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Identification and expressional analysis of two cathepsins from half-smooth tongue sole (Cynoglossus semilaevis)

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\textbf{A B S T R A C T}

Cathepsins are a family of lysosomal proteases that play an important role in protein degradation, antigen presentation, apoptosis, and inflammation. Cathepsins are divided into three groups, i.e., cysteine protease, serine protease, and aspartic protease. Cathepsin D and cathepsin L, which are aspartic protease and cysteine protease respectively, have been identified in a number of teleosts; however, the immunological relevance of fish cathepsins is largely unknown. In this study, we cloned and analyzed the expression profiles of a cathepsin D (CsCatD) and a cathepsin L (CsCatL) homologs from half-smooth tongue sole (Cynoglossus semilaevis). CsCatD is composed of 396 amino acid residues and shares 67.6–88.4% overall sequence identities with fish and human cathepsin D. Structurally CsCatD possesses an aspartic endopeptidase domain, which contains two conserved aspartic acid residues that form the catalytic site. CsCatL is 336 residues in length and shares 64.7–90.2% overall sequence identities with fish and human cathepsin L. CsCatL has an N-terminal cathepsin propeptide inhibitor domain followed by a Papain family cysteine protease domain, the latter containing four conserved catalytic residues: Gln-133, Cys-139, His-279, and Asn-303. Recombinant CsCatL purified from Escherichia coli exhibited apparent protease activity. Quantitative real time RT-PCR analysis detected constitutive expression of CsCatD and CsCatL in multiple tissues, with the lowest level found in heart and the highest level found in liver. Experimental challenge of tongue sole with the bacterial pathogen Vibrio anguillarum and megalocytivirus caused significant inductions of both CsCatD and CsCatL expression in kidney and spleen in time-dependent manners. Immunization of the fish with a subunit vaccine also enhanced CsCatD and CsCatL expression in the first week post-vaccination. These results suggest involvement of CsCatD and CsCatL in host immune reactions to bacterial and viral infections and in the process of antigen-induced immune response.

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1. Introduction

Cathepsins are a family of lysosomal proteases that can be classified into three groups based on the amino acid residues in their active sites: (i) cysteine protease, which include cathepsins B, C, F, H, K, L, O, S, T, U, V, W, and X; (ii) serine protease, which include cathepsins A and G; (iii) aspartic protease, which include cathepsins D and E [1]. Human cathepsin D is composed of 412 amino acid residues that contain a 20-residue propeptide and a 44-residue propeptide [2]. Cathepsin D is synthesized in rough endoplasmic reticulum as a preproenzyme, which is converted to the 52 kDa procathepsin D after removal of the propeptide. Procathepsin D is glycosylated at two N-linked glycosylation sites and modified with mannose 6-phosphate (M\textsubscript{6}P) at two asparagine residues [3]. The modified procathepsin D is targeted to lysosome by binding to M\textsubscript{6}P-receptors in trans-Golgi network that directs lysosomal segregation [4]. In the lysosomal compartment, the 44-residue propeptide was cleaved, resulting in a 48 kDa single chain intermediate active enzyme [5]. Like other aspartic proteases, cathepsin D has in the catalytic site two aspartate residues that are essential to enzyme activity. The physiological function of cathepsin D is to cleave intracellular proteins, including hormones and growth factors, and activate proenzymes and cellular factors involved in various cellular processes such as innate immune response and apoptosis [6–8]. In mice, cathepsin D is an essential enzyme, and cathepsin D deficiency results in death in the fourth week after birth [9].

Cathepsin L is a lysosomal cysteine protease of the peptidase C1 family. It is synthesized as an inactive proenzyme with an N-terminal propeptide that is removed upon activation. The propeptide region not only acts as an inhibitor of the enzyme activity but also required for the proper folding of the newly synthesized...
enzyme and transport of the proenzyme to lysosomes [10]. In mammals, cathepsin L functions mainly to degrade proteins in the lysosome and is known to be involved in antigen processing and Toll-like receptor (TLR) signaling [11]. Once secreted, cathepsin L can degrade extracellular matrix and thus participate in tumor growth and metastasis [12].

To date, a number of cathepsin D and cathepsin L sequences have been identified in teleosts, and active proteases of cathepsin D and cathepsin L have been purified from a large number of fish [13–22]. Some fish cathepsins D and L, such as those of zebrafish and rainbow trout, are known to be involved in oogenesis and embryogenesis [23,24]. However, except in a few cases, the potential immunological relevance of piscine cathepsins D and L remain unknown. In this study, we report the identification and expression analysis of cathepsin D (CsCatD) and cathepsin L (CsCatL) from half-smooth tongue sole (Cynoglossus semilaevis), an important economic fish species cultured widely in China. We found that the expressions of both CsCatD and CsCatL were regulated at transcription level by bacterial and viral infection. In addition, we also observed strong inductions of CsCatD and CsCatL expression by vaccination with a subunit vaccine. Our results suggest an immunological role for CsCatD and CsCatL in pathogen infection and vaccination.

2. Materials and methods

2.1. Fish

Half-smooth tongue sole (C. semilaevis) were purchased from a commercial fish farm in Shandong Province, China and maintained at 19 °C in aerated seawater. Fish were acclimatized in the laboratory for two weeks before experimental manipulation. Fish were anaesthetized with tricaine methanesulfonate (Sigma, St. Louis, MO, USA) prior to experiments involving blood collection with an overdose of tricaine methanesulfonate (Sigma, St. Louis, MO, USA) according to manufacturer’s recommendations and as described previously [26].

2.2. Cloning of CsCatD and CsCatL

A cDNA library of half-smooth tongue sole head kidney (HK) and spleen was constructed as described previously [25]. Clones of the library were randomly selected and subjected to DNA sequence analysis; one clone was found to contain the full length cDNA of CsCatD with 5'- and 3'- untranslated regions (UTRs); another clone contains the 5'-UTR, open reading frame (ORF), and partial 3'-UTR of CsCatD. The complete 3'-UTR of CsCatD was obtained by rapid amplification of cDNA ends (RACE) using the SMART RACE cDNA Amplification Kit (Clontech, Mountain View, CA, USA) according to manufacturer’s recommendations and as described previously [26]. The nucleotide sequences of CsCatD and CsCatL have been deposited in GenBank database under the accession numbers JF772215 and JF772216 respectively.

2.3. Sequence analysis

cDNA and amino acid sequences were analyzed using the BLAST program at the National Center for Biotechnology Information (NCBI) and the Expert Protein Analysis System. Domain search was performed with the simple modular architecture research tool (SMART) version 4.0 and the conserved domain search program of NCBI. The molecular mass and theoretical isoelectric point were predicted using EditSeq in DNASTAR software package (DNASTAR Inc, Madison, WI, USA). Multiple sequence alignment was created with the ClustalX program. Signal peptide search was performed with SignalP 3.0.

2.4. Plasmid construction

To construct pETCsCatL, which expresses His-tagged CsCatL, the coding region of CsCatL lacking the inhibitor domain and the signal peptide region was amplified by PCR with primers F1 (5'-GATATCCACCATGCGGATGAGATTTGAAAATGC-3'; underlined, EcoRV site) and R1 (5'-GGCATTACCACTGTTTAACTGCTTAATGCT-3'; underlined, EcoRV site); the PCR products were ligated with the T–A cloning vector pBS-T (Tiangen, Beijing, China), and the recombinant plasmid was digested with EcoRV to retrieve the CsCatL-containing fragment, which was inserted into pET259 [26] at the Swal site.

2.5. Purification of recombinant CsCatL (rCsCatL)

Escherichia coli BL21(DE3) (Bioteke, Beijing, China) was transformed with pETCsCatL. The transformants were cultured in LB medium at 37 °C to mid-log phase, and the expression of CsCatL was induced by adding isopropyl-β-D-thiogalactopyranoside to a final concentration of 1 mM. After growth at 30 °C for an additional 4 h, the cells were harvested by centrifugation, and His-tagged recombinant protein was purified using nickel-nitrilotriacetic acid columns (GE Healthcare, Piscataway, NJ, USA) as recommended by the manufacturer. The purified protein was dialyzed in PBS and concentrated with Amicon Ultra Centrifugal Filter Devices (Millipore, Billerica, MA, USA). The protein was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and visualized after staining with Coomassie brilliant blue. The concentration of purified protein was determined using the Bradford method with bovine serum albumin as the standard.

2.6. Activity of rCsCatL

rCsCatL was diluted in assay buffer [100 mM sodium phosphate, 1 mM ethylenediaminetetraacetic acid (EDTA), 0.025% Brij, 1.25 μM dithiothreitol (DTT), pH 6.0] to 4 μM, followed by adding DTT to 0.4 mM and incubation at 30 °C for 1 h. The protein was further diluted to various concentrations in assay buffer. The diluted protein or buffer alone (control) was mixed with 1 mM cysteine protease substrate Z-Arg-Arg-p-Nitro-Anilide (Sigma, St. Louis, MO, USA). The mixture was incubated at 30 °C for 2 h, and absorbance at 410 nm was determined.

2.7. CsCatD and CsCatL expression under normal physiological conditions

Brain, muscle, heart, gut, HK, spleen, gill, and liver were taken aseptically from four fish and used for total RNA extraction with the RNAPrep Tissue Kit (Tiangen, Beijing, China). One microgram of total RNA was used for cDNA synthesis with the SuperScrip II reverse transcriptase (Invitrogen, Carlsbad, CA, USA). Quantitative real time reverse transcriptase-PCR (qRT-PCR) was carried out in an Eppendorf Mastercycler (Eppendorf, Hamburg, Germany) using the SYBR ExScript qRT-PCR Kit (Takara, Dalian, China) as described previously [26]. Melting curve analysis of amplification products was performed at the end of each PCR to confirm that only one PCR product was amplified and detected. The expression level of CsCatD and CsCatL was analyzed using comparative threshold cycle method (2 -ΔΔCT) with β-actin as the control, which is known to be an appropriate RT-PCR reference for study gene expression in tongue sole [27]. All data are given in terms of mRNA levels relative to that of β-actin and expressed as means plus or minus standard errors of the means (SE).
**Fig. 1.** Alignment of the amino acid sequences of CsCatD (A) and CsCatL (B). Dots denote gaps introduced for maximum matching. The residues that are conserved among all aligned sequences are in gray. Numbers in brackets indicate overall sequence identities between CsCatD or CsCatL and the compared cathepsins. The conserved residues of the catalytic site are boxed. The GenBank accession numbers of the aligned cathepsin D sequences are as follows: *Lates calcarifer*, ABV59077; *Pelecanus onocrotalus*, ACN43675; *Takifugu rubripes*, NP_001072052; *Miichthys miiuy*, ADP89523; *Psetta maxima*, ABW70789; *Salmo salar*, ACH70630; *Oncorhynchus mykiss*, NP_001118183; *Tetraodon nigroviridis*, CAF91576; *Silurus asotus*, AAM62283; *Danio rerio*, CAK11131; *Chionodraco hamatus*, CAA07719; *Mus musculus*, BAE34900; *Homo sapiens*, NP_001900. The GenBank accession numbers of the aligned cathepsin L sequences are as follows: *Lates calcarifer*, ABV59078; *Hippoglossus hippoglossus*, ABJ99858; *Oryzias latipes*, NP_001098156; *Fundulus heteroclitus*, AAO64471; *Misgurnus mizolepis*, ABQ08058; *Cyprinus carpio*, BAD08618; *Oncorhynchus mykiss*, NP_001117777; *Salmo salar*, NP_001140018; *Danio rerio*, CAN88536; *Dicentrarchus labrax*, ACN93991; *Caligus clemensi*, ACO15730; *Engraulis japonicas*, BAC16538; *Osmerus mordax*, ACO09031; *Ictalurus punctatus*, NP_001187182; *Homo sapiens*, AA242984; *Mus musculus*, BARZ719.
| Cysteine protease domain | Inhibitor domain |
|-------------------------|------------------|
| **Cysteine protease domain** | **Inhibitor domain** |
| **CysCat** | **Inhibitor domain** |
| Laces calcifer | **Inhibitor domain** |
| Oryctes ikites | **Inhibitor domain** |
| Fundulus heteroclitus | **Inhibitor domain** |
| Mugil cephalus | **Inhibitor domain** |
| Cyprinus carpio | **Inhibitor domain** |
| Onchorhynchus mykiss | **Inhibitor domain** |
| Salmo salar | **Inhibitor domain** |
| Danio rerio | **Inhibitor domain** |
| Dicentrarchus labrax | **Inhibitor domain** |
| Calliscomenidae | **Inhibitor domain** |
| Engraulis japonicus | **Inhibitor domain** |
| Osmerus mordax | **Inhibitor domain** |
| Ictalurus punctatus | **Inhibitor domain** |
| Homo sapiens | **Inhibitor domain** |
| Mus musculus | **Inhibitor domain** |

**Fig. 1.** (continued)
2.8. CsCatD and CsCatL expression in response to bacterial and viral challenge

The fish bacterial pathogen Vibrio anguillarum C312 [25] was cultured in LB medium and resuspended in PBS to 1 × 10^9 colony forming units (CFU)/ml. Half-smooth tongue sole (~10.7 g) were divided randomly into groups of five fish and injected i.p. with 100 μl of V. anguillarum, 10^4 copies of the megalocytivirus RBIV-C1 [28] in 100 μl PBS or 100 μl PBS (control). Fish (four for each time point) were sacrificed at 1 h, 4 h, 12 h, 24 h, and 48 h post-challenge, and tissues were taken under aseptic conditions and used for qRT-PCR as described above.

2.9. CsCatD and CsCatL expression in response to vaccination

Purified recombinant DegQ, a protective antigen from the fish bacterial pathogen Vibrio harveyi [29], was prepared as described previously [29] and resuspended in PBS to 150 μg/ml. Half-smooth tongue sole were divided randomly into two groups (N = 32) and injected intraperitoneally with 100 μl of PBS (control) or DegQ. Fish were sacrificed at 2 h, 4 h, 12 h, 1 d, 7 d, 14 d, 21 d, and 28 d post-vaccination. CsCatD and CsCatL expression in HK was determined by qRT-PCR as described above.

2.10. Statistical analysis

All statistical analyses were performed with SPSS 15.0 software (SPSS Inc., Chicago, IL, USA). Data were analyzed with analysis of variance (ANOVA), and statistical significance was defined as P < 0.05.

3. Results and discussion

3.1. Characterization of CsCatD and CsCatL sequences

Sequence analysis showed that the cDNA of CsCatD contains a 5′-UTR of 80 bp, an ORF of 1191 bp, and a 3′-UTR of 369 bp. A putative polyadenylation signal, AATAAA, lies 179 bp upstream the poly-A tail constituted by 26 adenine residues. The deduced amino acid sequence of CsCatD is composed of 396 residues and has a molecular mass of 42.85 kDa and a theoretical pl of 7.87. CsCatD possesses a putative signal peptide formed by the N-terminal 18 residues. BLAST analysis showed that CsCatD shares 76.5–88.4% overall sequence identities with the cathepsin D of Takifugu rubripes, Paralichthys olivaceus, Scophthalmus maximus, Miichthys miuy, Takifugu rubripes, Salmo salar, Oncorhyhncus mykiss, Danio rerio, Silurus asotus, and Chionodraco hamatus (Fig. 1A). The overall sequence identities between CsCatD and mouse and human cathepsin D are 67.6% and 67% respectively. In silico analysis showed that CsCatD is an aspartate protease belonging to the peptidase family A1 (pepsin A, clan AA) with an aspartic endopeptidase domain (residues 115–335) and a cathepsin propeptide inhibitor domain (residues 28–87). Within the cysteine protease domain are four conserved catalytic residues: Gln-133, Cys-139, His-279, and Asn-303, of which, the Cys and His residues are known to form in part a catalytic dyad in mammalian cathepsin L, while the Gin and Asn residues play an accessory role in catalysis [30].

The cDNA of CsCatL contains a 5′-UTR of 36 bp, an ORF of 1011 bp, and a 3′-UTR of 328 bp. A putative polyadenylation signal, AATAAA, is located 13 bp upstream the poly-A tail. The ORF encodes a protein of 336 amino acids with a molecular mass of 37.94 kDa and a theoretical pl of 5.92. CsCatL possesses a putative signal peptide formed by the N-terminal 16 residues. BLAST analysis indicated that CsCatL shares 81.3–90.2% overall sequence identities with the cathepsin L of a number of fish, including L. calcarifer, Hippoglossus hippoglossus, Mugunurus mizolepis, Fundulus heteroclitus, S. salar, O. mykiss, Engraulis japonicas, and D. rerio (Fig. 1B). CsCatL is 64.7% and 60.4% identical to human and mouse cathepsin L respectively. Conserved domain search identified in CsCatL a Papain family cysteine protease domain (residues 115–335) and a cathepsin propeptide inhibitor domain (residues 28–87). Within the cysteine protease domain are four conserved catalytic residues: Gln-133, Cys-139, His-279, and Asn-303, of which, the Cys and His residues are known to form in part a catalytic dyad in mammalian cathepsin L, while the Gin and Asn residues play an accessory role in catalysis [30].
Take together, these results indicate that CsCatD and CsCatL are homologs of cathepsin D and cathepsin L respectively and possess structural features that are highly conserved in piscine and mammalian cathepsins.

3.2. Purification of recombinant rCsCatL and analysis of its activity

To examine the activity of CsCatL, rCsCatL lacking the inhibitor domain was purified from *E. coli* as a recombinant protein. SDS-PAGE analysis showed that the purified protein had a molecular mass matching that predicted for rCsCatL (28.6 kDa) (Supplementary data Fig. 1). Activity analysis showed that rCsCatL exhibited apparent cysteine protease activity in a concentration-dependent manner (Fig. 2).

3.3. Tissue-specific expression of CsCatD and CsCatL under normal physiological conditions

qRT-PCR analysis showed that under normal physiological conditions, both CsCatD and CsCatL mRNA levels were lowest in heart and highest in liver (Fig. 3). Between heart and liver, CsCatD mRNA was detected in increased order in gut, spleen, kidney, gill, muscle, and brain, while CsCatL mRNA was detected in increased order in gut, brain, spleen, kidney, gill, and muscle. These results are similar to those reported for the cathepsin D of turbot, catfish, and flounder, which exhibit expression in a wide range of tissues, with high levels found in liver [31–33]. Wide distribution of cathepsin L protein was also observed in Atlantic salmon [34]. However, in anchovy, cathepsin L mRNA was found predominately in the gut, with only trace amount in liver [35]. The difference may reflect the expressional difference between cathepsin L of different fish species.

3.4. Expression of CsCatD and CsCatL in response to bacterial and viral challenge

When tongue sole were challenged with the fish bacterial pathogen *V. anguillarum*, both CsCatD and CsCatL expressions in kidney, spleen, and liver increased to significant (*P* < 0.05) levels in a time-dependent manner. For CsCatD, maximum inductions occurred at 4 h, 1 h, and 4 h post-challenge in kidney, spleen, and liver respectively, while for CsCatL, maximum inductions in these tissues occurred at 4 h, 1 h, and 24 h post-challenge respectively (Fig. 4). Recent studies showed that mammalian cathepsin L is

![Fig. 4](image-url)

Expression of CsCatD and CsCatL in fish tissues in response to bacterial challenge. Half-smooth tongue sole were challenged with *Vibrio anguillarum* or PBS (control). CsCatD and CsCatL expression in kidney (A and D respectively), spleen (B and E respectively), and liver (C and F respectively) was determined by quantitative real time RT-PCR at various time points. Values are shown as means ± SE (*N* = 4).
involved in the infection of some viruses. For example, in one case, cathepsin L inhibitors blocked the entry of human coronavirus 229E, and in another case, cathepsin L is required for proteolytically processing of the Ebola virus glycoprotein during infection [36–39]. In our study, following challenge with the megalocytivirus RBIV-C1, CsCatD and CsCatL expressions in kidney and spleen were significantly ($P < 0.05$) upregulated, with peak inductions occurring at 12 h post-challenge in kidney and at 24 h post-challenge in spleen (Fig. 5). These results indicate that CsCatD and CsCatL are involved in host immune response during both bacterial and viral infections.

3.5. Expression of CsCatD and CsCatL in response to antigen stimulation

It is known that in mammals, cathepsin L and, to a lesser extent, cathepsin D are involved in MHC II-associated Ag presentation and regulation of CD4$^+$ T lymphocyte selection [12]. Mice defective in cathepsin L exhibit reduced number of CD4$^+$ T cells and were affected in the ability to degrade invariant chain, a critical step in MHC II-restricted antigen presentation, in cortical thymic epithelial cells, which are antigen presenting cells for positive selection of T lymphocytes in the thymus [12,40]. In our study, when tongue sole were vaccinated with a V. harveyi subunit vaccine DegQ, which is known to confer immunoprotection against V. harveyi infection [29], CsCatD and CsCatL expression in kidney was significantly ($P < 0.05$) upregulated from 2 h to 7 d and from 2 h to 14 d post-vaccination respectively (Fig. 6). However, for both CsCatD and CsCatL, dramatic inductions (including peak inductions) occurred at the early hours following vaccination, which favors the suggestion that CsCatL and CsCatD may, like what has been observed with mammalian cathepsins L and D, possibly participate in antigen presentation and processing.
In conclusion, the results of this study indicate that CsCatD and CsCatL expression was detectable in a wide range of tissues under normal physiological conditions and was significantly upregulated in kidney and spleen by bacterial and viral infections and by vaccination. These results support an immunological role for CsCatD and CsCatL in microbial infection and in vaccine-induced immune response.

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Appendix. Supplementary data

Supplementary data related to this article can be found online at doi:10.1016/j.fsi.2011.09.012.

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