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Short communication

Molecular characterization and expression analysis of the channel catfish cathepsin D genes

Tingting Feng [a,b], Hao Zhang [b], Hong Liu [b], Zunchun Zhou [b], Donghong Niu [b], Lilian Wong [b], Huseyin Kucuktas [b], Xiaolin Liu [a], Eric Peatman [b], Zhanjiang Liu [b,*]

[a] College of Animal Science and Technology, Northwest A&F University, Yangling, Shaanxi 712100, China
[b] The Fish Molecular Genetics and Biotechnology Laboratory, Department of Fisheries and Allied Aquacultures and Program of Cell and Molecular Biosciences, Aquatic Genomics Unit, Auburn University, Auburn, AL 36849, USA

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ABSTRACT

Cathepsin D is a lysosomal aspartic proteinase that participates in various degradation functions of the cell. In this study, we characterized the cathepsin D genes in channel catfish and found two genes encoding catfish cathepsin D, referred to as cathepsin D1 and D2 genes. These two genes are highly similar in genomic structure and organization, sharing a moderate level of amino acid sequence similarity (56%). Genomic Southern analysis suggested the presence of a single copy of each of the cathepsin D1 and D2 genes. Phylogenetic analysis provided strong evidence that two cathepsin D genes are present in most of the teleost lineage, with cathepsin D2 likely having been lost in some higher vertebrate lineages. The catfish cathepsin D1 and D2 genes are expressed in virtually all the 11 tested tissues (brain, gill, heart, head kidney, trunk kidney, intestine, liver, muscle, skin, spleen, and stomach) on the transcript level, but appear to exhibit greater levels of expression in immune-related tissues and organs. Upon infection with *Edwardsiella ictaluri*, the expression of the catfish cathepsin D genes showed the most significant changes in liver and head kidney, with time points and magnitude of transcript changes varying between the two genes. We additionally examined bacterially-mediated changes of expression in gill, intestine, and trunk kidney. The fact that bacterial infection can induce expression of the cathepsin D genes and that they appeared to be expressed naturally at higher levels in immune-related organs may suggest that they are an important component of the innate immune response of catfish against bacterial infections.

1. Introduction

Lysosomes are membrane-bound cytoplasmic organelles that serve as major degradative compartments in eukaryotic cells [1]. They play an important role in maintaining cellular homeostasis. Endogenous and exogenous macromolecules can be delivered to the lysosome through the biosynthetic and endocytic pathways [2]. Waste materials and cellular debris are degraded in lysosomes through acid hydrolysis. Proteases of the cathepsin family are among the most thoroughly studied lysosomal hydrolases [3]. There are over a dozen cathepsin proteases in a typical animal cell, and they are distinguished by their substrate specificities. For instance, Cathepsin A and G are serine proteases; cathepsin B, C, F, H, K, L1, L2, O, S, W, and Z are cysteine proteases; while cathepsin D and E are aspartyl proteases. Cathepsin D is a 40 kDa protein with an isoelectric point of 6.95, whereas cathepsin E is a dimeric peptide each having a molecular mass of 40 kDa with an isoelectric point of 4.6 and 4.65 [4]. With the exception of cathepsin K, which works extracellularly after secretion by osteoclasts during bone resorption, most cathepsin proteases are within the lysosomes. Cathepsin D plays an important role in the lysosomal-mediated degradation of proteins [5]. It has a broad peptide bond specificity similar to pepsin and has been shown to be involved in various physiological pathways, such as intracellular catabolic proteolysis [6,7], extracellular proteolysis and processing, secretion and activation of enzymes and hormones [8].

Cathepsin D has been cloned and sequenced in a number of mammalian species [9,10], and in several species of fish such as tilapia (*Tilapia nilotica* × *Tilapia aurea*) [11], rainbow trout (*Oncorhynchus mykiss*) [12], Antarctic icefish (*Chionodraco hamatus*) [13], seabream (*Sparus aurata*) [14], zebrafish (*Danio rerio*) [15], carp (*Cyprinus carpio*) [16], herring (*Clupea harengus*) [17], pufferfish

* Corresponding author. Tel.: +1 334 844 8727; fax: +1 334 844 4694.
E-mail address: zliu@acesag.auburn.edu (Z. Liu).

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(*Takifugu rubripes*) [18], Atlantic cod (*Gadus morhua*) [19], and turbot (*Scophthalmus maximus*) [20]. In most fish species, cathepsin D was found to be mainly expressed in the spleen and liver [19], but it is also abundant in skin mucosa [21] and muscle based on protein levels [11,17,22]. Because of its major functions in protein degradation in the lysosomes and because it produces parasin I, an antimicrobial peptide, it is believed that cathepsins are important for innate immunity [21]. In this study, we identified the channel catfish (*Ictalurus punctatus*) cathepsin D transcripts, and conducted phylogenetic analysis and transcript expression analysis related to bacterial infection.

2. Materials and methods

2.1. Identification of cDNAs encoding the channel catfish cathepsin D

BLASTN was used to search the channel catfish expressed sequence tags (EST) [23] using zebrafish cathepsin D gene as a query. After identification of catfish ESTs with sequence similarities to the zebrafish cathepsin D gene, the EST sequences were assembled using CAP3. The unique sequences obtained from the assembly were analyzed using BLASTX to confirm that the ESTs are related to cathepsin D genes. It was determined that cDNA clone CBCZ2535 contained the complete cDNA of cathepsin D1. Resequencing of this clone was carried out following standard protocols. A previous full-length cDNA project had obtained the complete sequence of catfish cathepsin D2. For both genes, a minimum of 4X coverage was achieved to ensure sequence accuracy. The full-length sequences were analyzed using the signalP 3.0 server, NetNGlyc 1.0 server and SMART program to predict the presence and location of signal peptide cleavage sites, N-Glycosylation sites and domains.

2.2. Bacterial challenge and collection of fish tissue samples

All experimental procedures involving fish were approved by the Institutional Animal Care and Use Committee of Auburn University under PRN 2008-1386. In the challenge, channel catfish, with an average body weight of 6.1 g and an average body length of 9.5 cm, kept at 27 °C in a flow-through system utilizing heated, dechlorinated municipal water were used for the challenge. A total of 480 fish were stocked into two separate experimental groups, a control group and a group for *Edwardsiella ictaluri* challenge. Immediately before challenge, 0 h control fish tissues were collected from 45 fish into three pools, with equal quantity of tissues from 15 fish each being pooled for tissue transcript expression analysis [24]. The 11 tissues collected included brain, gill, heart, head kidney, intestine (proximal and distal parts), liver, muscle (taken from the mid-dorsal region), skin, spleen, stomach, and trunk kidney. Fish were treated in each of two groups: (1) control group (phosphate-buffered saline, 100 μL PBS injected); (2) *E. ictaluri* challenged group (injection). To inoculate bacteria for the challenge, a single colony of *E. ictaluri* was isolated and cultured in BHI broth at 28 °C overnight. The bacterial culture was diluted with PBS (pH 7.4), and 1×10^5 CFU of bacteria in 100 μL PBS were injected intraperitoneally into the channel catfish. Injections were carried out under anesthesia using tricaine methanesulphonate (MS 222) at 100 mg L^{-1} using a 26 gauge needle. After bacterial challenge, the same 11 tissues were collected at 4 h, 24 h, 3 d, and 7 d after infection; and tissues of gill, head kidney, trunk kidney, liver, and intestine were used for analysis of expression with infection after preliminary tissue expression analysis.

2.3. RNA isolation and RT-PCR analysis

RNA was isolated following the guanidium thiocyanate method [25] using Trizol from Invitrogen (Carlsbad, CA) following manufacturer's instructions. The RNA was treated with DNAse I and column purified, and stored in a −80 °C freezer until analysis. The concentration of total RNA was quantified using an Ultrospec 1100 pro (Amersham Biosciences Fairfield, CT), with 260/280 between 1.8 and 2.0. Reverse transcription polymerase chain reaction (RT-PCR) was performed using iScript™ cDNA Synthesis kit (Bio-Rad Laboratories, Hercules, CA) using total RNA (500 ng/μL). Each cDNA template was diluted into 250 ng/μL and used directly in RT-PCR, along with gene-specific oligonucleotides designed from cathepsin D catfish ESTs. Primers (Table 1) were designed using the FastPCR software [26] and used at a concentration of 5 μM. In order to select optimum primer pairs, a melting curve analysis was carried out. Optimal primer pairs were selected based on their amplification specificity by melting curve analysis. The reactions were carried out using SsoFast™ EvaGreen® Supermix (Bio-Rad Laboratories) on a CFX96™ real-time PCR Detection System (Bio-Rad Laboratories). The reaction included an initial denaturing step of 30 s at 95 °C, following by 35 cycles of 95 °C for 5 s, 59 °C for 5 s, and a temperature regime ranged from 60 °C to 91 °C with 0.5 °C increments in every 5 s. In addition, we checked the amplicons by agarose gel with a 100 bp ladder in order to confirm the correct amplicon sizes and that a single product was amplified.

All statistical analyses were based on cathepsin gene expression levels normalized by 18S rRNA. The triplicate fluorescence intensities of the control and treatment products for each gene, as measured by crossing-point (C_t) values, were compared and converted to fold differences by the relative quantification method [27] using the Relative Expression Software Tool 384 v. 1 (REST) and assuming 100% efficiencies. Expression differences between control and treatment groups were assessed for statistical significance ($p < 0.05$) using a randomization test in the REST software. The mRNA expression levels of all samples were normalized to the levels of 18S rRNA gene in the same samples. Expression levels of 18S rRNA were constant between all samples. Each primer set amplified a single product as indicated by a single peak present for each gene during melting curve analysis.

2.4. Southern blot

Southern blot analysis was conducted to determine the genomic copy number of channel catfish Cathepsin D using genomic DNA.

Table 1
Primers used for the study of the catfish cathepsin D genes.

Gene	Amplicon sizes (bp)	Use	Forward primer (5′–3′)	Reverse primer (5′–3′)
Cathepsin D1	162	qRT-PCR	CCTGGGCATGGCTTACCCTCG	TGGGTCTGTGCCTCCCAGGAG
	676	Southern blot	CGGTCAAGTGACAGCAGCGGT	AGCCATGCCCAGGATGCCGT
Cathepsin D2	205	qRT-PCR	CTCTCGCTCAGGGCAAGCTGA	ATGGAGGGAACCCACAGGTTGG
	662	Southern blot	CTCTCGCTCAGGGCAAGCTGA	CTGCCAGTAAGCTTTCCGGGTG
18S	127	qRT-PCR	GAGAAACGGCTACCACATCC	GATACGCTCATTCCGATTACAG

Genomic DNA (15 μg) isolated from three channel catfish individuals was completely digested with 30U *Eco* R I, *Hind* III and *Pst* I (New England Biolabs, Beverly, MA) and was electrophoresed on a 0.7% agarose gel. The gels were then submerged in 0.25 N HCl for 10 min, denaturation buffer and neutralization buffer for 30 min, respectively. The DNA was transferred to an Immobilon nylon membrane (Millipore, Bedford, MA) by capillary transfer with 20× SSC buffer for 18 h. The DNA was fixed to the membrane with a UV cross-linker (Stratagene, La Jolla, CA) using the auto crosslink setting. The filters were hybridized with probes amplified with cathepsin D specific primers (Table 1). The random primed DNA labeling kit (Roche Applied Science, Indianapolis, IN) was used for the labeling of the cDNA fragment with ^{32}P-deoxycytidine triphosphate (Perkin Elmer, Boston, MA). Sephadex G50 spin columns (Amersham Biosciences, Piscataway, NJ) were used to remove unincorporated nucleotides. Probe was denatured at 95 °C for 5 min and added into hybridization tubes which had been under pre-hybridization for 2 h with hybridization solution. The filters were hybridized at 63 °C for 16 h in 30 ml hybridization solution (1% BSA, 1 mM EDTA at pH 8.0, 7% SDS, 0.5 M sodium phosphate, pH 7.2). The filters were washed with 2× SSC and 0.1% SDS at 60 °C and wrapped with Saran wrap, and exposed to Kodak BioMax MS film at −80 °C for 24 h for autoradiography.

2.5. Phylogenetic analysis

A phylogenetic tree was constructed using the Molecular Evolutionary Genetics Analysis (MEGA 4.0) software [28]. The cathepsin D protein sequences from various species were retrieved from GenBank and aligned by using ClustalW. The neighbor-joining method was used for the construction of the phylogenetic tree. Data was analyzed using Poisson distance correction and gaps were removed by complete deletion. The topological stability of the tree was evaluated by 10,000 bootstrap replications.

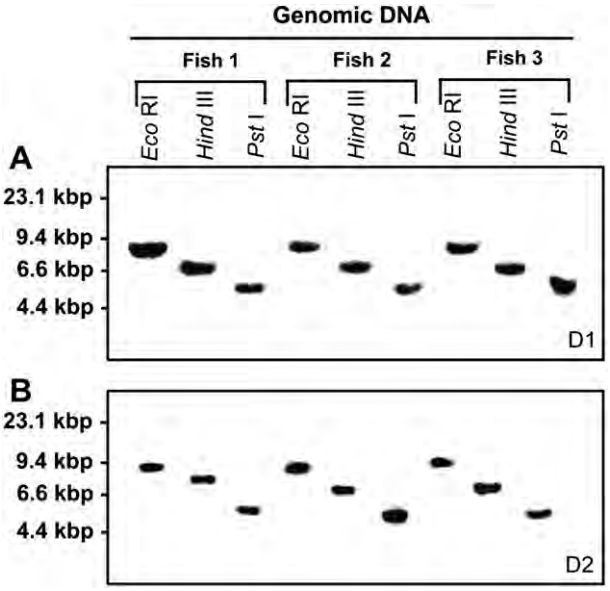

Fig. 2. A. Southern blot analysis of catfish cathepsin D1 using genomic DNA of three channel catfish individuals. Southern blot analysis procedures are detailed in the Materials and Methods section. Size markers (Kb) are indicated on the left. B. Southern blot analysis of catfish cathepsin D2 using genomic DNA of three channel catfish individuals. Southern blot analysis procedures are detailed in the Materials and Methods section. Size markers (Kb) are indicated on the left.

3. Results and discussion

3.1. Identification of the channel catfish cathepsin D gene transcripts

BLAST searches against all existing catfish EST and full-length cDNA databases using zebrafish cathepsin D as a query revealed the

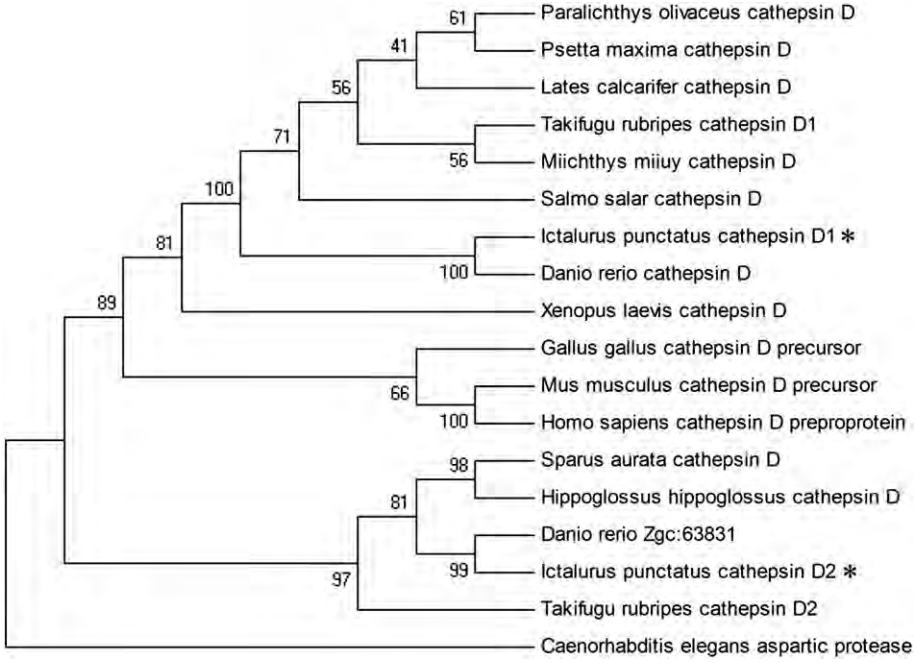

Fig. 1. Phylogenetic tree constructed using full-length cathepsin D of channel catfish and other species, GenBank accession numbers: *Paralichthys olivaceus* cathepsin D, ACN43675; *Psetta maxima* cathepsin D, ABW70789; *Lates calcarifer* cathepsin D, ABV59077; *Miichthys miiuy* cathepsin D, ADP89523; *Takifugu rubripes* cathepsin D1, NP_001072052, cathepsin D2, NP_001072053; *Danio rerio* cathepsin D, CAK11131; *Danio rerio* Zgc: 63831, AAI54325; *Xenopus laevis* cathepsin D, NP_001085308; *Gallus gallus* cathepsin D precursor, NP_990508; *Mus musculus* cathepsin D precursor, NP_034113; *Homo sapiens* cathepsin D preproprotein, NP_001900; *Sparus aurata* cathepsin D, AAB88862; *Salmo salar* cathepsin D precursor, ACH70630; *Hippoglossus hippoglossus* cathepsin D, ABI85390. Channel catfish cathepsin D is indicated by an asterisk. *Caenorhabditis elegans* protein (AAB06576) was used as an out-group.

presence of 36 cathepsin D-related ESTs. Assembly of the 36 ESTs indicated the presence of two distinct cathepsin D transcripts, and herein we will refer to these as cathepsin D1 and cathepsin D2. Complete sequences were verified in both cases by resequencing of individual cDNA clones.

Sequence analysis indicated that the catfish cathepsin D1 cDNA was 1626 bp long with an open reading frame (ORF) of 1191 nt (including termination codon) encoding 396 amino acids. A polyadenylation signal (AATAAA) was found at position 1609 nt, 17 bp upstream of the poly A tail. Analysis of amino acid sequences indicated that the catfish cathepsin D1 consists of a putative signal peptide of 18 aa, a propeptide of 29 aa, and a mature peptide of 320 aa. Two N-glycosylation sites were predicted to exist at aa position 131 and 249.

The catfish cathepsin D2 cDNA was 1389 bp containing an ORF of 1197 bp (including termination codon) encoding 398 amino acids. A polyadenylation signal (AATAAA) was found at position 1345 nt, 44 nt upstream of the poly A tail. Based on the amino acid sequence, catfish cathepsin D2 has a putative signal peptide of 19 aa, a propeptide of 29 aa, and a mature protein of 321 aa, one amino acid longer than the catfish cathepsin D1. Similar to the situation of cathepsin D1, two N-glycosylation sites were predicted to exist at aa position 133 and 251. Sequences of both cathepsin transcripts have been deposited in GenBank with accession numbers of JF415913 and GU588646 [29].

3.2. Phylogenetic analysis of channel catfish cathepsin D1 and cathepsin D2 genes

Phylogenetic analysis was conducted to identify the cathepsin-related sequences in catfish. As presented above, the two catfish cathepsin-like transcripts share 58% nucleotide identity and 56% amino acid identities. A phylogenetic tree was constructed based on the amino acid sequences of cathepsin D transcripts. As shown in Fig. 1, the catfish cathepsin D genes fell into two distinct clades: one clade included all the cathepsin D1 sequences from teleost species and the cathepsin genes from other vertebrate species, whereas the other clade included the catfish cathepsin D2 sequence along with cathepsin genes only from teleost species. The higher similarity of teleost cathepsin D1 to cathepsin D from higher vertebrates than to teleost cathepsin D2 suggests a duplication event in the common ancestor of fish and tetrapods followed by gene loss for cathepsin D2 in birds [30] and mammals. The description of only a single gene from several other fish species may be the result of insufficient study in these species, or similar gene silencing events.

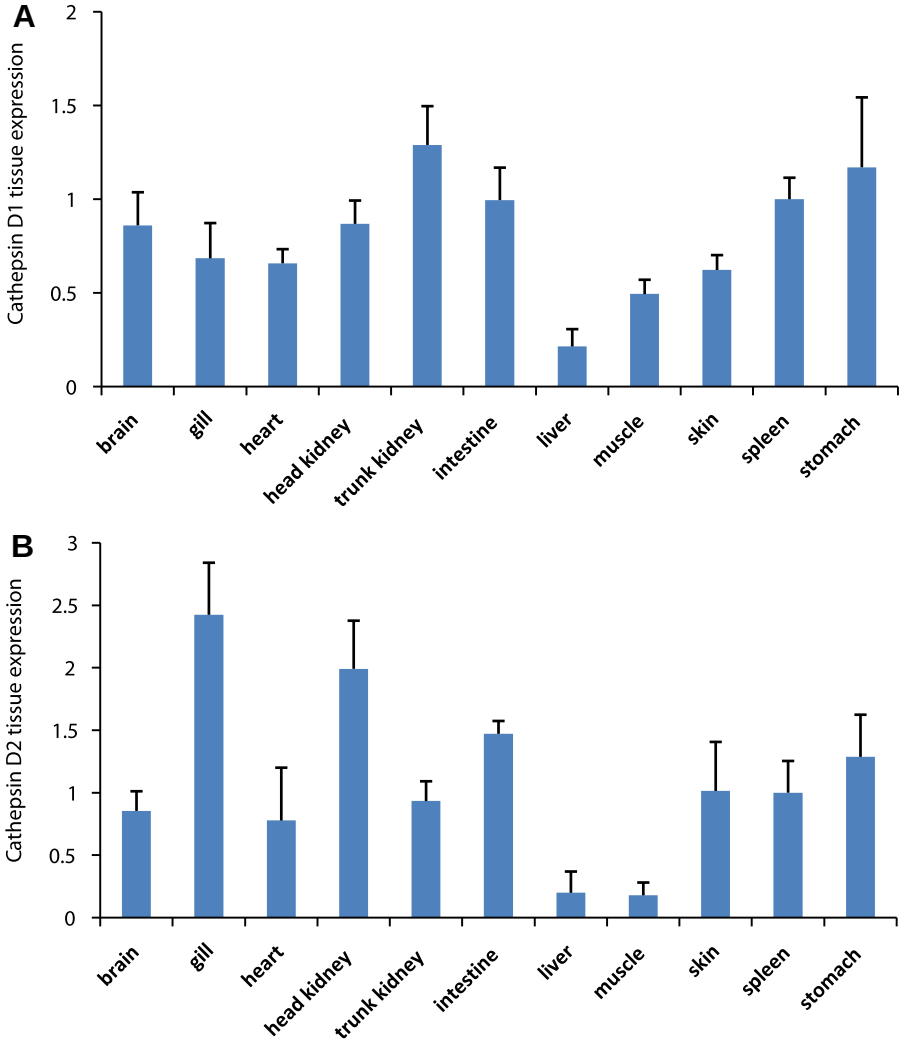

Fig. 3. A. Relative transcript expression of cathepsin D1 in channel catfish determined using qRT-PCR. The *Y*-axis represents 18S-normalized expression values. Tissue RNA expression values are presented relative to that of the spleen tissue (1×). B. Relative transcript expression of cathepsin D2 in channel catfish determined using qRT-PCR. The *Y*-axis represents 18S-normalized relative expression values. Tissue RNA expression values are presented relative to that of the spleen tissue (1×).

3.3. Copy numbers of channel catfish cathepsin D1 and D2

Genomic copy numbers of the channel catfish cathepsin D1 and D2 genes were determined by Southern blot analysis. As shown in Fig. 2, a single band was observed through hybridization of a cathepsin D probe for either cathepsin D1 or cathepsin D2, suggesting the presence of a single copy of the cathepsin D1 and D2 genes in the catfish genome. Similar patterns of Southern blot hybridization were observed for cathepsin D1 and D2 genes with all three restriction endonucleases, *Eco*R1, *Hin*d III, and *Pst* I. It is likely that the two genes are located in a genomic neighborhood sharing a high level of structural conservation. In zebrafish, cathepsin D1 and D2 are located on two chromosomes, chromosome 18 and 3, respectively. It appears that the catfish cathepsin D1 is likely the ortholog of the zebrafish cathepsin D1, not only because they share the highest sequence similarity and are placed into the same phylogenetic clade (which does not reflect orthology), but also because of their conserved synteny (which does reflect orthology). In zebrafish, the soluble carrier family 6 member 15 (SLC6A15) gene is in close proximity to cathepsin D1 gene, and a similar arrangement of these two genes exists in catfish. The genomic neighboring genes for catfish cathepsin D2 gene are not known at present time.

3.4. Transcript expression of channel catfish cathepsin D1 and D2

Tissue transcript expression analysis of the two catfish cathepsin genes was conducted using real-time RT-PCR. As shown in Fig. 3, the catfish cathepsin D1 and D2 genes are expressed in a wide range of tissues (Fig. 3A for cathepsin D1, Fig. 3B for cathepsin D2), but with a distinct pattern of expression. This divergence of expression patterns is in agreement of duplicated genes that often tend to share functional partitioning through tissue expression differences [31]. The catfish cathepsin D1 is expressed in all tested tissues with the highest expression in the trunk kidney and lowest expression in the liver; the catfish cathepsin D2 is also expressed in all tissues tested, but with the highest expression in the gill, and the lowest expression in the liver and muscle (Fig. 3).

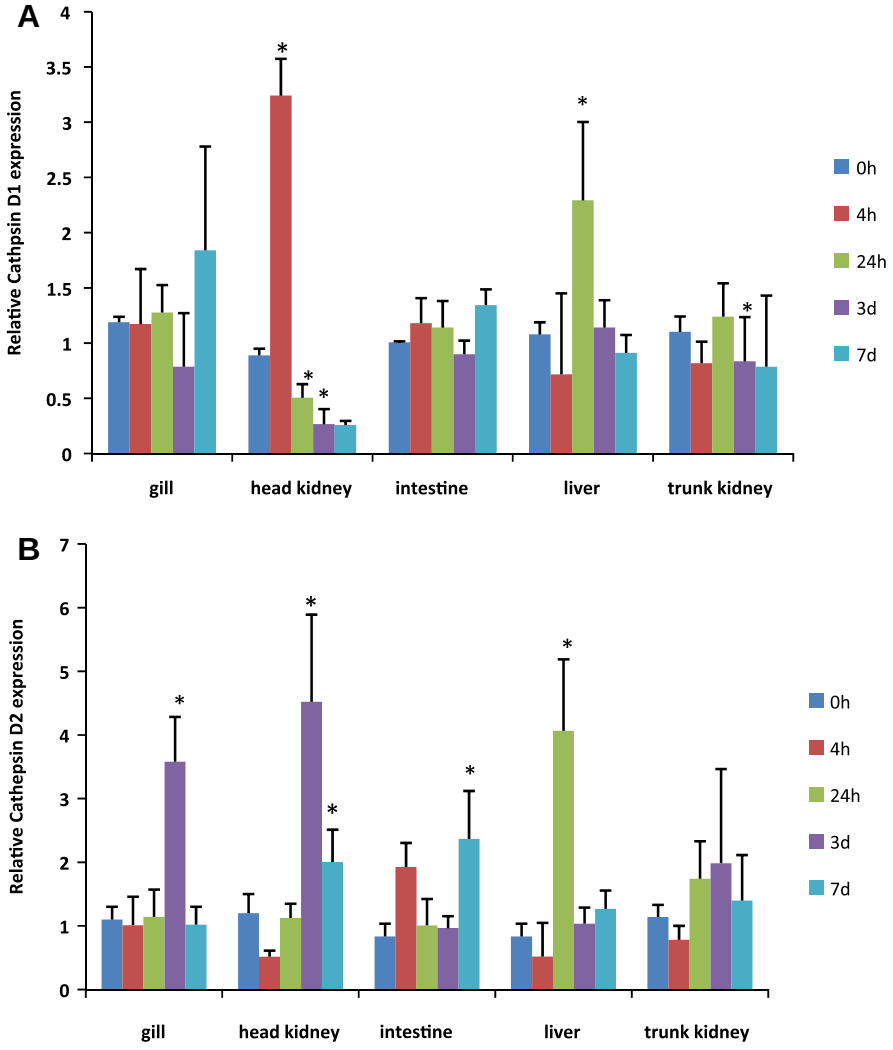

Fig. 4. A. Fold induction of channel catfish cathepsin D1 gene after *E. ictaluri* challenge. The RNA samples were collected at 0 h, 4 h, 24 h, 3 d, and 7 d post-challenge. Relative cathepsin D transcript expression was expressed as fold change over control samples taken at the same time interval as normalized to change in expression in the 18S control. The bars indicate mean expression of 3 tested pools (15 fish each) ± SE. Asterisks indicate statistical significance at the level of $P < 0.05$. B. Fold induction of channel catfish cathepsin D2 gene after *E. ictaluri* challenge. The RNA samples were collected at 0 h, 4 h, 24 h, 3 d, and 7 d post-challenge. Relative cathepsin D transcript expression was expressed as fold change over control samples taken at the same time interval as normalized to change in expression in the 18S control. The bars indicate mean expression of 3 tested pools (15 fish each) ± SE. Asterisks indicate statistical significance at the level of $P < 0.05$.

Cathepsin D was reported in other organisms to be constitutively expressed in almost all cell types, with much elevated transcript expression in certain forms of cancer and in maturing oocyte of egg-laying animals [21,32]. Consistent with the current study of catfish cathepsin D, cathepsin D genes from other teleost fish studied to-date were found to be expressed in a wide range of tissue types [6,12,13,15,17,18,20,22]. This is in agreement with the fact that the cathepsin D gene is a housekeeping proteinase in the lysosome. Nonetheless, there appeared to be a higher level of transcript expression of both cathepsin D1 and cathepsin D2 in organs and tissues involved in immune responses such as head kidney, spleen, gill, and intestines, but future studies are warranted to determine if the higher expression is related with immune functions.

As a first step to determine if cathepsin genes, as aspartyl proteases in the lysosomes, are responsive to bacterial infection, we have conducted transcript expression analysis of the catfish cathepsin genes after infection with *E. ictaluri*. Fold change was calculated relative to the corresponding timed, PBS-injected control. Expression of the catfish cathepsin D1 gene showed varied changes in the tested tissues following challenge (Fig. 4A). This transcript was significantly upregulated in head kidney at 4 h, and in liver by 24 h. Upregulation in head kidney was followed by significant down-regulation at 24 h and 3 d. Similarly, the expression of catfish cathepsin D2 was induced after infection of *E. ictaluri*, particularly in the gill, liver, intestine, and head kidney. The upregulation of gene expression appeared to be slower for cathepsin D2 than cathepsin D1, with the most rapid response in the liver at 24 h after infection (Fig. 4B).

Cathepsin D is a lysosomal endoproteolytic aspartic proteinase, and the lysosome is a membrane-bound acidic organelle that contains mature acid-dependent hydrolases and lysosomal-associated membrane proteins but lacks mannose-6-phosphate receptors [1]. These characteristics are shared with a group of cell type-specific organelles including major histo-compatibility complex class II compartments, platelet dense granules, basophil granules, and neutrophil azurophil granules [33]. This implies that the lysosome is biogenetically related to these specialized organelles which function in immunological and inflammatory reactions and suggests that cathepsin D could play a critical role as an immune proteinase in these processes. In this study, we found elevated transcript expression of both catfish cathepsin D genes following infection in several immune-related organs. The elevated expression could be a direct response of catfish to invading bacteria involving lysosomal protease degradation.

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References

[1] Kornfeld S, Mellman I. The biogenesis of lysosomes. Annu Rev Cell Biol 1989;5:483–525.
[2] Cuervo AM, Dice JF. Lysosomes, a meeting point of proteins, chaperones, and proteases. J Mol Med 1998;76:6–12.
[3] Liaudet-Coopman E, Beaujouin M, Derocq D, Garcia M, Glondu-Lassis M, Laurent-Matha V, et al. newly discovered functions of a long-standing aspartic protease in cancer and apoptosis. Cancer Lett 2006;237:167–79.
[4] Lapresle C, Puizdar V, Porchon-Bertolotto C, Joukoff E, Turk V. Structural differences between rabbit cathepsin E and cathepsin D. Biol Chem Hoppe Seyler 1986;367:523–6.
[5] Tang J, Wong RN. Evolution in the structure and function of aspartic proteases. J Cell Biochem 1987;33:53–63.
[6] Baricos WH, Zhou YW, Fuerst RS, Barrett AJ, Shah SV. The role of aspartic and cysteine proteinases in albumin degradation by rat kidney cortical lysosomes. Arch Biochem Biophys 1987;256:687–91.
[7] Dean RT. Direct evidence of importance of lysosomes in degradation of intracellular proteins. Nature 1975;257:414–6.
[8] Baldocchi RA, Tan L, King DS, Nicoll CS. Mass spectrometric analysis of the fragments produced by cleavage and reduction of rat prolactin: evidence that the cleaving enzyme is cathepsin D. Endocrinology 1993;133:935–8.
[9] Hetman M, Perschl A, Saftig P, Von Figura K, Peters C. Mouse cathepsin D gene: molecular organization, characterization of the promoter, and chromosomal localization. DNA Cell Biol 1994;13:419–27.
[10] Redecker B, Heckendorf B, Grosch HW, Mersmann G, Hasilik A. Molecular organization of the human cathepsin D gene. DNA Cell Biol 1991;10:423–31.
[11] Jiang ST, Wang YT, Chen CS. Purification and characterization of a proteinase identified as cathepsin D from tilapia muscle (*Tilapia nilotica* × *Tilapia aurea*). J Agric Food Chem 1991;39:1597–601.
[12] Brooks S, Tyler CR, Carnevali O, Coward K, Sumpter JP. Molecular characterisation of ovarian cathepsin D in the rainbow trout, *Oncorhynchus mykiss*. Gene 1997;201:45–54.
[13] Capasso C, Lees WE, Capasso A, Scudiero R, Carginale V, Kille P, et al. Cathepsin D from the liver of the antarctic icefish *Chionodraco hamatus* exhibits unusual activity and stability at high temperatures. Biochim Biophys Acta 1999;1431:64–73.
[14] Carnevali O, Centonze F, Brooks S, Marota I, Sumpter JP. Molecular cloning and expression of ovarian cathepsin D in seabream, *Sparus aurata*. Biol Reprod 1999;61:785–91.
[15] Riggio M, Scudiero R, Filosa S, Parisi E. Sex- and tissue-specific expression of aspartic proteinases in *Danio rerio* (zebrafish). Gene 2000;260:67–75.
[16] Goldmann T, Moorkamp A, Wiedorn KH, Suter L, Otto F. The prognostic value of the expression of collagenase IV, cathepsin D and metallothionein in squamous cell carcinomas of the skin determined by immunohistochemistry. Arch Dermatol Res 2001;293:115–20.
[17] Nielsen LB, Nielsen HH. Purification and characterization of cathepsin D from herring muscle (*Clupea harengus*). Comp Biochem Physiol B Biochem Mol Biol 2001;128:351–63.
[18] Kurokawa T, Uji S, Suzuki T. Identification of pepsinogen gene in the genome of stomachless fish, *Takifugu rubripes*. Comp Biochem Physiol B Biochem Mol Biol 2005;140:133–40.
[19] Wang PA, Stenvik J, Larsen R, Maehre H, Olsen RL. Cathepsin D from Atlantic cod (*Gadus morhua L.*) liver. Isolation and comparative studies. Comp Biochem Physiol B Biochem Mol Biol 2007;147:504–11.
[20] Jia A, Zhang XH. Molecular cloning, characterization and expression analysis of cathepsin D gene from turbot *Scophthalmus maximus*. Fish Shellfish Immunol 2009;26:606–13.
[21] Cho JH, Park IY, Kim HS, Lee WT, Kim MS, Kim SC. Cathepsin D produces antimicrobial peptide parasin I from histone H2A in the skin mucosa of fish. FASEB J 2002;16:429–31.
[22] Gildberg A. Aspartic proteinases in fishes and aquatic invertebrates. Comp Biochem Physiol B 1988;91:425–35.
[23] Wang S, Peatman E, Abernathy J, Waldbieser G, Lindquist E, Richardson P, et al. Assembly of 500,000 inter-specific catfish expressed sequence tags and large scale gene-associated marker development for whole genome association studies. Genome Biol 2010;11:R8.
[24] Liu H, Takano T, Abernathy J, Wang S, Sha Z, Jiang Y, et al. Structure and expression of transferrin gene of channel catfish, *Ictalurus punctatus*. Fish Shellfish Immunol 2010;28:159–66.
[25] Chomczynski P, Sacchi N. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal Biochem 1987;162:156–9.
[26] Kalendar R. FastPCR software for PCR primer and probe design and repeat search, www.biocenter.helsinki.fi/bi/programs/faspcr.htm; 2009.
[27] Pfaffl MW, Horgan GW, Dempfle L. Relative expression software tool (REST) for group-wise comparison and statistical analysis of relative expression results in real-time PCR. Nucleic Acids Res 2002;30:e36.
[28] Tamura K, Dudley J, Nei M, Kumar S. MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. Mol Biol Evol 2007;24:1596–9.
[29] Chen F, Lee Y, Jiang Y, Wang S, Peatman E, Abernathy J, et al. Identification and characterization of full-length cDNAs in channel catfish (*Ictalurus punctatus*) and blue catfish (*Ictalurus furcatus*). PLoS One 2010;5:e11546.
[30] Zuzarte-Luis V, Montero JA, Kawakami Y, Izpisua-Belmonte JC, Hurle JM. Lysosomal cathepsins in embryonic programmed cell death. Dev Biol 2007;301:205–17.
[31] Cresko WA, Yan YL, Baltrus DA, Amores A, Singer A, Rodriguez-Mari A, et al. Genome duplication, subfunction partitioning, and lineage divergence: Sox9 in stickleback and zebrafish. Dev Dyn 2003;228:480–9.
[32] Masson O, Prebois C, Derocq D, Meulle A, Dray C, Daviaud D, et al. Cathepsin-d, a key protease in breast cancer, is up-regulated in obese mouse and human adipose tissue, and controls adipogenesis. PLoS One 2011;6:e16452.
[33] Dell'Angelica EC, Mullins C, Caplan S, Bonifacino JS. Lysosome-related organelles. FASEB J 2000;14:1265–78.