We isolated a homolog of cathepsin D from the cDNA library of the olive flounder kidney. The olive flounder cathepsin D transcript consisted of 1,733 bp that encoded a polypeptide of 396 amino acids. The overall similarity between olive flounder cathepsin D and other cathepsin Ds was very high, with the highest amino acid sequence identity to barramundi perch (89%). RT-PCR revealed that cathepsin D was expressed in almost all tissues, with high expression in the liver, intestine, kidney, skin, and spleen. The accumulation of cathepsin D mRNA after bacterial infection, as determined by RT-PCR, was constitutive and increased greatly after bacterial infection.

Key words: Paralichthys olivaceus; cathepsin D; gene expression; RT-PCR

Aspartic proteases (EC 3.4.23) are a group of endopeptidases of the pepsin family that share the same catalytic apparatus and usually function at acidic pH levels. Four major groups of aspartic proteases have been identified in vertebrates: pepsins, cathepsin D, cathepsin E, and renins. These enzymes function primarily in the degradation of intracellular and endocytosed proteins. Among them, cathepsin D is the major, lysosomal aspartic protease. It has general proteolytic activities and a wide tissue distribution. Cathepsin D is apparently also involved in the processing of antigens, hormones, and neuropeptides. In mammals, it has been studied extensively, as it is known to be involved in pathological changes, including inflammatory states, apoptosis, and mutagenesis.

To date, the cathepsin D gene has been cloned and sequenced from some fish species, including trout, seabream, and Antarctic icefish. Furthermore, it has been identified in the mucus of wounded catfish, participating in the generation of a potent 19-residue linear antimicrobial peptide named parasin I from unacetylated histone H2A, but the limited research that has been undertaken on the role of cathepsin D has involved pathogenic bacterium infections in fish species.

In previous work, we constructed a cDNA library from the olive flounder kidney. Expressed sequence tag analysis enabled us to find cDNA clones (kidney-3-D2) whose amino acid sequences had significant similarity to barramundi perch cathepsin D. The full-length cDNA was amplified from the first-stranded cDNA of olive flounder kidney by 5'-RACE and 3'-RACE using primers (CathD-5'-RACE: 5'-GATGGAACGAGTGAAACGGGAAACC-3' and CathD-3'-RACE: 5'-GCTGCTGAGGGAACCGACCCAAANAAA-3') based on the kidney-3-D2 sequence. The complete cDNA of the olive flounder cathepsin D gene was compiled by overlapping the sequences of the EST clone and the 5'-RACE and 3'-RACE PCR products. The nucleotide and deduced amino acid sequences of the olive flounder cathepsin D cDNA (GenBank accession no. FJ172450) consisted of 1,733 bp, containing an open reading frame (ORF) of 1,191 bp encoding 396 amino acid residues. The deduced polypeptide had a molecular weight of 42.84 kDa and an expected isoelectric point of 5.99. The deduced amino acid sequence of olive flounder cathepsin D contained the active site aspartyl residues, which are conserved in the cathepsin D of other species (Fig. 1).

The cathepsin D aa sequences were aligned with the corresponding NCBI GenBank sequences of known cathepsin D molecules using the CLUSTALW program (Fig. 2A). Multiple alignment analysis revealed that the catalytic sites (DTG) of the deduced protein were well conserved. Cathepsin D exists as single-chain or two-chain enzyme in vivo, and proteolytic processing of the single-chain to two-chain form takes place in an insertional sequence specific for cathepsin D (Fig. 2A, shaded area in box). However, olive flounder cathepsin D lacked this insertion, suggesting that it exists as single-chain enzyme, like other aspartic proteases. A comparison of the cathepsin D sequences from olive flounder and other species was done using the Genetyx program ver. 8.0 (Genetyx, Tokyo, Japan). The overall similarity between olive flounder cathepsin D and other cathepsin Ds was very high, with the highest amino acid sequence identity to barramundi perch. A phylogenetic tree of olive flounder cathepsin D based on amino acid sequences was constructed by the neighbor-joining method using MEGA (version 3.0). The reliability of the tree was assessed by 1,000 bootstrapping. The tree showed that the olive flounder cathepsin D formed a cluster with low vertebrates, such as barramundi perch, zebrafish, and rainbow trout, which split with the mammalian cathepsin D (Fig. 2B).
Expression of olive flounder cathepsin D was conducted by RT-PCR analysis in various unchallenged tissues and in bacterial-challenged spleen and kidney from the olive flounder, *Paralichthys olivaceus*. Olive flounder were sampled from a fish farm in Pohang in the south-eastern region of South Korea, and were acclimated to laboratory conditions for 10 d. The brain, muscle, liver, intestine, stomach, kidney, skin, fin, spleen, gill, eye, and heart of the unchallenged olive flounder were collected and kept at −80°C until use. Total RNA samples were extracted using the TRIzol reagent (Invitrogen, Carlsbad, CA). First-strand cDNA synthesis was carried out using the Advantage RT-for-PCR Kit (BD Biosciences Clontech, Palo Alto, CA). PCR amplification of the cDNAs was carried out with primer pairs for *f-cathD* 5′-GGACCCACTCCAGAGACCCTGAAGAAC-3′ and 5′-CGGGTAGGCCATGCCGAGGATC-3′, and for *f-GAPDH* 5′-TCCCATGTTCTGCA-TCGCCGTA-3′ and 5′-ATTGAGCTCAGGGATGACCTTG-3′ (GenBank accession no. FJ172450) in the GeneBank database.

Expression of olive flounder cathepsin D was conducted by RT-PCR analysis in various unchallenged tissues and in bacterial-challenged spleen and kidney from the olive flounder, *Paralichthys olivaceus*. Olive flounder were sampled from a fish farm in Pohang in the south-eastern region of South Korea, and were acclimated to laboratory conditions for 10 d. The brain, muscle, liver, intestine, stomach, kidney, skin, fin, spleen, gill, eye, and heart of the unchallenged olive flounder were collected and kept at −80°C. Bacterial-challenging of olive flounders (*n* = 3) was conducted with Gram-negative bacteria, *Edwardsiella tarda*. The fish were anaesthetized with MS-222 (3-aminobenzoic acid ethyl ester, Sigma Aldrich, St. Louis, USA) and infected with *E. tarda* by injection of a sub-lethal dose (1:2 × 10⁶ cells) suspended in PBS buffer. Tissues were collected from three fish at 0, 1, 3, 6, 12, and 24 h post-injection, and frozen at −80°C until use. Total RNA samples were extracted using the TRIzol reagent (Invitrogen, Carlsbad, CA). First-strand cDNA synthesis was carried out using the Advantage RT-for-PCR Kit (BD Biosciences Clontech, Palo Alto, CA). PCR amplification of the cDNAs was carried out with primer pairs for *f-cathD* 5′-GGACCCACTCCAGAGACCCTGAAGAAC-3′ and 5′-CGGGTAGGCCATGCCGAGGATC-3′, and for *f-GAPDH* 5′-TCCCATGTTCTGCA-TCGCCGTA-3′ and 5′-ATTGAGCTCAGGGATGACCTTG-3′ (GenBank accession no. FJ172450) in the GeneBank database.

The signal peptide is gray boxed. One predicted N-linked glycosylation site is shown underlined. Amino acid (ATGAAA) is shown in bold and underlined. The nucleotide sequence is under no. FJ172450 in the GeneBank database.
infection in the spleen and kidney (Fig. 3B). IL-1β was not detectable in the unstimulated olive flounder and was induced only after bacterial infection, as in other fish species, whereas the expression level of cathepsin D was constitutive and increased greatly post-infection.

Our results suggest that cathepsin D might be involved in a number of physiological processes and in multiple pathways, in the olive flounder. Although it is not clear why cathensin D mRNA expression was controlled post-infection, we speculate that cathepsin D plays a potent role in host defense against infectious diseases, and is also an important component of innate immunity.

To understand better the immune related functions of cathepsin D, it is of prime importance to analyze the cathepsin D activity further, and the expression kinetics and localization of cathpesin D in response to infection.

![Fig. 2. Alignment and Phylogenetic Analysis of the Deduced Amino Acid Sequence of Olive Flounder Cathepsin D with That from Other Organisms.](image)

A. Identity sequences are displayed by dots (.), and deletions of amino acid residues are indicated by dashes (---). Amino acids (DTG) of conserved catalytic sites are shown in boxes, and the shaded areas in the box indicate an insertional sequence specific for cathepsin D. The amino acid identities of olive flounder cathepsin D with the aligned sequences are shown at the end of each sequence. GenBank accession numbers of the cathepsin D amino acid sequences are as follows: barramundi perch, ABV59077; fugu rubripes, NP_001072052; rainbow trout, AAC60301; zebrafish, CAK11131; Norway rat, NP_599161; Western clawed frog, NP_988964; chicken, NP_990508; human, CAA28955; pig, AAY42145; dog, CAJ14973; cattle, XP_609913; flounder, FJ172450. B. A phylogenetic tree of the aligned sequences was constructed using the neighbor-joining algorithm in MEGA (version 4.0). The degree of confidence for each branch point was determined by bootstrap analysis (1,000 repetitions).
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References


Fig. 3. RT-PCR Analysis of the Cathepsin D Gene Expression.
A, Expression of cathepsin D mRNA in various tissues of the olive flounder. BR, brain; MS, muscle; LV, liver; IT, intestine; ST, stomach; KD, kidney; SK, skin; FN, fin; SP, spleen; GI, gill; EY, eye; HR, heart. B, Semi-quantitative analysis of cathepsin D and IL-1β mRNA after artificial bacterial infection using the Gel-Doc system. Total RNA was extracted from the spleen and kidney sampled at 1, 3, 6, 12, and 24 h after infection with E. tarda. Open bar and solid bar represent the ratio of cathepsin D mRNA/GAPDH and the ratio of IL-1β mRNA/GAPDH respectively. Each value represents the mean ± SEM of RT-PCRs of three replicates.