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Short communication

Mucosal expression signatures of two Cathepsin L in channel catfish (*Ictalurus punctatus*) following bacterial challenge

Renjie Wang [a, 1], Lin Song [a, 1], Baofeng Su [c], Honggang Zhao [b], Dongdong Zhang [b], Eric Peatman [b], Chao Li [a, *]

[a] *Marine Science and Engineering College, Qingdao Agricultural University, Qingdao 266109, China*
[b] *School of Fisheries, Aquaculture, and Aquatic Sciences, Auburn University, Auburn, AL 36849, USA*
[c] *Key Laboratory of Freshwater Aquatic Biotechnology and Breeding, Ministry of Agriculture, Heilongjiang Fisheries Research Institute, Chinese Academy of Fishery Sciences, Harbin 150070, China*

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ABSTRACT

The mucosal surfaces of fish are the first line of host defense against various pathogens. The mucosal immune responses are the most critical events to prevent pathogen attachment and invasion. Cathepsins are a group of peptidases that involved in different levels of immune responses, but the knowledge of the roles of Cathepsin in mucosal immune responses against bacterial infection are still lacking. Therefore, in the present study we characterized the Cathepsin L gene family in channel catfish, and profiled their expression levels after challenging with two different Gram-negative bacterial pathogens. Here, two Cathepsin L genes were identified from channel catfish and were designated *CTSL1a* and *CTSL1*. Comparing to other fish species, the catfish *CTSL* genes are highly conserved in their structural features. Phylogenetic analysis was conducted to confirm the identification of *CTSL* genes. Expression analysis revealed that the *CTSL* genes were ubiquitously expressed in all tested tissues. Following infection, the *CTSL* genes were significantly induced at most timepoints in mucosal tissues. But the expression patterns varied depending on both pathogen and tissue types, suggesting that *CTSL* genes may exert disparate functions or exhibit distinct tissue-selective roles in mucosal immune responses. Our findings here, clearly revealed the key roles of *CTSL* in catfish mucosal immunity; however, further studies are needed to expand functional characterization and examine whether *CTSL* may also play additional physiological roles in catfish mucosal tissues.

1. Introduction

The mucosal surfaces are the first line of host defense against various pathogens, and the mucosal immune responses are the most critical events to prevent pathogen attachment and invasion. Especially for fish species, the mucosal surfaces (skin, gill and intestine) are constantly interacting with a wide range of pathogens present in the aquatic environment. It has been long hypothesized that observed differences in disease susceptibility among fish species and strains are due to the different ability of the fish host to prevent pathogen attachment and entry at the mucosal epithelial sites on the skin, gill and intestine [1–3]. However, the detailed molecular elements and actors involved in mucosal immune responses have always been overlooked, because most of the studies focused on the classical immmune tissues (liver, kidney and spleen). Therefore, characterizing the mucosal immune actors and their roles during pathogen infection can expand our knowledge of the immune network in catfish as well as teleost immunity.

Cathepsins are a group of peptidases that involve in different levels of immune responses, including apoptosis, inflammation, antigen processing and regulation of hormone processing [4]. Among different groups of cathepsins, cathepsin L (*CTSL*) is ubiquitously expressed in most immune tissues and cells, whereas many other cathepsins can be only found in specific cell types [5]. Cathepsin L is a lysosomal cysteine protease that exhibits strong endopeptidase activity, and involves in intracellular and extracellular protein degradation. In mammal mucosal surfaces, cathepsin L has been revealed to play vital roles in controlling normal mucosal epithelial homeostasis and supporting the host immune defense

* Corresponding author.
E-mail address: leoochao@163.com (C. Li).
[1] Both these authors contributed equally to the manuscript.

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against infection. In cathepsin L deficient mice, loss of cathepsin L activity showed the disorder of intestinal epithelial cells and initiation of intestinal epithelial disease [6]. Xu et al. reported that Cathepsin L supported airway lymphangiogenesis and protected against mycoplasmal infection in mice [7]. In human lung epithelial cells, Cathepsin L is involved in regulation of cell apoptosis [8]. Despite its important roles in mucosal health, the studies on immune roles of Cathepsin L in fish mucosal tissues after challenge are still lacking, though many studies have characterized Cathepsin L and its roles during pathogen infection in aquatic organisms. For example, Cathepsin L was induced in liver and blood cells in both lipopolysaccharide and *Edwardsiella tarda* challenged rock bream (*Oplegnathus fasciatus*) [9]. In *Cynoglossus semilaevis*, Cathepsin L was induced in kidney and spleen following *Vibrio anguillarum* and megalocytivirus infection [10].

The channel catfish (*Ictalurus punctatus*), one of the most dominant aquaculture species in the U.S., suffers from widespread disease outbreaks due to a number of bacterial pathogens. Especially, bacterial diseases of *Edwardsiella ictaluri*, the causative agent of enteric septicemia of catfish (ESC), and *Flavobacterium columnare*, known as the causative agent of columnaris disease, are two major Gram-negative bacterial pathogens that led to serious economic losses to channel catfish industry [11,12]. Recent RNA-seq studies of our group revealed that gene expression of Cathepsin L in catfish was significantly changed in intestine after *E ictaluri* infection [13], in skin after *Aeromonas hydrophila* infection and in gill following *F. columnare* challenge [14,15]. In addition, one Cathepsin L was identified in catfish and showed high expression levels in mucosal tissues by traditional RT-PCR analysis on agarose gels [16]. Nevertheless, despite the recognized importance of Cathepsin L in mucosal immunity, no studies have characterized Cathepsin L in genome-wide, and investigated the detailed immune roles in mucosal tissues after infection. In this regard, with the availability of the RNA-seq and genome databases, we in the present study sought to further identify the Cathepsin L, and determine the expression patterns of Cathepsin L in mucosal tissues after different bacterial infection, and lastly to understand its involvement in mucosal immunity.

2. Materials and methods

2.1. Sequence identification and analysis

The *CTSL* genes of the channel catfish were identified from the channel catfish transcriptome databases generated by our previous studies [13,15,17,18]. In brief, the *CTSL* sequences from the other fish species were used as queries to BLAST against the databases with a cutoff E-value of 1e-5. The candidate *CTSL* sequences were then verified by aligning against the catfish whole genome data (unpublished). The verified sequences were translated using ORF Finder (http://www.ncbi.nlm.nih.gov/gorf/gorf.html). The predicted amino acid sequences from ORFs were further verified by BLASTP (http://blast.ncbi.nlm.nih.gov/Blast.cgi) against NCBI nr protein database. The simple modular architecture research tool (SMART) was used to identify the conserved domains (http://smart.embl-heidelberg.de). Signal peptides of the *CTSL* genes were determined by SignalP 3.0 Server (http://www.cbs.dtu.dk/services/SignalP). ExPASy server was used to analyze the N-glycosylation sites [19].

2.2. Phylogenetic analysis

All the amino acids of *CTSL* from channel catfish and the other organisms were selected and retrieved from NCBI databases to construct the phylogenetic tree, including human, mouse, chicken, frog, turtle, medaka, fugu, tilapia, zebrafish and stickleback. Multiple protein sequence alignments were conducted using the ClustalW2 program [20]. Phylogenetic analyses were performed with MEGA 5 using the neighbor-joining method. Bootstrapping with 10,000 replications were conducted to evaluate the phylogenetic tree [21].

2.3. Bacteria challenge and sample collection

In order to characterize the immune roles of *CTSL* genes in the host defense against bacterial infection, *E. ictaluri* and *F. columnare* challenges were conducted following previously established protocols [13,22]. Channel catfish fingerlings were reared in the laboratory for at least two weeks prior to experimental challenge at a temperature of 28 °C. After a pre-challenge, the bacteria was re-isolated from a symptomatic fish and biochemically confirmed before being cultured. During challenge, symptomatic fish were confirmed to be infected with *E. ictaluri* and *F. columnare* respectively. During two bacterial challenge experiments, water circulation was off for 2 h and was then on until the termination of each experiments. For sample collection, the fish were euthanized with tricaine methanesulfonate (MS-222) at 200 mg/L (buffered with sodium bicarbonate). All samples were then stored in a −80 °C ultra-low freezer until preparation of RNA.

Briefly, *E. ictaluri* (MS-S97-773) was inoculated in a brain heart infusion (BHI) medium in a shaker incubator overnight at 28 °C. Fish were challenged in 30-L aquaria with 1 control group using sterilized media and 4 treatment groups using bacterial culture for treatment. Aquariums were randomly assigned according to the sampling timepoints-4 h, 24 h, 3 d and 7 d treatment, with forty fish in each aquarium. For the challenge, the fish were immersed for 2 h at a final concentration of 4×10^6 CFU/mL. Control fish were immersed in sterilized media alone for the same time. At 4 h, 24 h, 3 d and 7 d after challenge, tissues (skin, gill and intestine) from 30 fish (10 fish per replicate) were randomly collected from each aquarium at each time point; meanwhile, tissues from 9 control fish (3 fish per replicate) were also collected at the same time point.

The bacteria *F. columnare* (BGFS-27; genomovar II) was inoculated in a modified Shieh broth for 24 h in a shaker incubator (100 rpm) at 28 °C. Fish (siblings from the same catfish family used for *E. ictaluri* experiment) were randomly divided into 5 rectangular 30-L aquaria of which 1 aquarium was designated as control group and the other 4 were designated challenge groups (4 h, 24 h and 60 h). For the challenge, the fish were immersed for 2 h at a final concentration of 3 × 106 CFU/mL. Control fish were treated with identical procedures except that they were exposed to sterile modified Shieh broth. Gill and skin samples were collected at 4 h, 24 h and 60 h following challenge as well as that from the control group at the three samplings time points. At each time point, 18 fish were randomly selected and divided into 3 replicate pools (6 fish each pool), respectively.

2.4. Total RNA extraction and cDNA synthesis

Prior to RNA extraction, samples were ground with sterilized mortar and pestle in the presence of liquid nitrogen to a fine powder. Total RNA was extracted using Trizol® Reagent (Invitrogen, USA) following the manufacturer's protocol. RNA concentration and integrity of each sample was measured on a nanodrop 2000 (Thermo Electron North America LLC, FL). First strand cDNA was synthesized by iScript™ cDNA Synthesis Kit (Bio-Rad) according to manufacturer's protocol. The iScript chemistry uses a blend of oligo-dT and random hexamer primers. All the RNA from different timepoints was diluted to 250 ng/μl for cDNA synthesis and utilized for the quantitative real-time PCR (qPCR) reaction on a CFX96 real-time PCR Detection System (Bio-Rad Laboratories, Hercules, CA).