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# Isolation and Sequence Analysis of Two Ornithine Decarboxylase Antizyme Genes from Flounder (*Paralichthys olivaceus*)

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**Abstract** Ornithine decarboxylase (ODC) antizyme is a key regulatory protein in the control of cellular polyamines. We have isolated two distinct ODC antizyme cDNA clones (*AZS* and *AZL*) from a flounder (*Paralichthys olivaceus*) brain cDNA library. Their sequences revealed that both clones required translational frameshifting for expression. Taking +1 frameshifting into account, *AZS* and *AZL* products were 221 and 218 amino acid residues long, respectively, and shared 83.3% amino acid sequence identity. Comparison of the structure and nucleotide sequence of the antizyme genes showed that the genes were highly conserved in flounder, zebrafish, mouse, and human. A phylogenetic tree was constructed, based on the antizyme amino acid sequences from various species. The presence of the two types of antizyme mRNA species in brain, kidney, liver, and embryo was confirmed by using the reverse transcription-polymerase chain reaction (RT-PCR) and Northern blot analysis. Recombinant proteins of flounder ODC antizymes, containing His-Nus-S tag at the amino-terminus, were overexpressed as His-*AZL* and His-*AZS* fusion proteins in *Escherichia coli* BL21 (DE3) pLys by using the pET-44a(+) expression vector.

**Key words:** Ornithine decarboxylase, antizyme, polyamine, 26S proteasome, flounder (*Paralichthys olivaceus*)

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 [3, 4, 34]. ODC is one of the most highly regulated enzymes [8] and has a short half-life. The enzyme is downregulated by polyamines [31], which exert the most effective repression via antizyme [3]. Antizyme is an ODC inhibitory protein that is induced by a polyamine-dependent frameshift mechanism [26]. Antizyme binds to ODC and targets to rapid ubiquitin-independent degradation catalyzed by the 26S proteasome [6, 30], possibly after a conformational change in the ODC subunit [3, 29]. Antizyme is the only known non-ubiquitin signal for a substrate of the proteasome [24]. Another function of antizyme is to suppress cellular uptake of polyamines [27, 33]. These dual functions of antizyme effectively prevent an excessive accumulation of cellular polyamines.

Antizyme expression is regulated at the translational level by a polyamine-stimulated ribosomal frameshifting [3, 26]. Synthesis of antizyme requires translational frameshifting, resulting in bypassing a stop codon located shortly downstream of the initiation codon of open reading frame 1 (ORF1) [26]. The amino-terminal portion is encoded by open reading frame 1 (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 synthesizes a completely functional antizyme protein. Translation experiments *in vitro* showed that the frameshifting is stimulated by polyamines [10]. Antizyme binds to ODC subunits to form enzymatically inactive heterodimers [25]. The affinity of antizyme to ODC subunits is higher than that of ODC subunits to each other. Therefore, interaction between antizyme and ODC subunits results in the inactivation of ODC enzyme and the degradation of ODC subunits [24, 30]. Antizyme cDNA species and genes have been cloned from various sources, including *Homo sapiens* [10, 35], *Mus musculus* [12], *Drosophila melanogaster* [26], *Danio rerio* [32], *Xenopus laevis* [9], *Gallus gallus* [5], and *Rattus norvegicus* [28], and all these antizymes require translational frameshifting for their synthesis.

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**Table 1.** Primers used in this study.

| Primer | Nucleotide 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' | *AZS* expression (*Bam*H I), Forward |
| AZL-F2 | 5'-GTCGACATATGGTAAAATCCACC-3' | *AZL* expression (*Sal*I), Forward |
| AZS-R2 | 5'-CTCGAGGTCGTCATCAGAGGG-3' | *AZS* expression (*Xho*I), Reverse |
| AZL-R2 | 5'-CTCGAGCTCTTCGTCCGAAGAG-3' | *AZL* expression (*Xho*I), Reverse |

Zebrafish has two different types of antizyme: Antizyme small form (*AZS*) and antizyme large form (*AZL*). Two zebrafish antizymes have different expression and activities [32]. However, the knowledge of the molecular structure of antizyme in the marine fishes is extremely limited. Also, the nature of the two antizymes in these fishes and their roles in the control of the polyamine pathway are still unclear. Flounder (*Paralichthys olivaceus*), one of the most evolved teleosts, is a commercially important marine aquaculture species in Korea, and has been used for molecular levels study on various functional genes [2, 13, 21, 23]. Recently, a cDNA coding for flounder ODC was cloned, and its genetic characteristics and tissue expression have been reported [22].

In the present study, we isolated cDNAs encoding antizyme cDNA from flounder, identified the flounder *AZS* (GenBank accession number, AY257551) and *AZL* (GenBank accession number, AY257552) cDNAs from adult flounder, and characterized its expressions in adult tissues. These data are expected to provide a wider base of knowledge on the primary structure of antizyme at the molecular level and on functional diversity.

## 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, U.S.A.). 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, U.S.A.). The quantity of RNA was determined by measuring O.D. at 260 nm. The construction of the brain cDNA library was performed, using a ZAP-cDNA® Synthesis Kit (Stratagene, La Jolla, U.S.A.). The resulting library contained approximately $1\times10^5$ clones, and the library was then amplified up to $3\times10^9$/ml.

### Screening of ODC Antizyme cDNAs and DNA Sequencing

Conserved nucleotide sequences of vertebrate ODC antizymes were searched using the National Center for Biotechnology Information (NCBI) nucleotide and protein sequence database, and used for designing oligonucleotide primers. Oligonucleotide primers used in this study are summarized in Table 1. Oligonucleotide degeneracy primers for probe preparation for screening antizymes were synthesized at GenoTech (Taejeon, Korea). The probe used for screening ODC antizymes was amplified by PCR, using upstream (AZ-F) and downstream (AZS-R for *AZS*; AZL-R for *AZL*) 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, 60°C for 30 sec, and 72°C for 30 sec. Approximately, $1\times10^5$ 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 [1, 14, 15, 16, 17, 18]. Positive plaques were recovered from the second screening, and the phagemid containing the insert was excised according to the manufacturer's instructions (Stratagene, La Jolla, U.S.A.). The excised phagemid was sequenced by using an ABI PRISM™ DNA sequencing kit (Applied Biosystems, Foster, U.S.A.) and determined with ABI 377 Genetic Analyzer according to the manufacturer's instructions (Applied Biosystems, Fostercity, U.S.A.).

### Comparative Sequence Analysis of Flounder ODC Antizymes

To examine the molecular evolution of ODC antizymes, the following vertebrate ODC antizyme sequences were imported from the SwissProt databank/GenBank: *Homo sapiens AZ1* (human *AZ2*, NP004143), *H. sapiens AZ2* (human *AZ2*, O95190), *H. sapiens AZ3* (human *AZ3*,

NM016178), *Mus musculus AZ1* (house mouse *AZ1*, AB083045), *M. musculus AZ2* (house mouse *AZ2*, NM010952), *M. musculus AZ3* (house mouse *AZ3*, NM016901), *Rattus norvegicus* (Norway rat, NM139081), *Gallus gallus* (chicken, AAC97533), *Xenopus laevis* (African clawed frog, BAA06867), *Danio rerio AZS* (zebrafish *AZS*, AB017117), *D. rerio AZL* (zebrafish *AZL*, AB017118), *Paralichthys olivaceus AZS* (flounder, AY257551 in this study), and *P. olivaceus AZL* (flounder, AY257552 in this study). The nucleotide sequences were analyzed by 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). A phylogenetic tree was constructed, by the neighbor-joining (NJ) method, using the program Treecon [7, 36, 37] for the amino acid sequences of ODC antizymes from *H. sapiens*, *M. musculus*, *R. norvegicus*, *G. gallus*, *X. laevis*, *D. rerio*, and *P. olivaceus*.

### Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

In order to perform RT-PCR, total RNA was isolated from brain, kidney, muscle, liver, and embryo from mature flounder (N=10; size, 45 cm±10 cm; body weight, 900 g± 300 g; 3 years old). The Titan™ one-tube RT-PCR system (Roche, Mannheim, Germany) was used. Master mix 1 contained 0.2 mM dNTPs, 5 mM dithiothreitol, 50 pmol of upstream (AZS-F1; AZL-F1: Table 1) and downstream (AZS-R1; AZL-R1: Table 1) primers, template RNA, and 5 U 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 h at 50°C for reverse transcription followed by thermocycling. A temperature profile was on prereaction at 94°C for 5 min; 30 cycling reactions at 94°C for 40 sec, annealing temperature 65°C for 30 sec, and extension temperature 72°C for 1 min, and finally 7 min extension at 72°C. The temperature profile of flounder *AZS* was the same as the *AZL* profile, except for the annealing temperature at 52°C.

### Northern Blot Analysis

Total RNA was isolated from brain, liver, kidney, and embryo tissues. Five mg each of 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 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, and 2 µl of formaldehyde gel-loading dye (50% glycerol, 5 mM EDTA, pH 8.0, 0.25% bromophenol blue and xylene cyanol) were added to the mixture. Electrophoresis was performed by using 1.5% formaldehyde gel in 1× 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 20× SSC (DEPC treated) and incubated for 15 min twice. RNA was transferred to a NC membrane, using a capillary transfer method, and cross-linked by using a UV cross-linker with preset condition (1,200 µJ/cm$^2$ at 254 nm). Hybridization and detection were performed as described on DIG labeling and detection kit manuals (Boehringer Mannheim, Germany).

### Overexpression of ODC Antizyme Genes in *E. coli*

In order to express the ODC antizyme genes, the cloned antizyme cDNAs were subcloned into pGEM-T vector (Promega) by PCR, using a pair of oligonucleotides (AZS-F2 and AZS-R2 for *AZS*; AZL-F2 and AZL-R2 for *AZL*: Table 1), and the resulting plasmids were digested with *Bam*HI and *Xho*I for *AZS*, and *Sal*I and *Xho*I for *AZL*. Then, the excised fragment was ligated into pET-44a(+) expression vector (Novagen). The pET-44a(+) expression vector allows expression of a recombinant protein with an N-terminal fusion His-Nus-S tag. The resulting plasmids, pET-44a(+)-*AZS* and -*AZL*, were transformed into the *E. coli* strain BL21 (DE3) pLys. The cells harboring the ODC antizyme genes were cultured in LB medium (containing 50 µg/ml ampicillin) and induced by adding final concentration of 1 mM IPTG (isopropyl-β-D-thiogalactopyranoside) at a cell density corresponding to $OD_{600}$=0.5. Expressed proteins were analyzed by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) according to Laemmli [19].

## Results and Discussion

Fishes 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. ODC is a key enzyme in the biosynthesis of polyamines, as it regulates intracellular concentrations of polyamines, which are essential for cell growth and function. Recently, a cDNA coding for flounder ODC has been discovered, and its genetic characteristics and tissue expression were reported [22]. Antizymes effectively prevent an excessive accumulation of cellular polyamines by catalyzing degradation of ODC and by suppressing cellular uptake of polyamines. Antizymes have been shown to consist of an ancient gene family. Representatives with conserved structural, functional, and regulatory features are present from fungi to mammals [11]. Within vertebrate species, multiple isoforms are found, and human has at least four, mouse has three, and zebrafish has two antizymes.

In order to identify the ODC antizyme genes from flounder, degenerate oligonucleotide primers, encoding parts of the conserved domains, were prepared and used to produce a probe by using a flounder brain cDNA library, which was constructed by using a λ ZAP-II cDNA Synthesis Kit. Conserved nucleotide sequences of ODC antizymes were determined, using the NCBI nucleotide and protein sequence database, and the degeneracy primers were synthesized for the preparation of the probe for ODC antizymes screening. Using AZ-F and LT7 primers (Table 1), we have amplified two types of PCR products, using a flounder brain cDNA library as a template. Two PCR products, about 1,120 bp and 1,620 bp, were sequenced and turned out to be representing ODC antizyme gene sequences. However, there are some sequence differences between the two cDNA fragments, suggesting that flounder may have two different types of ODC antizyme genes. The probes used for screening two ODC antizymes were amplified by PCR, using upstream (AZ-F) and downstream (AZS-R for *AZS*; AZL-R for *AZL*) primers. Using the probes made with both PCR products, approximately $1\times10^5$ plaques were screened and several positive clones were obtained, and their nucleotide sequences were analyzed. After DNA sequence analysis, we identified two types of flounder ODC antizymes; antizyme small form (*AZS*) and antizyme large form (*AZL*).

The nucleotide and deduced amino acid sequences of cloned *AZS* cDNA (GenBank accession number AY257551) are shown in Fig. 1. The flounder *AZS* gene has 1,274 bp, including an open reading frame and 5'- and 3'-untranslated regions. The *AZS* cDNA consists of 40 bp of 5'-untranslated region (UTR), 663 bp of a coding region, encoding 221 amino acid residues, and 571 bp of 3'-UTR, followed by a poly(A) sequence. The 3'-UTR contains polyadenylation signals (attaaa). The nucleotide and deduced amino acid sequences of cloned *AZL* cDNA (GenBank accession number AY257552) are shown in Fig. 2. The flounder *AZL* gene

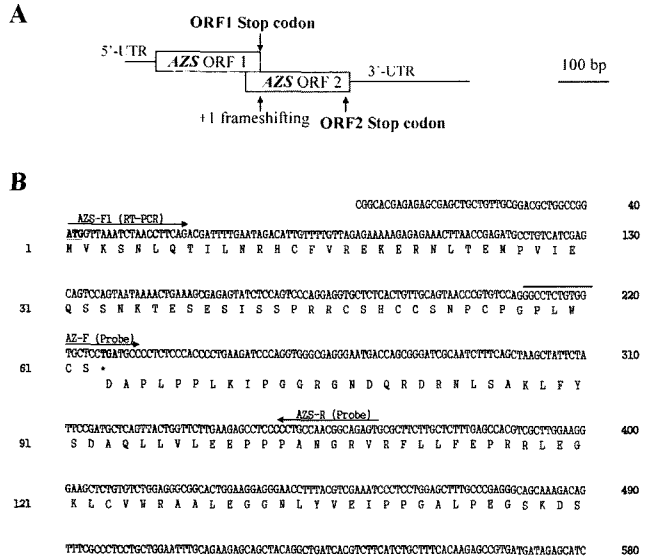


**Fig. 1.** (A) Structural location of flounder *AZS* cDNA. 5'-UTR indicates 5'-untranslated region, and 3'-UTR is the 3'-untranslated region. (B) Nucleotide and deduced amino acid sequences of the cDNA encoding flounder *AZS*.
The nucleotide sequence is numbered to the right and the amino acid sequence to the left. The *asterisk* indicates the stop codon. The potential initiator codons are underlined. The polyadenylation signal (AATAAA) is underlined. The *AZS* nucleotide sequence has accession number AY257551 in the GenBank database.

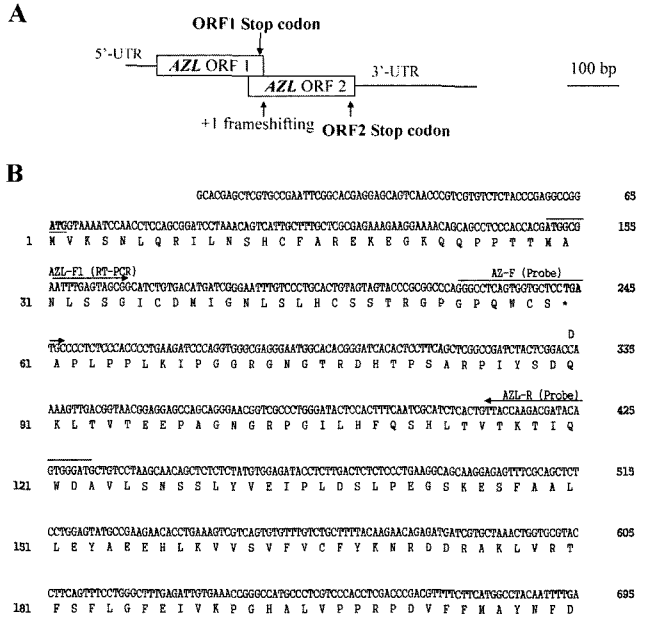


**Fig. 2.** (A) Structural location of flounder *AZL* cDNA. 5'-UTR indicates 5'-untranslated region, and 3'-UTR is the 3'-untranslated region. (B) Nucleotide and deduced amino acid sequences of the cDNA encoding flounder *AZL*.
The nucleotide sequence is numbered to the right and the amino acid sequence to the left. The *asterisk* indicates the stop codon. The potential initiator codons are underlined. The polyadenylation signal (AATAAA) is underlined. The *AZL* nucleotide sequence has accession number AY257552 in the GenBank database.

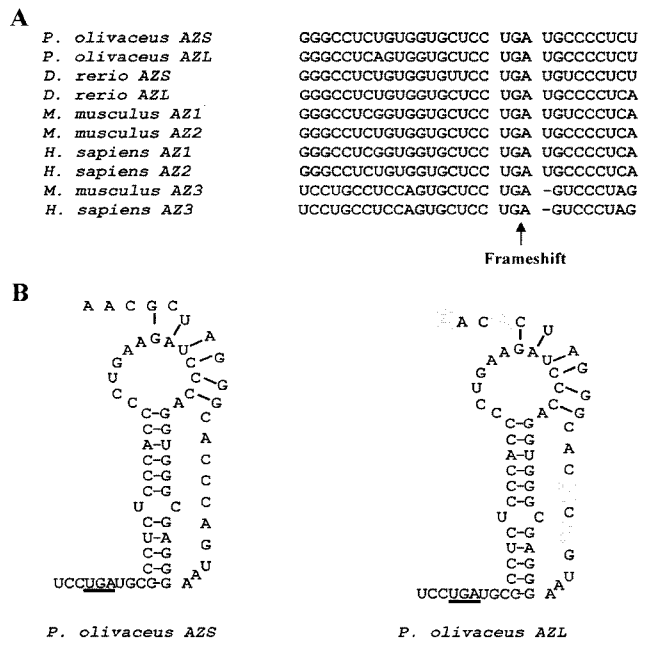


**Fig. 3.** Comparison of mRNA sequence around the frameshift site. (A) Comparison of the nucleotide sequences of the frameshift sites of different antizyme genes. (B) Comparison of the potential pseudoknots 3' adjacent to the shift site of flounder *AZS* and *AZL* mRNAs.

has 1,706 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. Also, the 3'-UTR contains polyadenylation signals (attaaa).

Flounder antizyme sequences were compared to the antizyme mRNA sequences of human, house mouse, and zebrafish. As shown in Fig. 3, nucleotides necessary for frameshifting and formation of the pseudoknot structure were highly conserved, implying that a ribosomal frameshift occurs during 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 antizymes (house mouse *AZ1*, human *AZ1*, house mouse *AZ2*, human *AZ2*, and zebrafish *AZL*) ORF1s, including the frameshift site (except zebrafish *AZS*, house mouse *AZ3*, and human *AZ3*) (Fig. 3).

The amino acid sequence identity was calculated, using Genedoc program [20]. Comparison of the flounder ODC

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H. sapiens AZ1       MVKSSLQRILNSHCFAREKEGDKPSATIHASRTMPLLSLHSRGGSSSESS-----RVSLHCCSNPGPGPRWCS DAPHPPLKIPGGRGNSQ   85
M. musculus AZ1      MVKSSLQRILNSHCFAREKEGDKRSATLHASRTMPLLSQHSRGGCSSESS-----RVALNCCSNLGPGPRWCS CVPHPPLKIPGGRGNSQ   85
Rattus norvegicus    MVKSSLQRILNSHCFAREKEGDKRSATLHASRTMPLLSQHSRGGCSSESS-----RVALHCCSNLGPGPRWCS DVPHPPLKIPGGRGNSQ   85
G. gallus            MVKSSLQRILNSHCFAREKEGNKS------TIMPAVLSLST----GQSSS-----RVPFNCCSNLGPGPRWCS DVPHPPLKIPGGRGNSQ   75
X. laevis            MVKSSLQRILNSHCFAREKEGNKR------NDAMPLLSIPS----SSESS-----RASFNCCSNLGPGPRWCS DVPHPPLKIPGGRGNSQ   75
D. rerio AZS         MVKSNLQTILNSHCFVREKESNIP--------KMPVIELTRN---KPESE-------SSHRCSNPCPGPLWCS DVPLPPLKIPGGRGNDQ   72
P. olivaceus AZS     MVKSNLQTILNRHCFVREKERNLT--------EMPVIEQSSN---KTESESISSPRRCSHCCSNPCPGPLWCS DAPLPPLKIPGGRGNDQ   79
D. rerio AZL         MVKSNLQRILNSHCFAREKEGKKQC-------ESSIMEALSSSITDRMAS------PTVCCSSTTGPGPLWCS DAPHPPLKIPGGRGNGA   77
P. olivaceus AZL     MVKSNLQRILNSHCFAREKEGKQQ--------PPTTMANLSSGICDMIGN------LSLHCSSTRGPGPQWCS DAPLPPLKIPGGRGNGT   76
H. sapiens AZ2       MINTQDSSILP-------------------------LSNCP----------------QLQCCRHIVPGPLWCS DAPHPLSKIPGGRGGG-   48
M. musculus AZ2      MINTQDSSILP-------------------------LSKCP----------------QLQCCRHIVPGPLWCS DAPHPLSKIPGGRGGG-   48
H. sapiens AZ3       MLPRCYKSITYK--------------------EEEDLTLQPR-----------------------SCLQCS ESL---VGLQEGKSTEQ   42
M. musculus AZ3      MLPCCYKSITYK--------------------EQEDLTLRP----------------HCCLPCSCLPCSCLQCS ESL---GGLQVGRSTAQ   52
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H. sapiens AZ1       RDHNLSANLFYSDDRLNVTEELT-SNDKTRILNVQSRLTDAKRINWRTVLSGGSLYIEIPGGALPEGSKDSFAVLLEFAEEQLRADHVFIC  176
M. musculus AZ1      RDHSLSASILYSDERLNVTEEPT-SNDKTRVLSIQSTLTEAKQVTWRAVWSGGGLYIELPAGPLPEGSKDSFAALLEFAEEQLQADHVFIC  176
Rattus norvegicus    RDHSLSASILYSDERLNVTEEPT-SNDKTRVLSIQCTLTEAKQVTWRAVWNGGGLYIELPAGPLPEGSKDSFAALLEFAEEQLRADHVFIC  176
G. gallus            RDHNLSANLFYSDNRLNVTEELT-SNNRTRILNVQSRLTDAKHISWRAVLNNNNLYIEIPSGALPEGSKDSFAVLLEFAEEQLQVDHVFIC  166
X. laevis            RDHNLSANLFYSDNRLNITEELT-SNNRTRILNVQSSLTDGKQVSWRAVLNNNNLYIEIPSGTLPDGSKDSFAILLEYAEEQLQVDHVFIC  166
D. rerio AZS         RDHSLSAKLFYSDAQLLVLEEAPQSNSRVRFLLFERRCSVSKHLVWRGALKGTNLYIEIPTGVLPEGSKDSFSLLLEFAEEKLQVDHVFIC  164
P. olivaceus AZS     RDRNLSAKLFYSDAQLLVLEEPPPANGRVRFLLFEPRRLEGKLCVWRAALEGGNLYVEIPPGALPEGSKDSFALLLEFAEEQLQADHVFIC  171
D. rerio AZL         RDHPSTTQTLYSDRKLTVTEEPA-GPGRPQILHFQSRPAAARLIQWEAVLRGDGLFVEIPCEPFPDGSKESFISLLEFAEEHLKVVSVFVC  168
P. olivaceus AZL     RDHTPSARPIYSDQKLTVTEEPA-GNGRPGILHFQSHLTVTKTIQWDAVLSNSSLYVEIPLDSLPEGSKESFAALLEYAEEHLKVVSVFVC  167
H. sapiens AZ2       RDPSLSA-LIYKDEKLTVTQDLPVNDGKPHIVHFQYEVTEVKVSSWDAVLSSQSLFVEIPDGLLADGSKEGLLALLEFAEEKMKVNYVFIC  139
M. musculus AZ2      RDPSLSA-LIYKDEKLTVTQDLPVNDGKPHIVHFQYEVTEVKVSSWDAVLSSQSLFVEIPDGLLADGSKEGLLALLEFAEEKMKVNYVFIC  139
H. sapiens AZ3       G-NHDQLKELYSAGNLTVLATDPLLHQDPVQLDFHFRLTSQTSAHWHGLLCDRRLFLDIPYQALDQGNRESLTATLEYVEEKTNVDSVFVN  134
M. musculus AZ3      EKDHSQLKELYSAGNLTVLSTDPLLHQDPVQLDFHFRLTPHSSAHWHGLLCDHRLFLDIPYQALDQGNRESLTATLEYVEEKTNVDSVFVN  144
                            :*. .* :    .       : ..          *     .  *::::*   : :*.::.:   **:.**: ..  **:

H. sapiens AZ1       FHKNREDRAALLRTFSFLGFEIVRPGHPLVPKRPDACFMAYTFERESSGEEEE----------------------------------------  228
M. musculus AZ1      FPKNREDRAALLRTFSFLGFEIVRPGHPLVPKRPDACFMVYTLEREDPGEED-----------------------------------------  227
Rattus norvegicus    FHKNRDDRAALLRTFSFLGFEIVRPGHPLVPKRPDACFMAYTFERDSSEEE-----------------------------------------  216
G. gallus            FHKNRDDRAMLLRTFRFLGFEIVIPGHPLVPKRPDACFMAYTFERDSSDED-----------------------------------------  216
X. laevis            FHKSRDDRASLLRTFSFMGFEIVRPGHPLVPTRPDAFFMAYRIERDSDGDE-------------------------------------------  214
D. rerio AZS         FHKSRDDRASLLRTFSFLGFEIVRPGHPLVPSRPDAFFMAYSIERDSSDDD-------------------------------------------  221
P. olivaceus AZS     FYKNREDRVKLVRTFSFLGFEMVKPGHALVPARPDVLFMAYNFDRDSSDED-------------------------------------------  218
D. rerio AZL         FYKNRDDRAKLVRTFSFLGFEIVKPGHALVPPRPDVFFMAYNFDRDSSDEE-------------------------------------------  217
P. olivaceus AZL     FYKNRDDRAKLVRTFSFLGFEIVKPGHALVPPRPDVFFMAYNFDRDSSDEE-------------------------------------------  217
H. sapiens AZ2       FRKGREDRAPLLKTFSFLGFEIVRPGHPCVPSRPDVMFMVYPLDQNLSDED-------------------------------------------  189
M. musculus AZ2      FRKGREDRAPLLKTFSFLGFEIVRPGHPCVPSRPDVMFMVYPLDQNLSDED-------------------------------------------  189
H. sapiens AZ3       FQNDRNDRGALLRAFSYMGFEVVRPDHPALPPLDNVIFMVYPLERDVGHLPSEPP----------------------------------------  187
M. musculus AZ3      FQIDRKDRGALLRAFSYMGFEVVRPDHPALPPWDNVIFMVYPLERDLGHPGQ-------------------------------------------  195
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**Fig. 4.** Multiple alignment of the deduced amino acid sequences of flounder ODC 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*.

antizymes with other species is shown in Fig. 4. The flounder antizymes have high similarity in amino acid residues with other species, having 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. Both mammalian *AZ1* and zebrafish *AZS* have the activity of binding to ODC and show acceleration of ODC degradation. However, both mammalian *AZ2* and zebrafish *AZL* show only the activity of binding to ODC, but do not have the activity of accelerating ODC degradation, at least *in vitro* [32, 38].

A molecular phylogenetic tree of the vertebrate antizymes is shown in Fig. 5. It shows the evolutionary divergence of the ODC genes of zebrafish, frog, house mouse, Norway rat, chicken, and human. The flounder ODC antizyme proteins are related to the zebrafish ODC antizymes more closely than to the human ODC antizymes, as reflected in the sequence identity. Phylogenetic analysis could not clearly separate same orthologs among *AZ1*, *AZ2*, *AZS*, and *AZL*. However, the results of this study provide phylogenic evidence on the antizymes, which may be essential for understanding the molecular evolution of this gene in vertebrates.

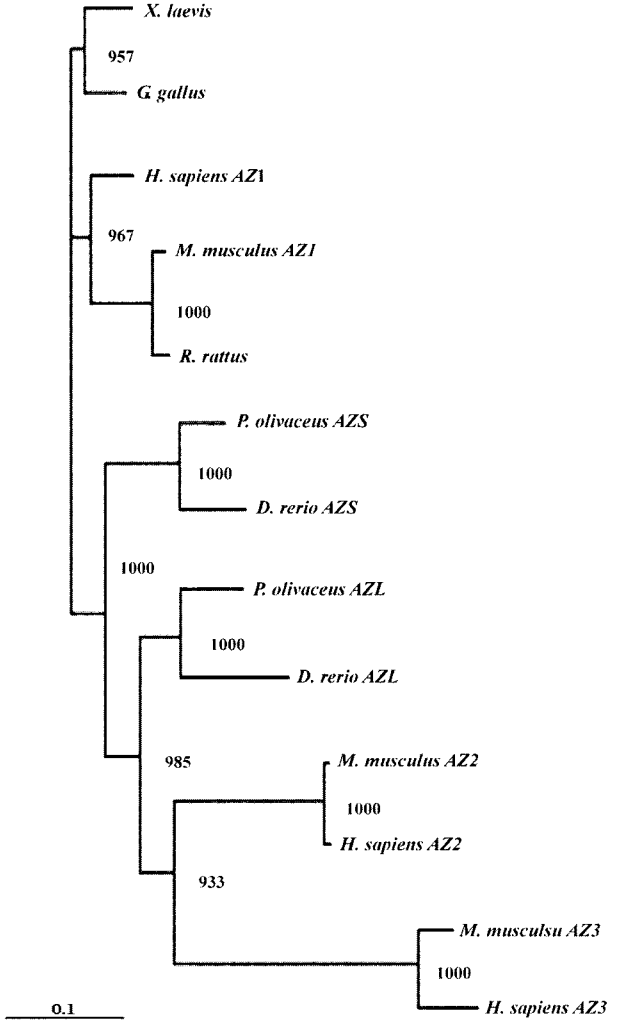


**Fig. 5.** A molecular phylogenetic tree of ODC antizyme, based on the NJ method.
The values shown on each internal branch are the percentage support determined from a bootstrap analysis with 1,000 replications.

A comparison of the amino acid sequence of vertebrate ODC antizymes indicated that the flounder ODC antizymes, *AZL* and *AZS*, and those of other species are highly conserved. Flounder antizymes are similar to each other in many regards. They have a significant sequence homology (83.3% identity and 88.2% similarity). Potential internal initiators are located at codon 26 of *AZS* mRNA (Fig. 1) and at codons 29 and 39 of *AZL* mRNA (Fig. 2). 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 [3]. The details of this regulation are not fully understood.

The tissue distribution of the flounder ODC antizyme genes was investigated by RT-PCR, using total RNA isolated from flounder tissues as template. As shown in Fig. 6, an approximately 600 bp DNA fragment was amplified

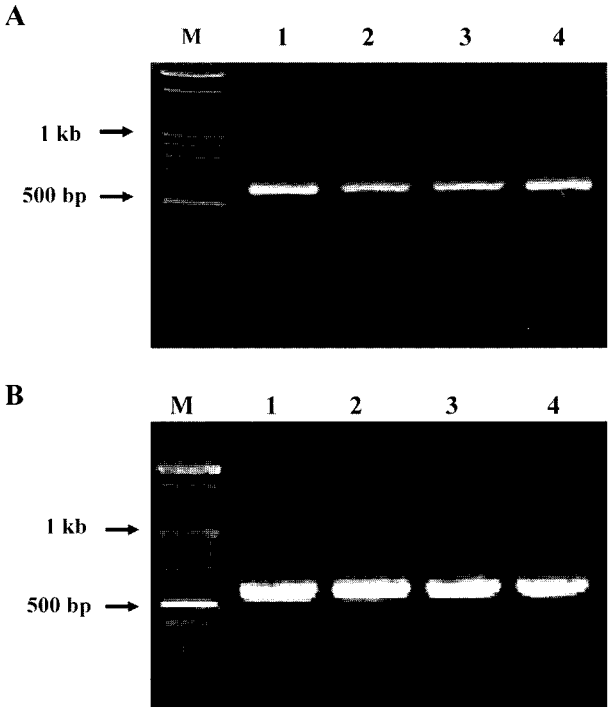


**Fig. 6.** Pattern of expression detected by RT-PCR analysis of *AZS* (A) and *AZL* (B).
M indicates DNA molecular size marker; lane 1, total RNA template for RT-PCR isolated from flounder brain; lane 2, liver; lane 3, kidney; lane 4, embryo.

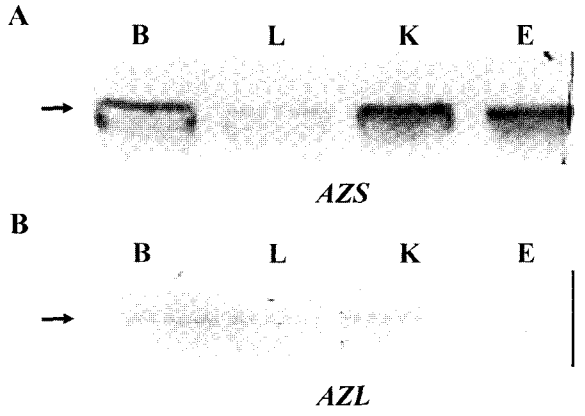


**Fig. 7.** Northern blot analysis of antizyme mRNA species.
Each lane was loaded with total RNA (5 μg). B indicates brain; L, liver; K, kidney; E, embryo.

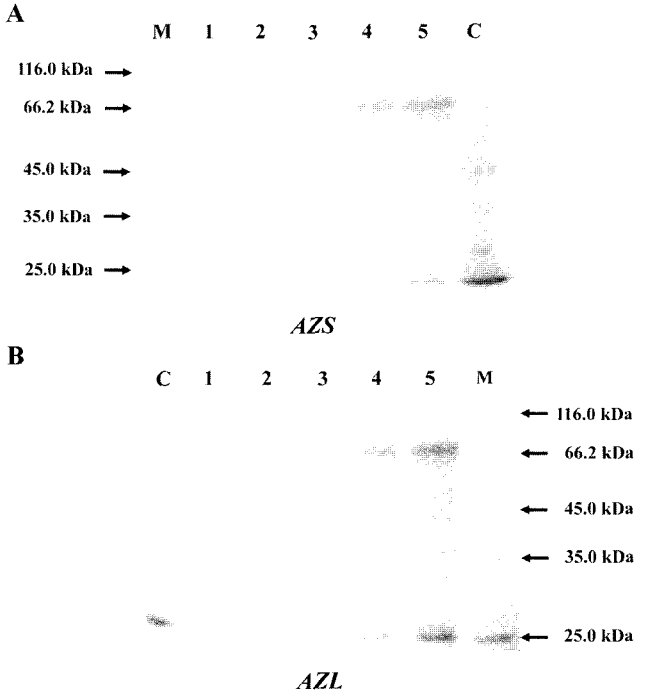


**Fig. 8.** Analysis of the expressed proteins, using SDS-PAGE of His-*AZS* (A) and His-*AZL* (B).
M indicates protein molecular size markers; C, proteins from uninduced cell extracts of BL 21 (DE3) pLys. Lanes 1–5, proteins from induced cell extracts 0, 10, 30, 60, and 120 min after IPTG induction, respectively.

from all total RNAs extracted from the brain, liver, kidney, and embryo tissues. 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.

Also, as shown in Fig. 7, the tissue-specific expression of *AZS* and *AZL* genes was confirmed by Northern blot analysis. The result of Northern blotting provided evidence for the expression of ODC antizymes in tissues from the flounder brain, liver, kidney, and embryo. Northern blot analysis showed that two antizymes were expressed in all tissues investigated, but mRNA expression was more strongly detected in *AZS* than that of *AZL*. Thus, both flounder *AZS* and *AZL* mRNAs have a wide tissue distribution, but *AZL* mRNA is less abundant. The flounder ODC antizyme genes were expressed in all tissues examined, indicating that the flounder ODC mRNA has a wide tissue distribution [22].

To express the flounder ODC antizyme genes in a prokaryotic system, each of *AZS* and *AZL* genes was subcloned into pET-44a(+) expression vector, which allows expression of recombinant protein with N-terminal fusion His-Nus-S tag. The resulting plasmids, pET-44a(+)-*AZS* and pET-44a(+)-*AZL*, were transformed into *E. coli* BL 21(DE3) pLys strain, respectively, the expression of the recombinant protein was induced by the addition of IPTG, and the expression patterns of the *AZL* and *AZS* genes were analyzed by using SDS-PAGE. As shown in Fig. 8, the optimal induction of a recombinant *AZL* and *AZS* protein was achieved at 1 h after IPTG induction. Furthermore, the molecular masses of the expressed His-*AZS* and His-*AZL* fusion proteins turned out to be approximately 69 kDa and 70 kDa, respectively: The pET-44a(+) expression vector has an N-terminal fusion tag (about 62 kDa for *AZS* and 63 kDa for *AZL*).

In conclusion, our study provides phylogenic information on the ODC antizyme that is essential for understanding the molecular evolution of this enzyme's gene in vertebrates. It may be necessary to conduct a comparative analysis of the structure, expression, and function of the ODC antizyme gene in order to elucidate the mechanism responsible for controlling the polyamine biosynthetic pathway and the intracellular polyamine concentrations. Fishes 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 ODC antizymes make this species a very useful model for studying the mechanism of polyamine participation in the ODC regulation system during development.

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