly(A) RNA was isolated with a method described in Micro-Fast Track™ 2.0 Kit manual. The quantity of total RNA and poly(A) RNA were determined spectrophotometrically.

Construction of brain cDNA library

The complementray DNA (cDNA) library was constructed using a ZAP-cDNA synthesis kit (Stratagene). The single stranded cDNAs were made by reverse transcription reaction with a pair of primers which containing oligo dT and *EcoR* I adapter. Then, double stranded cDNAs were synthesized using T4 DNA Polymerase I and RNase H. The synthesized double stranded cDNAs were blunt-ended at each terminus by incubating cDNAs with *Pfu* DNA Polymerase. After addition of *EcoR* I adaptor to each cDNA terminus, the resulting cDNAs were digested with *Xho* I and ligated into ZAP-III XR vector (Lambda ZAPII vector digested with *EcoR* I-*Xho* I, CIAP treated). After 48 h incubation at

4℃, the ligation mixture was mixed with Gigapack III Gold packaging extract.

Probe preparation and screening of antizyme cDNAs

Conserved nucleotide sequences of antizyme among the vertebrate species were determined using NCBI (National Center for Biotechnology Information) nucleotide and protein sequence database and used for the designing of oligonucleotide primers. Oligonucleotide primers for screening antizymes were synthesized from GenoTech (Taejeon, Korea). PCR was carried out using a pair of the 'LT7' (5' TTGTAATACGACTCACTATAGGGC 3') and 'AZ-F' (5' GGGCCTC(T/G)GTGGTGCTCCTGATG 3') primers. The main PCR program was 94℃ for 30 sec, 60℃ for 30 sec, and 72℃ for 1 min 30 sec in a 30 cycle reaction. Probe for screening antizymes [*AZS* for 'AZ-F' and 'AZS-R' (5' ACTCTG-CCGTTGGCAGGG 3'); *AZL* for 'AZ-F' and 'AZL-R' (5' ATCCC-ACTGTATCGTCTTGGTAA 3')] were labeled with a DIG (digoxigenin) oligonucleotide 3' end labeling kit (Roche). Reaction mixture (10X labeling buffer, 6.25 mM $CoCl_2$ solution, 100 pmol oligonucleotide, 50 μM of DIG-ddUTP solution and 50 U terminal Transferase) was made to

total volume of 20 $\mu\ell$ and incubated at 37℃ for 15 min and rapidly transferred on ice. one $\mu\ell$ glycogen solution and 200 $\mu\ell$ of 0.2 mM EDTA solution (pH 8.0) were mixed with above reaction mixture. DIG labled probes were quantified and used for the immunoscreening procedure. Approximately, 1 X 10^5 plaques from the cDNA library were screened with above probes and isolated several positive plaques. These plaques were recovered and further confirmed by the second screening. Positive plaques were recovered from the second screening and the phagemid containing the insert was excised according to the manufacturer's instructions (Stratagene).

Plasmid DNA purification and Sequencing

Plasmid DNA was purified using Wizard Plus SV minipreps DNA purification system (Promega). Sequencing reaction was performed using an ABI PRISM™ DNA sequencing kit and an ABI 377 Genetic Analyzer (Perkin Elmer). Sequencing reaction mixture (4 $\mu\ell$ of big dye ready reaction mixture, 500 ng of template DNA, 3.2 pmole of primer, and water to 20 $\mu\ell$ volume) was prepared and placed into a thermal cycler. Thermal cycling

with 25 cycle of program (96℃ 10 sec, 50℃ 5 sec, 60℃ 4 min) was performed. After that, reaction mixture was precipitated with ethanol and dissolved in 20 $\mu\ell$ TSR (template suppressing reagent) following with 2 min incubation at 95℃. Tube was cooled down on ice and DNA sequence was analyzed using an ABI 377 sequencing analyzer. Nucleotide and its deduced protein sequences were analyzed using the EMBL and Genbank databases.

Comparative sequence analysis of flounder antizymes

To define molecular evolution, several vertebrates antizyme sequences were imported from the SwissPort data bank and GenBank as follow: *Homo sapiens AZ1* (human *AZ2*, NP004143), *Homo sapiens AZ2* (human *AZ2*, O95190), *Homo sapiens AZ3* (human *AZ3*, NM016178), *Mus musculus AZ1* (house mouse *AZ1*, AB083045), *Mus musculus AZ2* (house mouse *AZ2*, NM010952), *Mus musculus AZ3*, NM016901), *Rattus norvegicus* (Norway rat, NM139081), *Gallus gallus* (chicken, AAC97533), *Xenopus laevis* (African clawed frog, BAA06867), *Danio rerio AZS* (zebrafish *AZS*, AB017117), *Danio rerio AZL* (zebrafish *AZL*, AB017118), *Paralichthys olivaceus AZS* (flounder, AY257551), and *Paralichthys olivaceus AZL* (flounder, AY257552). All the DNA sequence data

were analyzed using the Internet-based programs such as Blastn and Blstx program in the GeneBank database and aligned with the program of Clustal W. 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 was 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 in brain, kidney, muscle, liver, and embryo from mature flounder (n=10; size: 45±10 ㎝, body weight; 900±300 g; 3 years old). Titan™ one tube RT-PCR system (Roche, Germany) was used. Master mix 1 contained 0.2 mM dNTPs, 5 mM dithiothreitol, 50 pmol of upstream (for *AZS*-F2: 5' ATGGTTAAATCTAACCTTCAG 3'; for *AZL*-F2: 5' GATGGCGAATTTGAGTAGCG 3') and downstream (for *AZS*-R2: 5' GGATGACCCGGTCTCAC 3'; for *AZL*-R2: 5' CT-CGAGGGGCTGTCCAGAG 3') primers, template RNA, and 5U of Rnase inhibitor. Master mix 2 consisted of 5X 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 h at 50℃ for reverse transcription followed by thermocycling. The temperature profile of flounder *AZL* was on prereaction at 94℃ for 5 min; 30 cycling reactions denaturation temperate at 94℃ for 40 sec, annealing temperate 65℃ for 30 sec, and extension temperate 72℃ for 1 min; and finally 7 min extension at 72℃. The temperature profile of flounder *AZS* was the same as *AZL* profile except annealing temperate at 52℃.

Northern blot analysis

Total RNA was isolated from brain, liver kidney, and embryo tissues. Five μg of each total RNA was separated by electrophoresis on a 1.5% formaldehyde gel. Denaturing gel loading mixture (RNA sample, 5× formaldehyde gel running buffer, 3.5 $\mu\ell$ of 37% formaldehyde, 10 $\mu\ell$ of formamide, and water to 20 $\mu\ell$) was made and incubated at 65℃ for 15 min. After incubation, the mixture was rapidly cooled down on ice. 2 $\mu\ell$ 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)-propanesul fonic 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).

Analysis of expressed protein on SDS-PAGE

For expression in the prokaryotic system, the *AZS* and *AZL* genes were subcloned in pET-44a(+) expression vector (Novagen) using restriction sites (*AZS* for *BamH* I and *Xho* I ; *AZL* for *Sal* I and *Xho* I). The pET-44a(+) vector is designed for high-level expression of peptide sequences fused with 495 amino acid of Nus · Tag™ protein and showed in Figure 1. pET-44a(+)-AZ plasmid was transformed into the *E. coli* strain BL21 (DE3) pLys. The cell harboring a plasmid which contains antizyme genes was cultured overnight in 10 ㎖ of LB/amp (50 ㎍/㎖) broth at 37℃ in shaking incubator.

The cell was induced by adding IPTG (isopropyl-β-D-thiogalacto-pyranoside) to a final concentration of 1 mM at mid-log growth (OD_{600} of 0.5-1.0).

At various induction periods, one millilitre of the culture was centrifuged 14,000 rpm for 1 min at room temperature and supernatant was removed by aspiration. Each pellet was resuspended in 100 ㎕ of 1×SDS-loading buffer (50 mM Tris-Cl, pH 6.8, 10% glycerol, 2.0% SDS, 100 mM dithiothreitol, 0.1% bromophenol blue), and heated to 100℃ for 3 min. The sample was centrifuged 14,000 rpm for 1 min at room temperature, and stored on ice until all of the samples collected and ready to load on a gel. Samples (20 ㎕) were loaded on a 10% SDS-PAGE (10% seperating gel, pH 8.8 overlayed 4% stacking gel, pH 6.8). Electrophoresis was performed at 200 mA for 2 h. The gel was stained with coomassie brilliant blue and destained with destaining solution (7% acetic acid, 15% methanol).

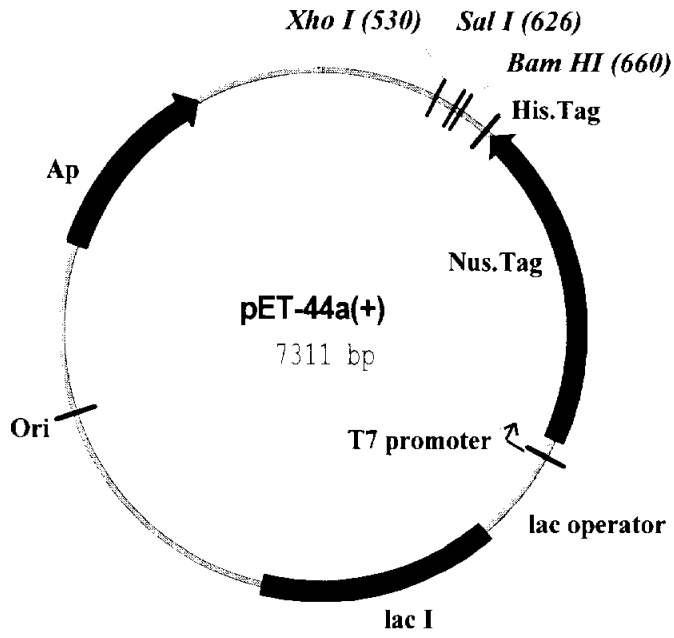


Figure 1. Map of pET-44a(+) vector (Novagen). The pET-44a(+) vectors system is a versatile system for the expression, purification, and detection of fusion proteins produced in *E.coli*. The system is based on inducible, high-level expression of peptide sequences fused with Nus · Tag™ and His · Tag.

RESULTS

Nucleotide sequences of flounder Ornithine decarboxylase antizymes

We have cloned two types of antizymes using a flounder brain cDNA library as a template by the polymerase chain reaction. As shown in Figure 2, Two PCR products, about 1110 bp and 1500 bp, were obtained using AZ-F and LT7 primers. After DNA sequence analysis, two PCR products represented antizyme genes. However, there are some sequence differences between two clones, suggesting that these clones may be two different antizyme genes. Using the probes made with both PCR products, we have obtained several positive clones and analyzed their nucleotide sequences.

The nucleotide and deduced amino acid sequences of cloned *AZS* cDNA were determined as shown in Figure 3. The flounder *AZS* gene has 1274 bp encoding 221 amino acid residues. The *AZS* cDNA consists of 40 bp of 5'-untranslated region (UTR), 663 bp of a coding region and 571 bp of 3'-UTR, followed by a poly (A) sequence. The 3' UTR contains polyadenylation signals (attaaa).

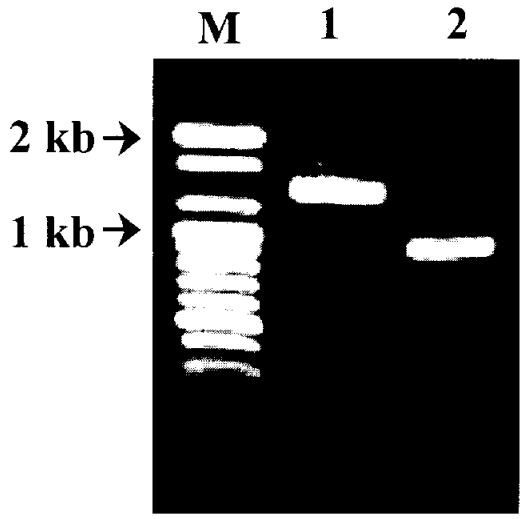
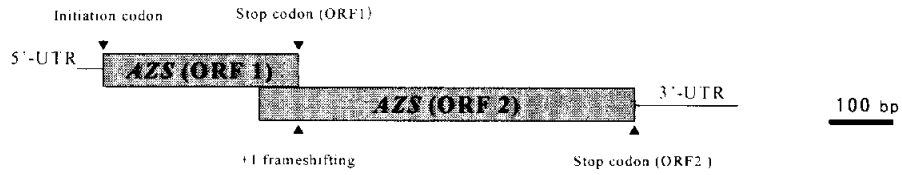


Figure 2. PCR products amplified with AZ-F and LT7.
M indicates DNA molecular weight marker; lane 1, 1500 bp; lane 2, 1100 bp

A



B

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1                                                    CGGCACGAGAGAGCGAGCTGCTGTCGGGACGCTGGCCGG
   AZS-F1 (RT-PCR)
41 ATGGTTAAATCTAACCTTCAGACGATCTTGAATAGACATTGTTTTGTTAGAGAAAAAGAGAGAAACTTAAGCGAGATGCCTGTCATCGAG
   M  V  K  S  N  L  Q  T  I  L  N  R  H  C  F  V  R  E  K  E  R  N  L  T  E  M  P  V  I  E   30
                                                                                  AZ-F
121 CAGTCCAGTAATAAAATGAAAGCGACAGTATCCTCAGTCCCAGGAGGTGCTCTCACTGTTGCAGTAACCCTGTGTCCAGGGCCCTGTGG
   Q  S  S  N  K  T  E  S  E  S  I  S  S  P  R  R  C  S  H  C  C  S  N  P  C  P  G  P  L  W   60
   AZ-F (Probe)
181 TGCTGCTGATGCCCCTACTCCCACCCCTGAAGATCCCAGGTGGCGAGGGAATGACCAGCGGGATCGCAATCTTTCAGCTAAGCTATTCTAT
   C  S  *
         D  A  P  L  P  P  L  K  I  P  G  G  R  G  N  D  Q  R  D  R  N  L  S  A  K  L  F  Y   90
                                                AZS-R (Probe)
241 TCCGATGCTCAGTTACTGGTTCTTGAAGAGCCTCCCCCTGCCAACGGCAGATGTCGCTCCTGCTCTTGAGCCACGTCGCTTGGAAGGG
   S  D  A  Q  L  L  V  L  E  E  P  P  P  A  N  G  R  V  R  F  L  L  F  E  P  R  R  L  E  G  120

301 AAGCTCTGTGTCTGGAGGCGGCACTGGAAGGAGGGAACCTTACGTCGAAATCCCTCCTGGAGCTTTGCCCGAGGGCAGCAAAGACAGT
   K  L  C  V  W  R  A  A  L  E  G  G  N  L  Y  V  E  I  P  P  G  A  L  P  E  G  S  K  D  S  150

361 TTTGGCCTCCTCGCTGGAATTTGGAGAAGAGCAGCTACAGGCTGATCACGTCTTCATCTGCTTTCACAAGAGCCGTGATGACAGACGCATCC
   F  A  L  L  L  E  F  A  E  E  Q  L  Q  A  D  H  V  F  I  C  F  H  K  S  R  D  D  R  A  S  180
                                                AZS-R1 (RT-PCR)
421 CTCCTACGCACATTCAGCTTCCTGGGGCTTCGAGATTGTGAGACCGGGTCATCCCTCGTCCCCCTCCGCCCTGACGCTTTCCTCATGGCT
   L  L  R  T  F  S  F  L  G  F  E  I  V  R  P  G  H  P  L  V  P  S  R  P  D  A  F  F  M  A  210

481 TACAGCATCGAACGAGACTCCTCTGATGACGACTAGATAACGCTCAGCAGTTACAAACGTTTGTTGTACTTCTTTTGATCCATACACACA
   Y  S  I  E  R  D  S  S  D  D  D  *                                                        221

541 AATCTTCATTTTTGGGGAAATGAAAATATTATTCAAGTTCAGTATGGTGAGGTTTCCTGCTCCTTTGTGTTTGTTTGACCTTACCTCC
601 CACGTGCTGTGCTTCAATGATACTTGCACTAGGGTAAGATGTTAATACTACCAGCAATGTAAAGCAAGCAGCCCACATAATACCAGGGCGT
661 TGTGTCCTCGACCACCTGTATGACCCTTCGTGATGTGTAGTTCTGACTCCTGCTGAGCCTCCCACCCCCCCCCCCCCTCAGAATAAACCC
721 TGATCAGAATAAACCCTGATCACCTCCACAGTGTAATATTTATATAAATTCAAACTCATCATACTCATGTAAGTAGATATGTCTTCATGG
781 CAACTATCAGTTGATTCTCCAGCTTTGTATTGACTTTTTTTTAAACTGTGTGCATGATTTTCATTTGTGGTTTTAATTCACTTGATAAGAA
841 CCTGCATGTTGTTAACTGTACTAAACAAAAGCTGCTCACCTACAAAAAAAAAAAAAAAAAAAAAAAA
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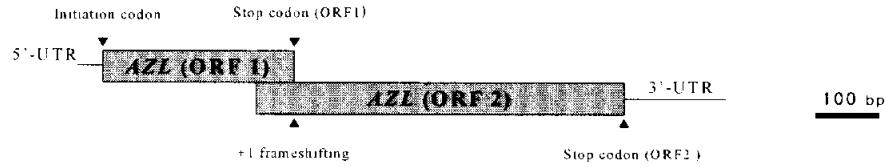
Figure 3. (A) Structure of flounder *AZS* gene. 5'-UTR indicates 5'-untranslated region and 3'-UTR, 3'-untranslated region. (B) The nucleotide and deduced amino acid sequences of cDNA encoding flounder *AZS*. The nucleotide numbers are shown on the left side and amino acid sequence to the right side. An *asterisk* indicates the stop codon. The potential initiator codons are underlined. The consensus polyadenylation signal is underlined.

The nucleotide and deduced amino acid sequences of cloned *AZL* cDNA were shown in Figure 4. The flounder *AZL* gene has 1706 bp encoding 218 amino acid residues. The *AZL* cDNA consists of 65 bp of 5'-untranslated region (UTR), 654 bp of a coding region and 987 bp of 3'-UTR, followed by a poly(A) sequence. The 3' UTR contains polyadenylation signals (attaaa).

Flounder antizyme sequences were compared to the antizyme mRNA sequences of human, house mouse, Norway rat, chicken, frog, zebrafish. As shown in figure 5, nucleotides necessary for frameshifting and the formation of the pseudoknot structure were highly conserved, implying that a ribosomal frameshift occurs in the translation of all these antizyme mRNA species. The first AUGs would initiate translation of an ORF (ORF1) that overlaps the longer downstream ORF (ORF2) such that a +1 translational frameshifting event in the overlap would generate a protein product analogous to the products of antizyme genes from higher eukaryotes. Furthermore, the last 13 nucleotides of ORF1 (G UGG UGC UCC UGA) are identical to the last 13 nucleotides of ORF1s, including the frameshift site (except zebrafish *AZS*) (Figure 5).

A



B

```
                                                                    AZL-F1
  66 ATGGTAAAATGCAACCTCCAGCGGATCGCTAAACAGTCATTGCTTTGCTCGCGAGAAAGAAGGAAAACAGCAGCCTCCCACCACGATGGCG
     M  V  K  S  N  L  Q  R  I  L  N  S  H  C  F  A  R  E  K  E  G  K  Q  Q  P  P  T  T  M  A   30
     AZL-F1 (RT-PCR)                                                          AZ-F (Probe)
 156 AATTTGAGTAGCGGCATCTGTGACATGATCGGCAATTTGTCGCTGCACTGTAGTAGTACCCGCGGCCCAGGCGCTTAGTGGTGCTCCTGAT
     N  L  S  S  G  I  C  D  M  I  G  N  L  S  L  H  C  S  S  T  R  G  P  G  P  Q  W  C  S  *
                                                                          D   60

 246 GCCCCTCTCCCACCCCTGAAGATCCCAGGTGGGCGAGCGAATGGCACACCGGATCACACTCTTCAGCTCGGCCGATCTACTCGGACCAA
     A  P  L  P  P  L  K  I  P  G  G  R  G  N  G  T  R  D  H  T  P  S  A  R  P  I  Y  S  D  Q   90
                                                                 AZL-R (Probe)
 336 AAGTTGACGGTAACGCAGGAGGCAGCAGGGAACGGTCGCCCTGGGATACTCCACTTTCAATCGCATCTCACTGTTACCAAGACGATACAG
     K  L  T  V  T  E  E  P  A  G  N  G  R  P  G  I  L  H  F  Q  S  H  L  T  V  T  K  T  I  Q  120
     AZL-R
 426 TGGGATGCTGCGCTTAAGCAACAGCTCTCTCTATGTGGAGATACCTCTCGACTCTTCCCCTGAAGCCAGTAAGGAGAGTTTCGCAGCTCTC
     W  D  A  V  L  S  N  S  S  L  Y  V  E  I  P  L  D  S  L  P  E  G  S  K  E  S  F  A  A  L  150

 516 CTGGAGTATGCCGAAGAACACCTGAAAGTCGTCAGTGTGTCTGTCTTTACAAGAACAGAGATGATCGTGCTAAACTGGTGCGTACC
     L  E  Y  A  E  E  H  L  K  V  V  S  V  F  V  C  F  Y  K  N  R  D  D  R  A  K  L  V  R  T  180

 606 TTCAGTTTCCTGGGCTTTGAGATTGTGAAACCGGGCCATGCCCTCGTCCACCTCGACCCGACGTTTTCTTCATGGCCTACAACTTTGAC
     F  S  F  L  G  F  E  I  V  K  P  G  H  A  L  V  P  P  R  P  D  V  F  F  M  A  Y  N  F  D  210
                                 AZL-R2 (RT-PCR)
 696 CGGGACTCTTCGGACGAAGAGTAGCCGCCTGGACAGCGCGTCCACCCCATCCTCACTACCGAATATTCCCCTCCCCACTATATGTTTGCC
     R  D  S  S  D  E  E  *                                                                   217

 786 TACCCTGTCCCAGTTTATCCATCTCCACCCAGCGTCTGTGTTAACCCCTCAGCTTCAGCGCTCTGAATGCATGAAGTATTCAACCCCCC
 876 CCCCCCCATGTTTTTATTCAAGGGAATATGTAAGCTGTGCCTCTCCTGTTCCCATCATCCATCTTGCGTATTTAATACACTGTTTTC
 966 ATTCAAGATTGATTGGTGTTGTCACCTAAATACGAGGGAAACTGTTTTTTTGGACTTGATCACATTGCAGTTGTCATAGCTCGTTGTC
1056 TTATTTCCATCTTTTGTCAGTCTTTGTTGTTATTCCTTTTATGATAGATTAAGGAGGAGAAATCTTAATTTTTGAATGTACAAC
1146 AAAAGTGCATCAGGTTTACACTCTGGTACTCCAGCTTTGTTTCTTTTGGATTGGCTAAAAGATGACTCCAAATTTTGTCCAAACACTT
1236 AAACGTACATTCAGCCGCGACCAGTTTTGTTTGTAGCACTGATACCCTTCTTAAGTATTAGTATTTTTAAAACTTTACGCTTGAGCGACA
1326 GCCCCAATAATTGGTACTTCATAGCGGAATACTGAATCCATTTAGCCTAGACGTGGAGAGGAGAGATGAATACTGTTGCACGTGTTGGTT
1416 TCGAGATCCTATGACTGTGTCTGTACAAAGCGAAAGCAAAGTGAATAACCCAAAGTGCTTTTGCTTCTTCGTGCCATGCATATCTGACA
1506 ATATTGTAGTGTTTCGTTTTGGAGGTGCAAAAACGTTGCTGTGAATGGACTTTTTCTTTGTTTTGTCATTTTTGATTAGCATTCGTTT
1596 TCAACTTTTGTCCCTCATGACAATAAGGAGTTCATTTTCACCTGCATAGTCGTACCTGTTGTGCGTCTCTGATAATAAACAAACAAA
1686 AACAAAAAAAAAAAAAAAAAA
```

Figure 4. (A) Structure of flounder *AZL* gene. 5'-UTR indicates 5'-untranslated region and 3'-UTR, 3'-untranslated region. (B) The nucleotide and deduced amino acid sequences of cDNA encoding flounder *AZL*. The nucleotide numbers are shown on the left side and amino acid sequence to the right side. An *asterisk* indicates the stop codon. The potential initiator codons are underlined. The consensus polyadenylation signal is underlined.

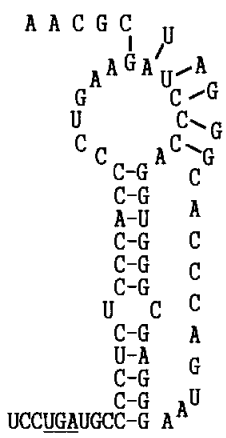
A

P. olivaceus AZS	GGGCCUCUGUGGUGCUCC UGA UGCCCCUCU
P. olivaceus AZL	GGGCCUCAGUGGUGCUCC UGA UGCCCCUCU
D. rerio AZS	GGGCCUCUGUGGUGUUCC UGA UGUCCCUCU
D. rerio AZL	GGGCCUCUGUGGUGCUCC UGA UGCCCCUCA
M. musculus AZ1	GGGCCUCGGUGGUGCUCC UGA UGUCCCUCA
M. musculus AZ2	GGGCCUCUGUGGUGCUCC UGA UGCCCCUCA
H. sapiens AZ1	GGGCCUCGGUGGUGCUCC UGA UGCCCCUCA
H. sapiens AZ2	GGGCCUCUGUGGUGCUCC UGA UGCCCCUCA
M. musculus AZ3	UCCUGCCUCCAGUGCUCC UGA -GUCCCUAG
H. sapiens AZ3	UCCUGCCUCCAGUGCUCC UGA -GUCCCUAG

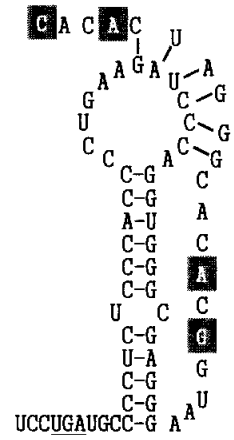
▲

Frameshift

B



P. olivaceus AZS



P. olivaceus AZL

Figure 5. Comparison of mRNA sequences around the frameshift site. (A) Comparison of the nucleotide sequences of the frameshift sites of different antizyme genes. (B) Comparison of the potential pseudo-knots 3' adjacent to the shift site of flounder *AZS* and *AZL* mRNA.

Sequence identity and Phylogenetic tree

The amino acid sequence identity was calculated using Genedoc program (Khal *et al.*, 1997). Comparison of the flounder antizymes with other species is shown in Figure 6. The flounder antizymes have a high similarity in amino acid residues with other species, greater than 50% sequence identity. By this analysis flounder *AZS* shows 78.8% and 54.1% sequence identity with zebrafish *AZS* and *AZL*, respectively. Also, flounder *AZL* shows 56.6% and 73.1% sequence identity with zebrafish *AZS* and *AZL*, respectively. A molecular phylogenetic tree of the vertebrate antizymes was shown in Figure 7.

Tissue Distribution of *AZS* and *AZL*

In order to determine the expression of the antizyme genes at the transcription level, total RNA was isolated from flounder brain, liver kidney, and embryo tissues.

Reverse transcription-polymerase chain reaction (RT-PCR) was performed using the above RNAs as a template. The resulting RT-PCR products were analyzed on agarose gel electrophoresis. As shown in Figure 8, approximately 600 bp DNA fragment was amplified from all total RNAs extracted from the brain, liver, kidney, and embryo tissues, indicating that antizyme genes were expressed in all tissues examined.

Also, the tissue-specific expression of *AZS* and *AZL* genes was confirmed by Northern blot analysis. The result of Northern blotting was shown in Figure 9.

Figure 6. Multiple alignment of the deduced amino acid sequence of flounder antizymes with known ODC antizyme protein sequences taken from GenBank; the accession numbers are given in the text. Amino acid residues that are identical in the ODC antizymes are marked with an *asterisk*.

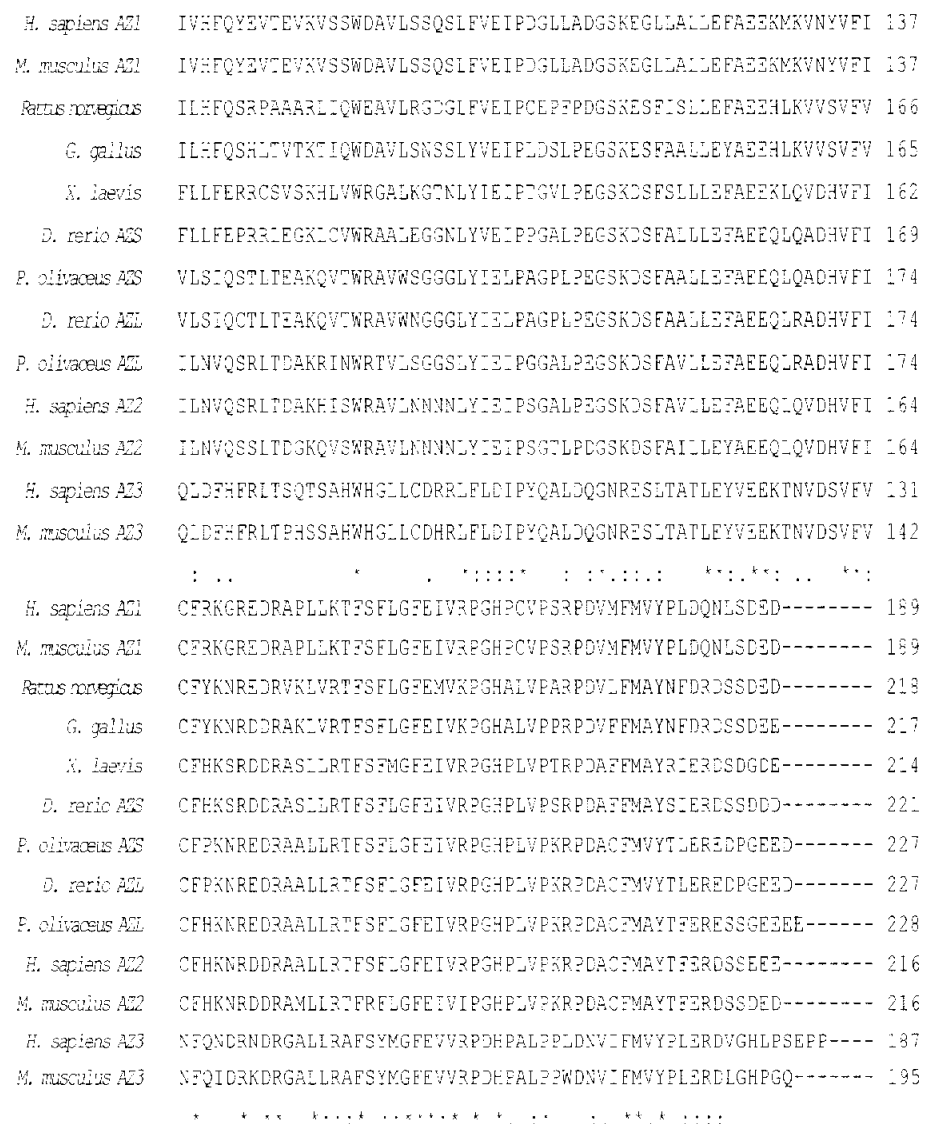


Figure 6. continued.

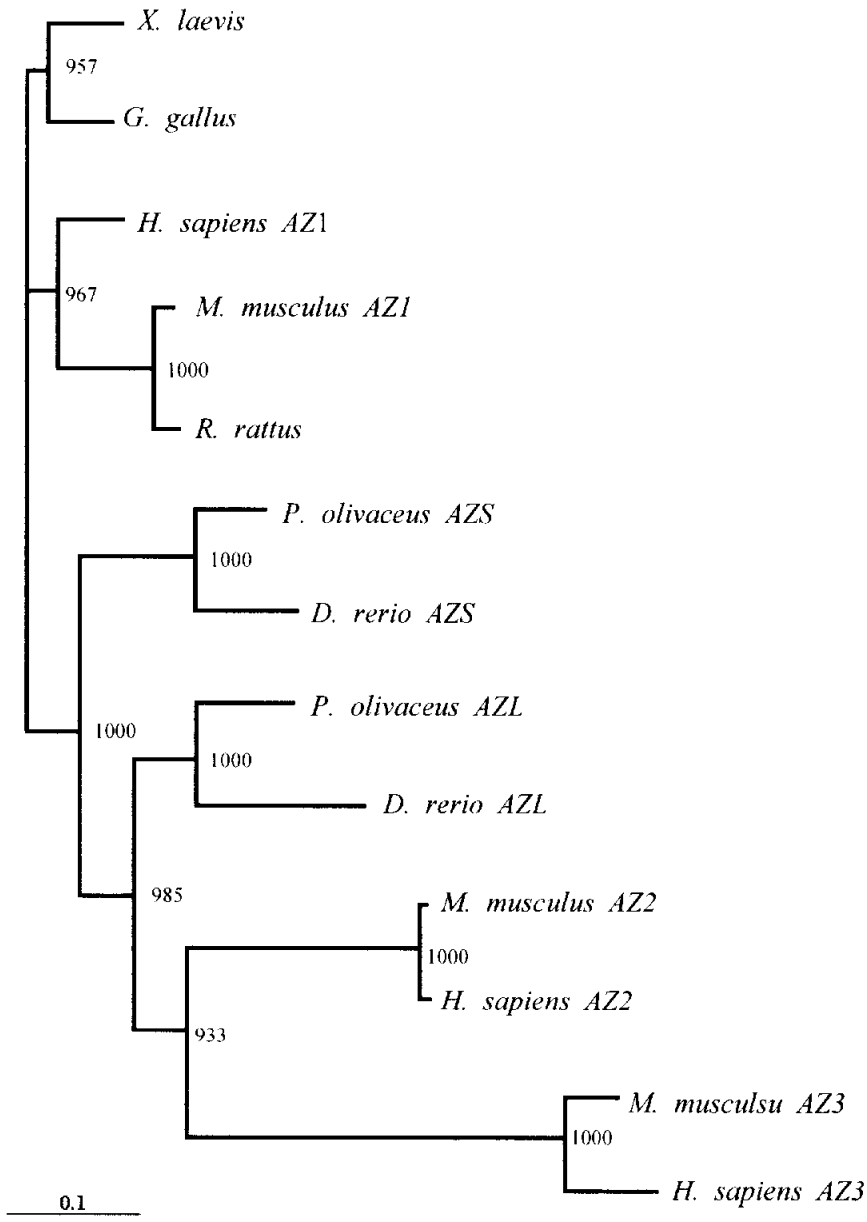


Figure 7. A molecular phylogenetic tree of ODC antizyme based on the NJ method. The values shown on each internal branch are the percentage support determined from a bootstrap analysis with 1,000 replications.

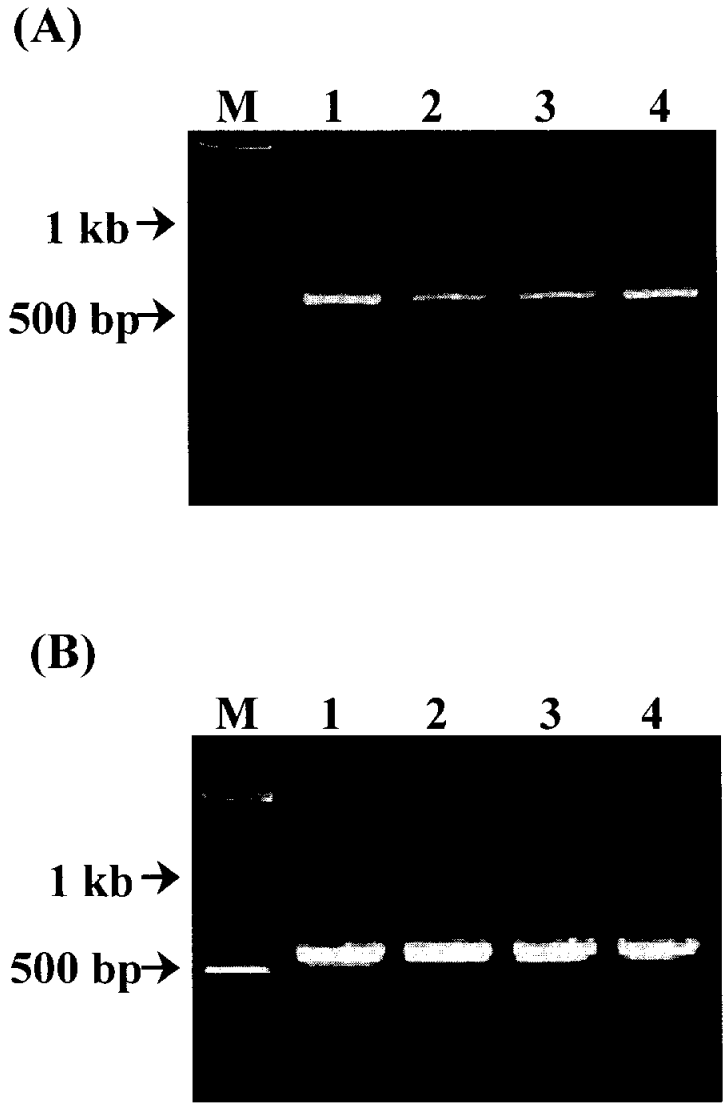


Figure 8. The patterns of RT-PCR analysis of *AZS* (A) and *AZL* (B). M indicates DNA molecular weight marker; lane 1, brain; lane 2, liver; lane 3, kidney; lane 4, embryo.

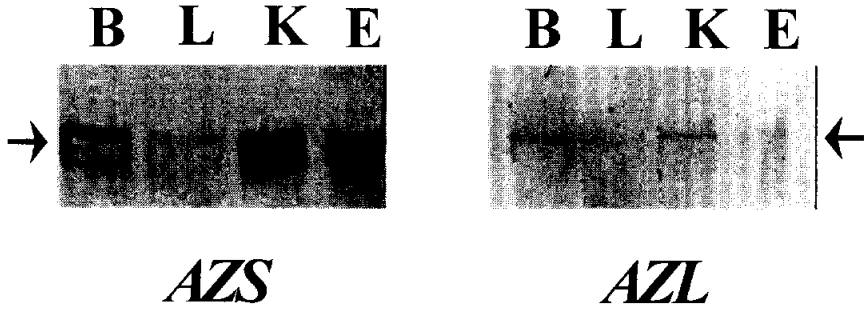


Figure 9. Northern blot analysis of antizyme mRNA species
Each lane was loaded with total RNA (5μg). B indicates brain; L, liver; K, kidney; E, embryo.

Overexpression of flounder *AZS* and *AZL* gene in *E.coli*

In order to express flounder antizyme genes into the prokaryotic system, each of *AZL* and *AZS* genes were subcloned into the expression vector, pET-44a(+), which allows expression of recombinant protein with N-terminal fusion His-tag, and transformed into *E. coli* BL 21(DE3) pLys strain. The expression of the recombinant protein was induced by the addition of IPTG.

The expression pattern of the *AZL* and *AZS* genes were shown in Figure 10 and the molecular weights of the expressed His-*AZS* and His-*AZL* fusion proteins were turned out to be approximately 69.3 kDa and 70 kDa, respectively.

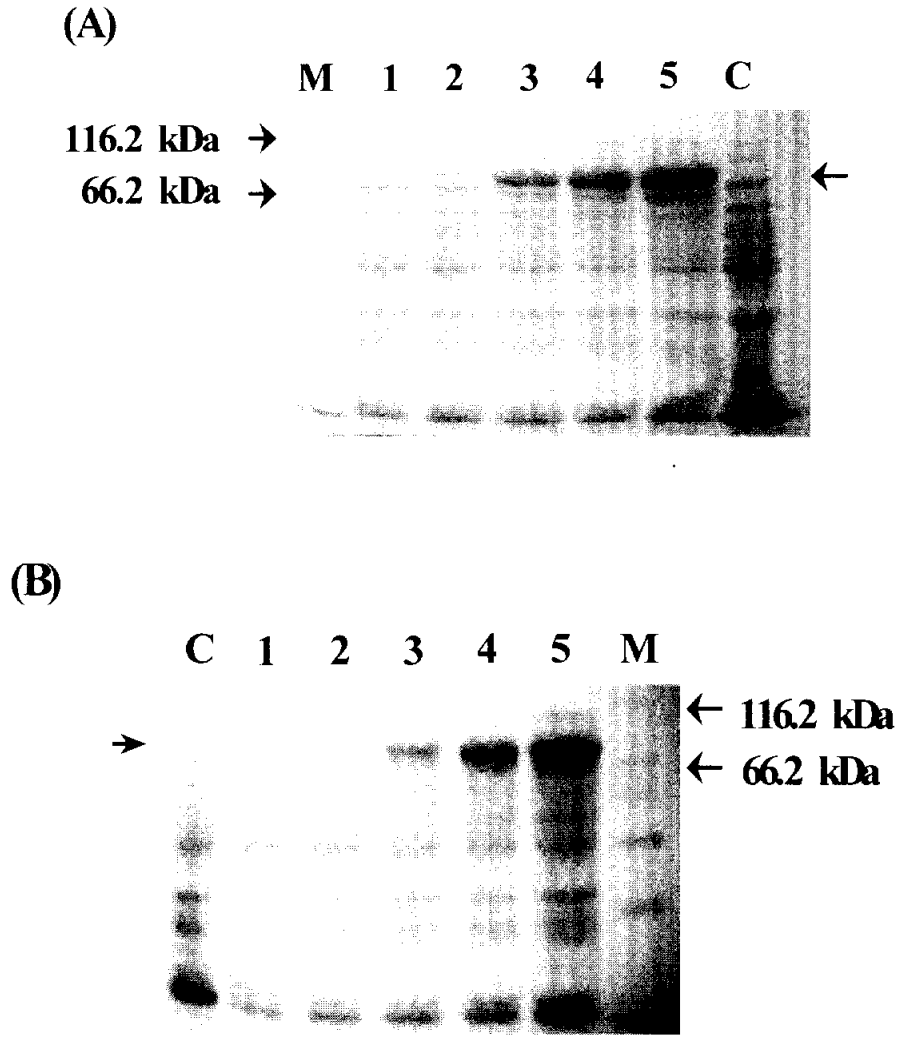


Figure 10. Patterns of SDS-PAGE of His-*AZS* (A) and His-*AZL* (B). M indicates protein molecular weight marker; C, protein from uninduced cell extracts of BL 21 (DE3) pLys. lane 1~5, proteins from induced cell extracts 0 h, 10 min, 30 min, 1 h, 2 h of IPTG induction, respectively.

DISCUSSION

We have cloned and characterized two types of antizyme genes, *AZL* and *AZS*, from the flounder. A comparison of the amino acid sequence of vertebrate ODC antizyme indicated that the flounder ODC antizymes are highly conserved with those of other species. Flounder antizyme, *AZS* and *AZL*, are similar to each other in many regards. They have a significant sequence homology (83.3% identical and 88.2% similar). It has been established that *AZS* plays an important role in regulating *in vivo* polyamine levels. *AZS* binds to ODC and accelerate ODC degradation. On the other hand, *AZL* does not accelerate ODC degradation.

Phylogenetic analysis could not clearly separate same orthologs among *AZ1*, *AZ2*, *AZS*, and *AZL*. Theses findings suggest that fish is the most primitive vertebrate, and genetic information obtained from fish can reveal the origin and diversion of genes with similar function in other organisms. The results of this study provide phylogenic evidence on the antizymes, which may be essential to understand the molecular evolution of this gene in vertebrates.

RT-PCR provided evidence for the expression of both *AZS* and *AZL* genes. Thus, the DNA banding patterns resulting from both flounder *AZS* and *AZL* mRNAs have a wide tissue distribution in flounder.

Northern blot analysis was showed that two antizymes were expressed in all tissues investigated, but mRNA expression was more strongly detected in *AZS* than that of *AZL* mRNA level. Thus, both flounder *AZS* and *AZL* mRNAs have a wide tissue distribution but *AZL* mRNA is less abundant.

The ODC antizyme genes were expressed into the prokaryotic expression system. The overexpression of cloned antizymes were analysed using SDS-PAGE. The optimal induction of a recombinant *AZL* and *AZS* protein was achieved at 1hr after IPTG induction.

In conclusion, we have cloned and characterized the flounder antizyme genes. Flounder ODC antizyme mRNA was detected in brain, liver, kidney, and embryo tissues. The present study will certainly be helpful for future studies in understanding the roles that each of two antizymes plays *in vivo*.

국문초록

세포 내 polyamine들은 ornithine decarboxylase (ODC) antizyme에 의해 feedback mechanism에 의해서 조절되며, ODC는 polyamine의 생합성 과정에 중요한 작용을 하는 효소이다. ODC antizyme은 26S proteasome에 의한 ODC 분해를 가속화시키는 단백질이다. 본 연구에서 두개의 다른 ODC antizyme (*AZS*와 *AZL*)을 넙치에서 분리하였다. 이들 유전자의 특징으로 *AZS*와 *AZL* 발현되기 위해서는 translational frameshifiting을 필요로 한다. 이러한 +1 frameshifting을 고려하면, *AZS*와 *AZL* 유전자는 각각 221과 218개의 아미노산 잔기를 가진 단백질로 전사되며, 이들은 아미노산 서열상 83.3%의 동질성을 가진다. 넙치, zebrafish, 쥐, 사람의 antizyme 유전자의 구조와 엄기 서열을 비교하면 높은 보존성을 가진 것을 알 수 있었다. 다양한 종으로부터 antizyme의 아미노산 서열을 기초로 phylogenetic tree를 구축하였다. RT-PCR과 Northern blot 분석을 통하여 뇌, 신장, 간, 배아 조직에서 두 antizyme mRNA가 발현함을 증명하였으며, 또한 antizyme의 유전자를 다량발현 시키기 위해서 pET-44a(+) expression vector에 subcloning 한 다음에, His · Tag에 fusion된 antizyme 단백질을 *E. coli*에서 효율적으로 다량발현 되는 것을 확인하였다.

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어느 듯 2년이란 시간이 흘러 또 하나의 마침표를 찍게 되었습니다. 이렇듯 하나의 마침표를 찍게 만들어 주시고 항상 부모님과 같은 사랑과 격려를 주시며 부족한 저를 바른 길로 이끌어주신 지도교수이신 김영태 교수님께 머리 숙여 깊은 감사를 드립니다. 또한 저의 부족한 논문을 끝까지 읽고 수정해주신 이원재 교수님과 윤철원 교수님께 감사를 드립니다. 대학원 석사과정동안 많은 관심과 학문적으로 가르침을 주셨던 김진상 교수님, 이명숙 교수님, 송영환 교수님, 이훈구 교수님, 최태진 교수님께 감사 드립니다.

학부과정을 마치고 이곳 생화학 실험실에 들어 온 저를 기본부터 차근차근 가르쳐 주신 재형이 형, 2년 동안 셀 수 없이 많이 싸웠던 재광이 형, 무형선배, 보영이 누나, 우리 꼴통 소연이, 윤여사 (주연이), 실험실에서 잠시나마 나를 바닥생활에서 벗어나게 해준 조선생 (우성이)과 나의 못된 성격 때문에 가장 많이 고생했을 우리 실험실 석사 막둥이자 동기인 미영이에게도 고맙다는 말과 앞으로 어디서 무엇을 하든지 서로를 잊지 말고 지내자는 말을 전하며, 우리 실험실 식구들 중 가장 막둥이들인 희진이, 지현이, 가영이, 유진이에게 고맙다는 말과 앞으로도 재미나게 실험실 생활을 하자는 말을 전합니다. 그리고 언제나 큰형처럼 보살펴 주신 정민, 종규 선배님과 지희 누나와 나의 동기들인 오서방 정환이, 홍바람 재홍이, 슈렉 인영이와 후배인 재성이, 재경이, 경규, 재기에게 고맙다는 말을 전하며 나머지 7호관에서 함께 생활하며 지낸 수많은 사람들과 5호관에서 자기가 학부는 늦게들어왔지만 대학원은 먼저 들어왔으니 자기가 선배라고 우기며 생활하는 종오에게 정말 고맙다는 말 전합니다. 학교 밖에서 나를 도와준 나의 진정한 친구들인 정래, 재호, 기수, 대은이에게 진한 우정에 고맙다는 말을 전하며 지금은 서울에서 열심히 공부하고 있는 수미에게도 고맙다는 말을 전합니다. 또한 2년 동안 나의 스트레스를 확실히 풀어주신 오킴스 큰 사장님, 작은 사장님 정말 고맙습니다.

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