Fish and Shellfish Immunology 93 (2019) 623–630

Contents lists available at ScienceDirect

Fish and Shellfish Immunology

journal homepage: www.elsevier.com/locate/fsi

Full length article

Characterization, expression, enzymatic activity, and functional identification of cathepsin S from black rockfish *Sebastes schlegelii*

Guang-hua Wang[a,1], Shu-wen He[a,1], Xue Du[a], Bing Xie[a], Qin-qin Gu[a], Min Zhang[a,c,*], Yong-hua Hu[b,c,d,**]

[a] Marine Science and Engineering College, Qingdao Agricultural University, Qingdao, 266109, China
[b] Institute of Tropical Bioscience and Biotechnology, Chinese Academy of Tropical Agricultural Sciences, Haikou, 571101, China
[c] Laboratory for Marine Biology and Biotechnology, Pilot National Laboratory for Marine Science and Technology, Qingdao, China
[d] Hainan Provincial Key Laboratory for Functional Components Research and Utilization of Marine Bio-resources, Haikou, 571101, China

ARTICLE INFO

Keywords:
Cathepsin S
Sebastes schlegelii
Bacterial infection
Immune defense

ABSTRACT

Cathepsin S belong to the cathepsin L-like family of cysteine cathepsins. It is well known that Cathepsin S participate in various physiological processes and host immune defense in mammals. However, in teleost fish, the function of cathepsin S is less investigated. In the present study, a cathepsin S homologue (SsCTSS) from the teleost fish black rockfish (*Sebastes schlegelii*) were identified and examined at expression and functional levels. In silico analysis showed that three domains, including signal peptide, cathepsin propeptide inhibitor I29 domain, and functional domain Pept_C1, were existed in the cathepsin. SsCTSS possesses a peptidase domain with three catalytically essential residues (Cys25, His162, and Asn183). Phylogenetic profiling indicated that SsCTSS are evolutionally close to the cathepsin S of other teleost fish. The expression of *SsCTSS* in immune-related tissues was upregulated in a time-dependent manner upon bacterial pathogen infection. Purified recombinant SsCTSS (rSsCTSS) exhibited apparent peptidase activity, which was remarkably declined in the presence of the cathepsin inhibitor E−64. rSsCTSS showed strong binding ability to LPS and PGN, the major constituents of the outer membranes of Gram-negative and Gram-positive bacteria, respectively. rSsCTSS also exhibited the capability of agglutination to different bacteria. The knockdown of *SsCTSS* attenuated the ability of host to eliminate pathogenic bacteria. Taken together, our results suggested that SsCTSS functions as cysteine protease which might be involved in the antibacterial immunity of black rockfish.

1. Introduction

Cathepsin is a class of lysosomal proteases, which play critical roles in a variety of physiological and pathological conditions in both prokaryotes and eukaryotes, such as fertilization, cell division, differentiation, molting, tissue remodeling, immune responses, antigen processing, and apoptosis [1–7]. Cathepsins can be classified into three groups based on the amino acid residues in their active sites, i.e., cysteine protease (cathepsins B, C, F, H, K, L, O, S, T, U, V,W, and X), which is the greatest group, serine protease (cathepsins A and G), and aspartic protease (cathepsins D and E) [8,9]. The nascent cysteine cathepsin is an inactive zymogen, after modification and excision by other proteases or by an autocatalytic mechanism, a mature peptide with proteolytic activity is generated [8]. Cysteine cathepsins can form a V-shaped substrate-binding cleft through a papain-like fold, and the variations in shape and residual make cysteine cathepsin members present difference in substrate preference and catalytic properties [8,10].

Based on sequence similarity, length, and structure of the proregion, cysteine cathepsins can be further divided into the L-like (L, V, K, S, W, F, and H) and B-like subfamily (B, C, O, and X) [11,12]. Cathepsin S (CTSS) belongs to L-like cathepsins subfamily and was first discovered in bovine lymphnodes [13]. Specially, CTSS is stable at a neutral or slight alkaline pH and can retain its activity in outside of lysosomes, which endow its ability of degradation of extracellular matrix [14,15]. In mammal, CTSS is highly expressed in immune cells, such as antigen-presenting cells and B cells [16], and involves in immune responses against microbial infection, such as antigen presentation [3], TLRs signaling [17], inflammation and infection [18], and regulation of

* Corresponding author. Marine Science and Engineering College, Qingdao Agricultural University, Qingdao, 266109, China.
** Corresponding author. Institute of Tropical Bioscience and Biotechnology, Chinese Academy of Tropical Agricultural Sciences, Haikou, 571101, China.
E-mail addresses: lissazhang06@163.com (M. Zhang), huyonghua@itbb.org.cn (Y.-h. Hu).
[1] These two authors contribute equally to this work.

https://doi.org/10.1016/j.fsi.2019.08.012
Received 18 June 2019; Received in revised form 4 August 2019; Accepted 6 August 2019
Available online 07 August 2019
1050-4648/ © 2019 Elsevier Ltd. All rights reserved.

mature cathepsin L in B lymphocytes [19]. In teleost, CTSS homologues have been identified in several species, such as *Fundulus heteroclitus* [20], *Ictalurus punctatus* [21], *Paralichthys olivaceus* [22], *Oplegnathus fasciatus* [23], *Miichthys miiuy* [24], *Lutjanus argentimaculatus* [25], *Larimichthys crocea* [26], *Sciaenops ocellatus* [27], *Pelteobagrus fulvidraco* [28]. However, the function of CTSS, especially immune roles against pathogen infection, in teleost, are largely unknown.

Black rockfish (*Sebastes schlegelii*), an economic fish species that is cultured worldwide. However, the aquaculture of black rockfish has been severely challenged by pathogens infection [29]. Studies on immune-associated factors of black rockfish should be paid more attention. As far as we know, there are few research reports about cathepsin in black rockfish so far. In this study, cathepsin S (SsCTSS), from black rockfish was identified and analyzed, and the expression patterns and biological properties of SsCTSS were examined.

2. Materials and methods

2.1. Fish

Black rockfish (average 10.2 ± 1.3 g) were purchased from a commercial fish farm in Shandong Province, China and maintained at 20 °C in aerated seawater. Fish were acclimatized in the laboratory for two weeks before experimental manipulation. Before experiment, fish were randomly sampled and verified to be absent of pathogens in tissues as reported previously [30]. Before tissue collection, fish were euthanized with an overdose of tricaine methanesulfonate (Sigma, St. Louis, MO, USA) as reported previously [31].

2.2. Bacterial strains

Vibrio anguillarum C1 was previously isolated from diseased black rockfish and kindly provided by Doctor Cheng of Qingdao Agricultural University. *Escherichia coli* DH5α and *Trans*etta (DE3) were purchased from Transgene (Beijing, China). *Staphylococcus aureus* 1D00101 was purchased from China General Microbiological Culture Collection Center (Beijing, China). All strains were cultured in Luria-Bertani broth (LB) medium at 28 °C (for *V. anguillarum*) or 37 °C (for all others).

2.3. Cloning of cathepsin S (SsCTSS)

A cDNA library of black rockfish head kidney and spleen was constructed as reported previously [32]. Blast analysis indicated that one of the expressed sequence tags shares high sequence identities with known cathepsin S (*SsCTSS*), with 5′- and 3′- untranslated regions (UTRs).

2.4. Sequence analysis

The cDNA and amino acid sequence of SsCTSS was analyzed using the BLAST program at the National Center for Biotechnology Information (NCBI). Signal peptide search and domain search were performed with the simple modular architecture research tool (SMART) version 4.0. The molecular mass and theoretical isoelectric point (pI) were predicted using DNAman software package (Lynnon Biosoft, Quebec, Canada). The secondary structure of SsCTSS could be browsed on the Pole Bioinformatique Lyonnais (PBIL) server (https://prabi.ibcp.fr/htm/index.php). The presumed 3D protein structural model of SsCTSS was established using protein homology/analogy recognition engine V 2.0 (Phyre2) (http://www.sbg.bio.ic.ac.uk/phyre2/html/page.cgi?id=index).

Table 1
PCR primers used in this study.

Primers	Sequences (5′– 3′)	Target genes
EF1AF1	5′- AACCTGACCACTGAGGTGAAGTCTG-3′	*EF1A*
EF1AR1	5′- TCCTTGACGGACACGTTCTTGATGTT-3′	
*SsCS*RTF1	5′-GCAGCAGCCGCTTTTGA-3′	*SsCS*
*SsCS*RTR1	5′-GCTTCCCCGTTGTCTTGG-3′	
*SsCS*F2	5′-CAGCAGTCTTATGCCACTCTCA-3′	*SsCS*
*SsCS*R2	5′-CTACATGATGGGGTAGCACGAAT-3′	

2.5. Quantitative real time reverse transcription-PCR (RT-qPCR) analysis of SsCTSS expression

2.5.1. SsCTSS expression in fish tissues under normal physiological conditions

Blood, liver, gills, spleen, kidney, heart, muscle, brain, and intestine were taken aseptically from five fishes 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 Superscript II reverse transcriptase (Invitrogen, Carlsbad, CA, USA). RT-qPCR was carried out in a LightCycler 96 system (Roche Applied Science, North Carolina, USA) using the SYBR ExScript RT-qPCR Kit (Takara, Dalian, China). The primers used to amplify *SsCTSS* are SsCSRTF1/SsCSRTR1 (Table 1). Melting curve analysis was carried out at the end of each PCR to confirm the specificity of PCR products. The black rockfish elongation factor 1α (SsEF1A) gene was used as an internal control (GenBank accession no: MN218774), which was previously proved as an appropriate internal control for RT-qPCR normalization [33]. The expression level of *SsCTSS* were analyzed using comparative threshold cycle method ($2^{-\Delta\Delta CT}$). The primers used to amplify *SsEF1A* are SsEF1AF1/SsEF1AR1 (Table 1). All data are given in terms of relative mRNA levels to that of tissue in which *SsCTSS* expression was the lowest.

2.5.2. SsCTSS expression in fish tissues in response to bacterial infection

To examine *SsCTSS* expression in response to bacterial infection, *V. anguillarum* was cultured as above and resuspended in PBS to 1×10^7 colony forming units (CFU)/ml. Black rockfish were divided randomly into two groups (40 fish/group), and injected intraperitoneally (i.p.) with 100 μl of *V. anguillarum* or PBS (control). Fish were sacrificed at 4 h, 8 h, 12 h, 24 h, 48 h and 72 h post-infection (hpi), then kidney, liver, and spleen were taken under aseptic conditions and used for RT-qPCR analysis as described above.

2.6. Plasmid construction and protein purification

To construct the plasmid that expresses SsCTSS, the coding sequence without signal peptide and inhibitor domain of cathepsin S was amplified with PCR primers SsCSF2/SsCSR2, (Table 1). The PCR products were cloned into the expression vector pEASY-Blunt E1 (Transgen). The His-tagged recombinant plasmid (pBCS) were verified by sequencing and transformed into the competent cells Transetta (DE3) (Transgen). The positive transformants were cultured at 20 °C in LB broth containing ampicillin (50 mg/ml) until OD_{600} reached about 0.8. Then isopropyl β-D-thiogalactoside (IPTG) was added to the LB medium at a final concentration of 0.5 mM. After further incubation at 20 °C for 18 h, the bacterial cells were harvested and resuspended in 5 ml binding buffer (20 mM sodium phosphate, 500 mM NaCl, 20 mM imidazole, pH 7.4) for sonication. The recombinant fusion protein, His-tagged rSsCTSS, were purified under native condition using nickel nitrilotriacetic acid columns (GE Healthcare, Piscataway, NJ, USA) according to the manufacturer's instructions. The purified protein was dialyzed for 24 h against PBS at 4 °C. The eluted samples were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The concentration of the protein was measured with the Bradford method using bovine serum albumin (BSA) as the standard.

2.7. Proteolytic activity assay

Proteolytic assay was conducted as previously reported with modifications as follows. The assay was performed in a reaction solution of 0.1 M citrate-phosphate buffer (McIlvaine buffer, pH 7.5), 1 mM EDTA, 0.025% BRIJ, and 4 mM dithiothreitol (DTT). rSsCTSS of different concentrations in the reaction solution with or without 10 μM cathepsin inhibitor E − 64 (Sigma–Aldrich, USA) were added with 1 mM cathepsin S substrate Z-Phe-Arg-p-nitro-anilide (Enzo Life Sciences, Switzerland). For the control sample, the reaction solution along was added with 1 mM substrate. After incubation at 30 °C for 2 h, OD_{410} was measured. The effects of temperature was measured as described above except that 1 M of protein was incubated at various temperature as indicated.

2.8. Interaction of rSsCTSS with PAMPs

Interaction of rSsCTSS with PAMPs was determined by enzyme-linked immunosorbent assay (ELISA) as follows: briefly, LPS or PGN (Sigma) was dissolved at a concentration of 50 μg/μl in 50 mM sodium bicarbonate (pH 9.8). One hundred microliter of LPS or PGN solution was added to a 96-well ELISA plate (Sangon, Shanghai, China), and coating with 0.05% (w/v) poly-lysine. After fixing the cells with 0.05% (v/v) gluteraldehyde and blocking with 1% (w/v) bovine serum albumin, the cells were reacted with 0.25 mM rSsCTSS or PBS (control) in PBS buffer at 4 °C for 4 h, then treated with mouse anti-His antibody (Tiangen, Beijing, China) for 2 h at room temperature. After washing three times with PBS, horse-radish peroxidase-conjugated goat anti-mouse IgG (Tiangen, Beijing, China) was added to the plates. Color development was performed using the TMB Kit (Tiangen, Beijing, China). The plates were read at 450 nm with a Precision Microplate Reader (Molecular Devices, Toronto, Canada). Positive readings were defined as at least twice of that of the control.

2.9. Binding assay with bacteria

The purified rSsCTSS (1 mg/ml) and rSmSOCS3 (1 mg/ml, a negative control) [34] were labeled with fluorescein isothiocyanate (FITC) using a ReadiLink™ Antibody Labeling Kit (Solarbio) according to the manufacturer's instructions. FITC-labeled rSsCTSS (10 μl) was mixed with 30 μl of *V. anguillarum* (5×10^7 CFU/ml), *E. coli* (5×10^7 CFU/ml), and *S. aureus* (5×10^7 CFU/ml), consecutively, and incubated at room temperature for 1 h. The microbes were washed three times with 1.0 ml of 10 mM PBS (pH 7.4), harvested by centrifugation at 5000 × *g* at room temperature for 1 min, and resuspended in 1.0 ml of 10 mM PBS (pH 7.4). Aliquots (10 μl) of the microbial suspensions were applied to microscope slides and observed under a LEICA DM2500 imager fluorescence microscope. The same concentration of rSmSOCS3 was used as a negative control.

3. RNA interference

The small interfering (si) RNA of *SsCTSS* was synthesized by Ribobio (Guangzhou, China) and named *SsCTSS*-Ri (5′- AGGCAAATATGGCAA CAAAdTdT-3′). The control siRNA (named *SsCTSS*-RiC) was provided by the company, and its sequence was not disclosed to customers.

The *in vivo* interfering effect of *SsCTSS*-Ri was determined by a method reported previously [35]. *V. anguillarum* was cultured in LB broth at 28 °C to an optical density at 600 nm (OD_{600}) of 0.8. Then, the cells were washed with phosphate-buffered saline (PBS) and re-suspended in PBS to a concentration of 1×10^5 CFU/ml. Healthy black rockfish were randomly divided into two groups (15 fishes per group) named A and B. Groups A and B were injected i.p. with *SsCTSS*-Ri and *SsCTSS*-RiC (10 μg/fish) respectively. At 24 h post-injection, the fish were injected intraperitoneally with 1×10^5 CFU of *V. anguillarum*. The spleen was collected aseptically from the fish at 12 and 24 h post-infection. To examine bacterial loads in fish, the tissues were homogenized in PBS, and the homogenates were diluted in PBS and plated in triplicate on LB agar plates. The plates were incubated at 28 °C for 48 h, and the colonies that emerged on the plates were counted.

3.1. Statistical analysis

All statistical analyses were performed with SPSS 17.0 software (SPSS Inc., Chicago, IL, USA). Data were analyzed with analysis of variance (ANOVA), and statistical significance was defined as $P < 0.05$.

3.2. Ethics statement

The experiments involving live animals were conducted in accordance with the “Regulations for the Administration of Affairs Concerning Experimental Animals” promulgated by the State Science and Technology Commission of Shandong Province. The study was approved by the ethics committee of Qingdao Agricultural University.

4. Results

4.1. Cloning and sequence analysis of SsCTSS

A cathepsin S homologue (named SsCTSS) was obtained from the cDNA library of black rockfish. The full-length cDNA of *SsCTSS* (GenBank accession no: MN218774) contains a 5′-UTR of 26 bp, an open reading frame (ORF) of 1014 bp, and a 3′-UTR of 302 bp (Fig. S1). The 3′-UTR is followed by a putative polyadenylation signal AATAAA, which lies 20 bp upstream from the poly-A tail. The ORF encodes a putative protein of 337 amino acid residues with a predicted molecular mass of 37.1 kDa and a theoretical isoelectric point of 6.16. Similarly, three domains, signal peptide (1–25), I29 domain (34–94), and Pept_C1 domain (122–336), were found in SsCTSS (Fig. S1). In the mature peptide, three conserve motifs including the cysteine active site (QGSCGSCWFSA$^{140-150}$), histidine active site (VNHGVLAVGYG$^{282-292}$) and asparagine active site (YWLVKNSWG$^{299-307}$) were identified. Amino acid sequence alignment indicated that SsCTSS shares high overall identities (84–88%) with cathepsin S homologues of *Oplegnathus fasciatus*, *Epinephelus coioides*, *Dicentrarchus labrax*, *Lutjanus argentimaculatus*, *Scophthalmus maximus*, *Miichthys miiuy*, and share moderate overall identities (62-58%) with cathepsin S homologues of *Homo sapiens,* and *Mus musculus* (Fig. 1A). SsCTSS also harbors the ERF/WNIN motif in its propeptide regions.

The secondary structure analyses showed SsCTSS possesses 3 β-sheets, 3 beta hairpins,5 beta bulges, 11 strands, 15 helices, 13 helix-helix interacs, 24 beta turns, 6 gamma turns, 3 disulphides and other structures (Fig. S2). The tertiary structure predicted by Phyre2 showed SsCTSS was 91% identical to c2c0yA with 100% confidence, and the image was colored by rainbow from N to C terminus (Fig. S3).

4.2. Phylogenetic analysis of CTSS

To further verify the gene identification, and determine the phylogenetic relationship of SsCTSS gene with those of other species, a neighborjoining phylogenetic tree was constructed by MEGA7. Phylogenetic analyses showed that the higher vertebrates and the lower vertebrates (teleost) CTSS fall into two distinct clusters. SsCTSS was placed in the teleost cluster, within which SsCTSS formed a subcluster with the CTSS of several fish species (Fig. 1B).

4.3. Expression of SsCTSS under normal physiological conditions

RT-qPCR was carried out to examine the expression profile of *SsCTSS* in the blood, brain, gills, heart, intestine, kidney, liver, muscle, spleen of black rockfish. The results showed that *SsCTSS* expression was detected in all the examined tissues, with high levels found in brain,

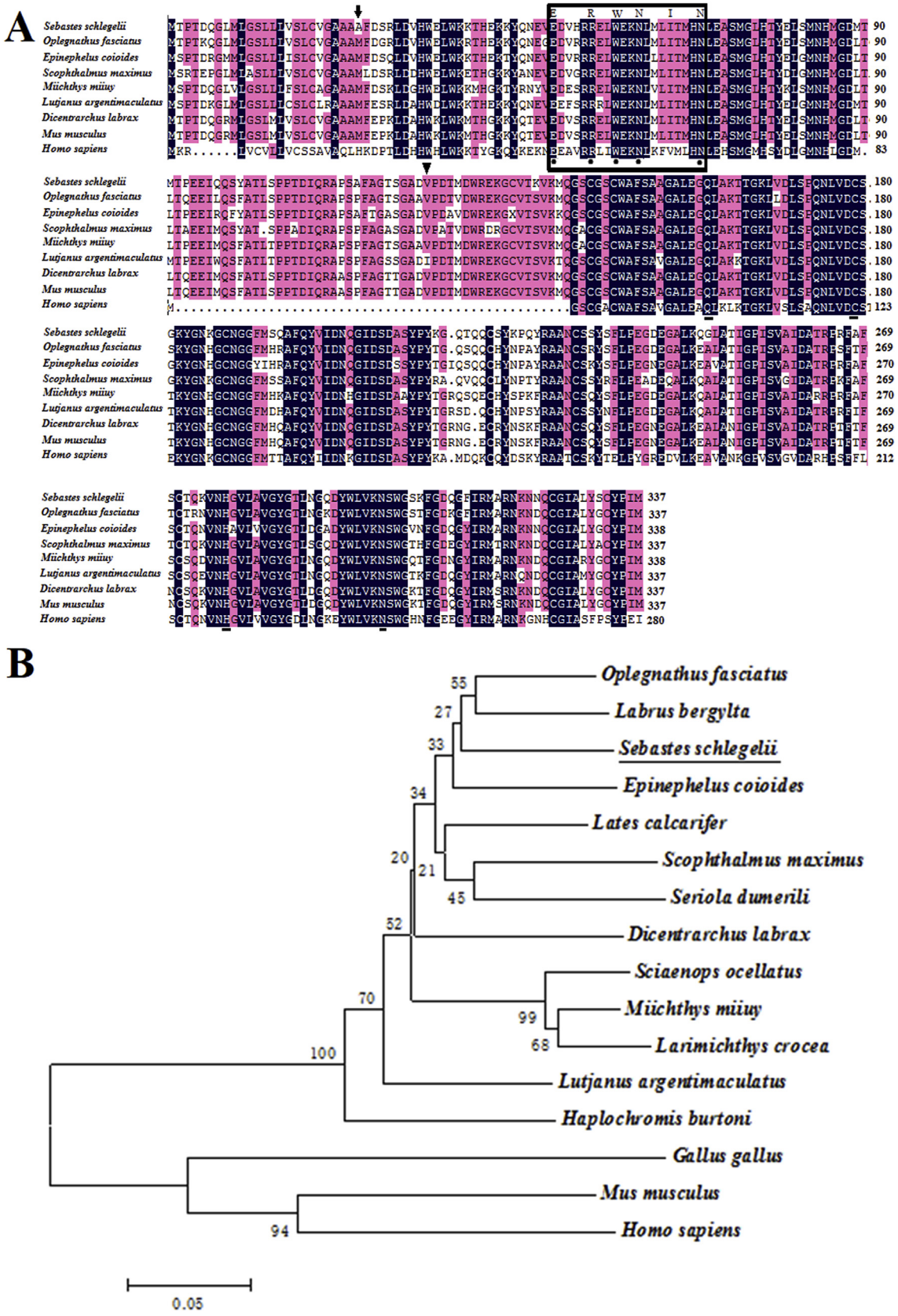

Fig. 1. Homology analysis and phylogenetic analysis of CTSS. A, alignment of the deduced amino acid sequences of CTSS homologues. Dots denote gaps introduced for maximum matching. The consensus residues are in black, the residues that are ≥75% identical among the aligned sequences are in pink. The ERF/WNIN motifs are boxed. The Gen Bank accession numbers of the aligned sequences are as follows. *Dicentrarchus labrax*, CBJ56264.1; *Epinephelus coioides*, APG79660.1; *Homo sapiens*, NP_001186668.1; *Lutjanus argentimaculatus*, ACO82388.1; *Miichthys miiuy*, ADP55137.1; *Mus musculus*, XP_006501038.2; *Oplegnathus fasciatus*, BAK55650.1; *Scophthalmus maximus*, ARR29132.1. B, phylogenetic analysis of SsCTSS. The phylogenetic trees were constructed with MEGA 7.0 software (http://www.megasoftware.net/) using the neighbor-joining method. SsCTSS was marked by underline. Numbers beside the internal branches indicate bootstrap values based on 1000 replications. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

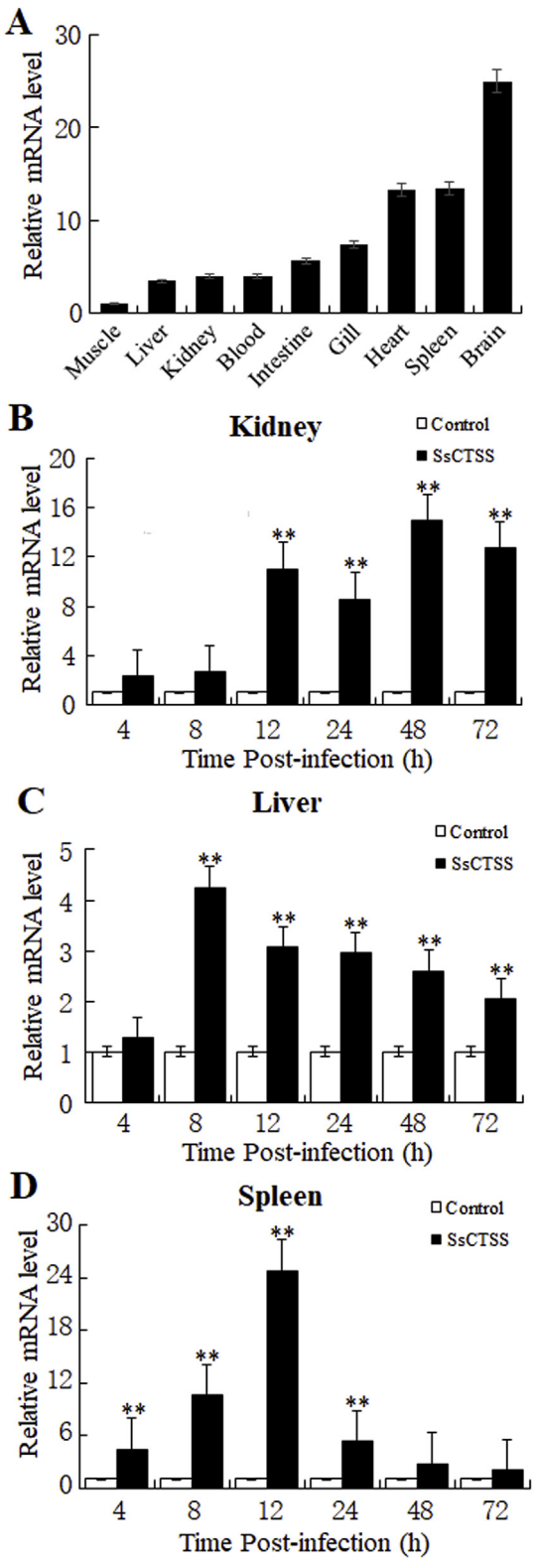

Fig. 2. *SsCTSS* expression under normal physiological conditions and pathogen infection conditions. A, *SsCTSS* expression in different tissues under normal physiological conditions. The expression of *SsCTSS* in the blood, brain, muscle, kidney, spleen, intestine, heart, gills and liver of rock blackfish was determined by quantitative real time RT-PCR (RT-qPCR). The expression level of *SsCTSS* in muscle was respectively set as 1. B, *SsCTSS* expression in immune-related tissues in response to bacterial challenge. Black rockfish were infected with *Vibrio anguillarum* or PBS (control), then *SsCTSS* expression in kidney, liver, and spleen were determined by RT-qPCR at 4 h, 8 h, 12 h, 24 h, 48 h, and 72 h after infection. In each case, the expression level of the control fish was set as 1. Values are shown as means ± SEM (N = 3). N, the number of times the experiment was performed. **, $P < 0.01$; *, $P < 0.05$.

and low levels in muscle (Fig. 2A).

4.4. Expression of SsCTSS in response to bacterial infection

To examine whether *SsCTSS* expressions were modulated by bacterial infection, black rockfish were experimentally challenged with the fish pathogen *V. anguillarum*, and *SsCTSS* expressions in kidney, liver, and spleen were analyzed by RT-qPCR at 4, 8, 12, 24, 48, and 72 hpi. The results showed that the expression levels of *SsCTSS* in kidney were significantly upregulated after *V. anguillarum* infection at 12, 24, 48 and 72 hpi, with the highest expression level at 48 hpi (14.9-fold) (Fig. 2B). *SsCTSS* expression in spleen was significantly upregulated at 4, 8, 12, 24, and 48 hpi, with the highest expression level at 12 hpi (26.5-fold) (Fig. 2D). However, the expression of *SsCTSS* in liver was upregulated only at 8 and 12 hpi, with the highest expression level at 8 hpi (4.2-fold) (Fig. 2C). These results indicated that *SsCTSS* could be involved in anti-bacterial infection.

4.5. Proteolytic activity of rSsCTSS

rSsCTSS were expressed and purified from *E. coli* and were used to examine for proteolytic activity. The purified proteins displayed a single bind on SDS-PAGE with molecular masses comparable to the predicted molecular weight of SsCTSS (Fig. S4). Proteolytic activity assay revealed that rSsCTSS were able to hydrolyze substrate Z-Phe-Arg-p-nitro-anilide in a dose-dependent manner. The activity of rSsCTSS were both remarkably reduced in the presence of cysteine protease inhibitor E−64 (Fig. 3A). To measure the effect of temperature on the activity of rSsCTSS, the proteolytic assay was conducted under various temperature. The results showed that rSsCTSS displayed increased activity as the temperature rose from 25 °C to 50 °C, and decreased activity as the temperature rose from 50 °C to 60 °C, and the optimal temperature of rSsCTSS was 50 °C (Fig. 3B).

4.6. Binding and gathering activity of rSsCTSS

To characterize the PAMP recognition capacity of rSsCTSS, ELISA was used to measure the binding of rSsCTSS to LPS and PGN, the major constituents of the outer membranes of Gram-negative and Gram-positive bacteria, respectively. The results showed that compared with control, rSsCTSS exhibited apparent binding abilities to LPS and PGN. The binding activity of rSsCTSS to both compounds increased with raising concentrations of rSsCTSS (from 20 to 160 mg/ml) (Fig. 4).

To inspect the binding ability of rSsCTSS to bacteria, rSsCTSS was labeled by FITC and incubated with Gram-negative bacteria (*E. coli*, *V. anguillarum*) and Gram-positive bacteria (*S. aureus*), then green fluorescence was detected by microscope. As showed in Fig. 5, rSsCTSS was able to bind to all the three bacteria. Moreover, rSsCTSS exhibited obvious agglutination effect to *E. coli* and mild agglutination effect of *S. aureus*. However, rSmSOCS3, a control protein, did not exhibit any binding and gathering effect to three bacteria (Fig. 5).

4.7. Knockdown of SsCTSS and its antibacterial effects

Since, as observed above, SsCTSS plays a role in anti-bacterial immune, we further examined the effect of *SsCTSS* knockdown on bacterial invasion. For this purpose, *SsCTSS*-Ri and *SsCTSS*-RiC (RNAi control) were synthetized and injected into fish. The expression of *SsCTSS* was determined by RT-qPCR and the results showed that in *SsCTSS*-Ri-administered fish, the expression *SsCTSS* was significantly

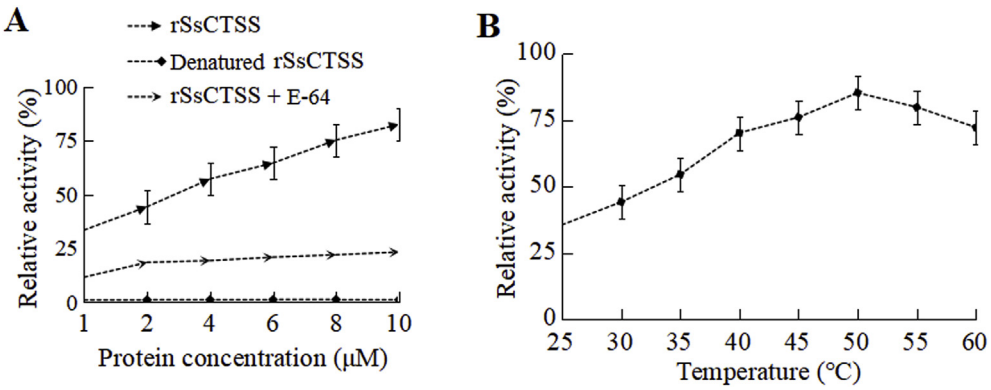

Fig. 3. Proteolytic activity of rSsCTSS. A, Cathepsin substrate Z-Phe-Arg-p-nitro-anilide was incubated with recombinant cathepsins or with denatured cathepsins or with recombinant cathepsins including cathepsin inhibitor E−64. The activity was determined by measuring absorbance at OD_{410} and expressed as percentages of the maximum activity of rSsCTSS. B, the effects of temperature on the activity of rSsCTSS were determined against Z-Phe-Arg-p-nitro-anilide. Data represent means ± SD (N = 3). N, the number of times the experiment was performed.

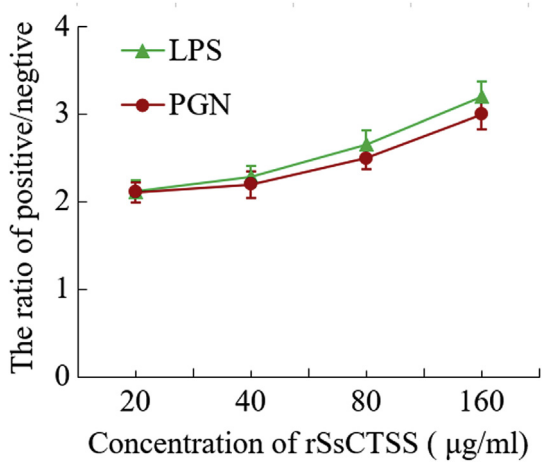

Fig. 4. Binding of rSsCTSS to LPS and PGN. LPS or PGN was coated in 96-well plate and then incubated with different concentrations of rSsCTSS. The binding of rSsCTSS to LPS and PGN was determined by ELISA.

reduced compared to that in the control (Fig. 6A). To examine the effect of *SsCTSS* knockdown on bacterial infection, fish treated with *SsCTSS*-Ri or *SsCTSS*-RiC were infected with *V. anguillarum*, and bacterial numbers were determined at 12 and 24 h post-infection. The results showed that cells administered with *SsCTSS*-Ri exhibited significantly increased bacterial amounts compared to control (Fig. 6B).

5. Discussion

In this study, we identified and analyzed cathepsin S, SsCTSS, from black rockfish, and examined its expression and biological property. The deduced amino acid sequences of SsCTSS exhibited a typical cathepsin domain architecture, including a putative signal peptide followed by a propeptide region and a papain family cysteine protease domain. Three conservative catalytic activity regions, a cysteine active site, a histidine active site, and an asparagine active site [36], were existed in SsCTSS. The characteristic ERWNIN motif of endopeptidase cathepsins was also found in the propeptide regions of SsCTSS. rSsCTSS was able to hydrolyzea substrate Z-Phe-Arg-p-nitro-anilide in a dose-dependent manner, indicating that they are functional orthologs of mammalian cathepsins. This temperature-dependent activity profile was similar to those of cathepsin L-like proteases in some teleost fish, such as *S. ocellatus* [26], *C. carpio* [37], and *E. japonica* [38]. Sequence alignment revealed that the teleost CTSS homologues are highly conserved and that the amino acid sequence of SsCTSS shared high overall sequence identity with other teleost CTSS homologues. Phylogenetic analysis indicated that SsCTSS was close with the CTSS from *Lates calcarifer* and *O. fasciatus*. The high sequence identity, together with the phylogenetic analysis and the structural features, indicated that SsCTSS

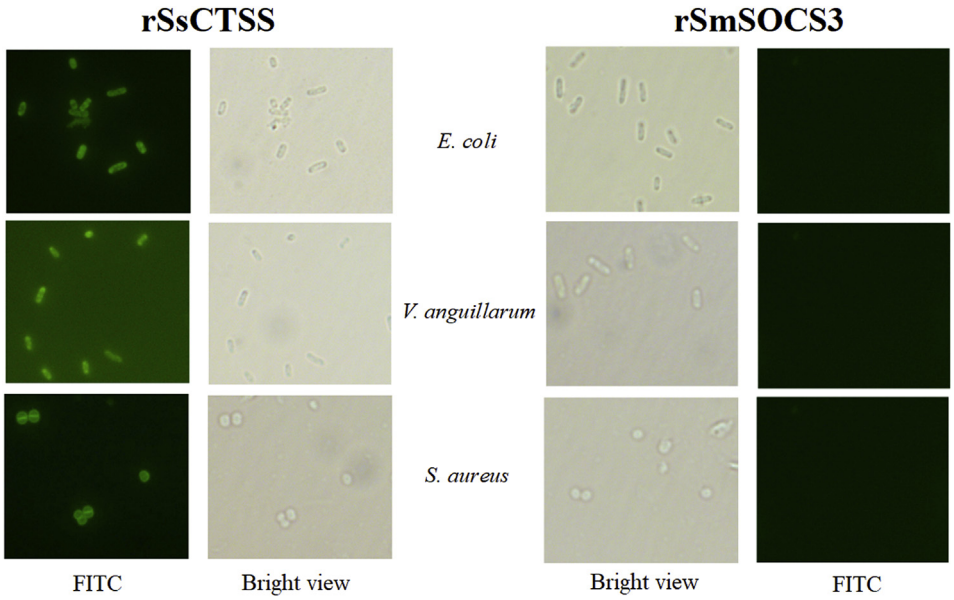

Fig. 5. Binding and gathering activity of rSsCTSS to bacteria. rSsCTSS was labeled by FITC and incubated with *Escherichia coli*, *Vibrio anguillarum* and *Staphylococcus aureus*. Bacteria was washed three times and detected under a LEICA DM2500 imager fluorescence microscope. rSmSOCS3, as control protein.

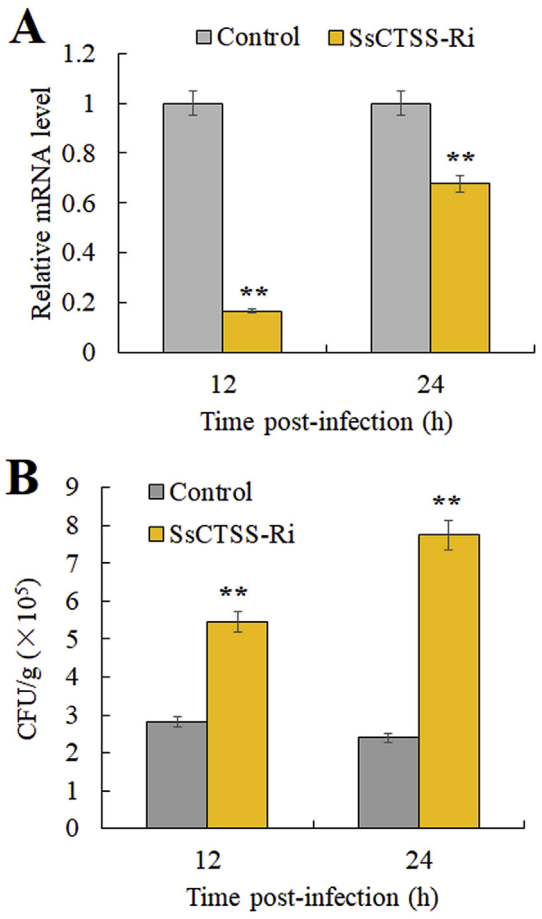

Fig. 6. Effect of *SsCTSS* knockdown on bacterial infection. *SsCTSS*-Ri and *SsCTSS*-RiC (RNAi control) were synthetized and injected into rock blackfish, the expression of *SsCTSS* in spleen was determined by RT-qPCR (A). After injecting for 24 h, fish were infected with *Vibrio anguillarum*, and the amounts of bacteria in spleen were determined at 12 and 24 h post-infection (B). Values are shown as means ± SEM (N = 3). N, the number of times the experiment was performed. **, $P < 0.01$.

is a new member of the vertebrate CTSS subfamily.

In mammals, CTSS is implicated in major histocompatibility complex class II antigen presentation. Thereinto, CTSS is mostly expressed in immune cells, such as macrophages, such as dendritic cells and B cells [16,39]. In teleost, CTSS expression was detected in multiple tissues [22,24,26], which is consistent with our observations. In *S. ocellatus*, cathepsin S expression was highest in brain [26]. Similarly, in our study, the organization with the highest expression of SsCTSS was brain, followed by spleen, which imply a possible involvement of SsCTSS during angiogenesis and immune defense in black rockfish.

It is well known that CTSS play important roles in innate immunity, protecting the host against pathogen infection [17,19,40]. Previous studies in teleost showed that CTSS were significantly induced after stimulation with pathogens. For example, expression of CTSS in red drum was significantly induced by *Edwardsiella tarda* infection [26]. In other several species, such as *P. olivaceus* [21], *O. fasciatus* [22], and *L. argentimaculatus* [24], the expression of cathepsin S homologues were elevated by bacterial infection or LPS stimulations. Consistent with these results, in our present study, RT-qPCR analysis showed that when black rockfish were challenged with the pathogen *V. anguillarum*, *SsCTSS* expressions in the head kidney, liver, and spleen were significantly enhanced in a manner that depended on tissue type and the infection stage. In two primary immune organs, head kidney and spleen, expression level induced by pathogen infection are higher and duration are longer, which points to an implication of SsCTSS in immune response of black rockfish to bacterial infection.

To further understand the immunological roles of SsCTSS, we determined the binding ability of rSsCTSS to microbial ligands. The experimental results showed that rSsCTSS exhibited strong effect with LPS and PGN, which are the major constituents of the outer membranes of Gram-negative and Gram-positive bacteria, respectively. Moreover, SsCTSS possessed the capability of gathering bacteria. Similar results were reported in turbot cathepsin Z [41]. Up to now, reports about exploration to immunological roles of CTSS are very limit. Other cathepsin, such as cathepsin C, was recently reported that its overexpression in vitro significantly delayed the cytopathic effect progression evoked by SGIV and inhibited the viral genes transcription [42]. In this study, knockdown of *SsCTSS* was conducted and the results showed that following exposure to *V. anguillarum* infection, fish with *SsCTSS* knockdown displayed more bacteria than the control fish, which indicated that played a functional role in anti-bacterial infection.

In conclusion, in this study we identified and characterized a cysteine cathepsin, SsCTSS, from black rockfish. We provide an insight into the functionality of teleost CTSS by showing that expression of SsCTSS is required for responding to bacterial infection, and exhibits apparent proteolytic activity to substrate Z-Phe-Arg-p-nitro-anilide. CTSS not only possessed the capability of binding and gathering with bacteria, but also involved in anti-bacterial infection. These observations suggest that a potential involvement of SsCTSS as a bioactive protease in antibacterial immunity of black rockfish.

Acknowledgments

This work was supported by the National Natural Science Foundation of China (grants no. 31572652 and 31872607), the Central Public-interest Scientific Institution Basal Research Fund for Chinese Academy of Tropical Agricultural Sciences (No. 1630052018001, 1630052018005), and the “First class fishery discipline” programme in Shandong Province, China.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fsi.2019.08.012.

References

[1] T.S. Uinuk-ool, T.N. Kuroda, F. Figueroa, A. Sato, I.E. Samonte, W.E. Mayer, et al., Phylogeny of antigen-processing enzyme: cathepsins of a cephalochordate, an agnathan and a bony fish, Scand. J. Immunol. 58 (2003) 436–448.
[2] D.B. Guiliano, X. Hong, J.H. McKerrow, M.L. Blaxter, Y. Oksov, J. Liu, et al., A gene family of cathepsin L-like proteases of filarial nematodes are associated with larval molting and cuticle and eggshell remodeling, Mol. Biochem. Parasitol. 136 (2004) 227–242.
[3] L.C. Hsing, A.Y. Rudensky, The lysosomal cysteine proteases in MHC class II antigen presentation, Immunol. Rev. 207 (2005) 229–241.
[4] A.K. Dixit, P. Dixit, R.L. Sharma, Immunodiagnostic/protective role of Cathepsin L cysteine proteinases secreted by Fasciola species, Vet. Parasitol. 154 (2008) 177–184.
[5] U. Repnik, V. Stoka, V. Turk, B. Turk, Lysosomes and lysosomal cathepsins in cell death, Bba-Proteins Proteom 1824 (2012) 22–33.
[6] S.N. Wei, Y.B. Huang, X.H. Huang, J. Cai, Y. Yan, C.Y. Guo, et al., Characterization of cathepsin B gene from orange-spotted grouper, *Epinephelus coioides* involved in SGIV infection, Fish Shellfish Immunol. 36 (2014) 194–205.
[7] L.S. Dai, Y. Sun, Y.X. Sun, B.J. Zhu, C.L. Liu, Characterization and function of a cathepsin B in red crayfish (Procambarus clarkii) following lipopolysaccharide challenge, Fish Shellfish Immunol. 56 (2016) 162–168.
[8] V. Turk, V. Stoka, O. Vasiljeva, M. Renko, T. Sun, B. Turk, et al., Cysteine cathepsins: from structure, function and regulation to new frontiers, Bba-Proteins Proteom 1824 (2012) 68–88.
[9] J.D. Colbert, S.P. Matthews, G. Miller, C. Watts, Diverse regulatory roles for lysosomal proteases in the immune response, Eur. J. Immunol. 39 (2009) 2955e65.
[10] S. Turk, B. Turk, V. Turk, Papain-like lysosomal cysteine proteases and their inhibitors: drug discovery targets? Biochem. Soc. Symp. 70 (2003) 15–30.
[11] K.M. Karrer, S.L. Peiffer, M.E. Ditomas, Two distinct gene subfamilies within the family of cysteine protease genes, Proc. Natl. Acad. Sci. U. S. A 90 (1993) 3063–3067.
[12] A. Rossi, Q. Deveraux, B. Turk, A. Sali, Comprehensive search for cysteine

cathepsins in the human genome, Biol. Chem. 385 (2004) 363e372.
[13] D. Bromme, A. Steinert, S. Friebe, S. Fittkau, B. Wiederanders, H. Kirschke, The specificity of bovine spleen cathepsin S. A comparison with rat liver cathepsins L and B, Biochem. J. 264 (1989) 475–481.
[14] G.P. Shi, J.S. Munger, J.P. Meara, D.H. Rich, H.A. Chapman, Molecular cloning and expression of human alveolar macrophage cathepsin S, an elastinolytic cysteine protease, J. Biol. Chem. 267 (1992) 7258e7262.
[15] S. Conus, H.U. Simon, Cathepsins and their involvement in immune responses, Swiss Med. Wkly. Times 20 (2010) 140: w13042.
[16] A. Rudensky, C. Beers, Lysosomal cysteine proteases and antigen presentation, Ernst Schering Res. Found. Workshop 56 (2006) 81–95.
[17] F. Matsumoto, S.I. Saitoh, R. Fukui, T. Kobayashi, N. Tammura, K. Konnio, et al., Cathepsins are required for Toll-like receptor 9 responses, Biochem. Biophys. Res. Commun. 367 (2008) 693–699.
[18] S. Memmert, A. Damanaki, A.V.B. Nogueira, S. Eick, M. Nokhbehsaim, A.K. Papadopoulou, et al., Role of Cathepsin S in periodontal inflammation and infection, Mediat. Inflamm. (2017) 4786170.
[19] K. Honey, M. Duff, C. Beers, W.H. Brissette, E.A. Elliott, C. Peters, et al., Cathepsin S regulates the expression of cathepsin L and the turnover of gamma-interferon-inducible lysosomal thiol reductase in B lymphocytes, J. Biol. Chem. 276 (2001) 22573–22578.
[20] M. Fabra, J. Cerda, Ovarian cysteine proteinases in the teleost *Fundulus heteroclitus*: molecular cloning and gene expression during vitellogenesis and oocyte maturation, Mol. Reprod. Dev. 67 (2004) 282–294.
[21] N.Y. Kim, S.J. Ahn, A.R. Lee, J.S. Seo, M.S. Kim, J.K. Kim, et al., Cloning, expression analysis and enzymatic characterization of cathepsin S from olive flounder (*Paralichthys olivaceus*), Comp. Biochem. Physiol. B 157 (2010) 238–247.
[22] J.W. Kim, J.M. Jeong, H.J. Park, E.G. Kim, H.N. Kim, Y.S. Chae, et al., Molecular identification and expression analysis of cathepsins O and S from rock bream, *Oplegnathus fasciatus*, Fish Shellfish Immunol. 31 (2011) 578–587.
[23] Y.N. Sun, T.J. Xu, J.X. Wang, Y.Z. Cheng, R.X. Wang, Sequence and expression analysis of cathepsin S gene in the miiuy croaker *Miichthys miiuy*, Fish Physiol. Biochem. 37 (2011) 761–765.
[24] J. Zhou, L. Li, Z.H. Cai, Identification of putative cathepsin S in mangrove red snapper *Lutjanus argentimaculatus* and its role in antigen presentation, Dev. Comp. Immunol. 37 (2012) 28–38.
[25] Q. Li, J. Ao, Y. Mu, Z. Yang, T. Li, X. Zhang, et al., Cathepsin S, but not cathepsin L, participates in the MHC class II-associated invariant chain processing in large yellow croaker (*Larimichthys crocea*), Fish Shellfish Immunol. 47 (2015) 743–750.
[26] B.G. Sun, H. Chi, Cathepsin S of *Sciaenops ocellatus*: identification, transcriptional expression and enzymatic activity, Int. J. Biol. Macromol. 82 (2016) 76–82.
[27] X. Dong, Z. Ye, L. Song, B. Su, H. Zhao, E. Peatman, et al., Expression profile analysis of two cathepsin S in channel catfish (*Ictalurus punctatus*) mucosal tissues following bacterial challenge, Fish Shellfish Immunol. 48 (2016) 112–118.
[28] Y. Wang, X. Liu, S. Lv, J. Ren, F. Ke, Identification and activity of a paralog of cathepsin S from yellow catfish (*Pelteobagrus fulvidraco*) involved in immune response, Fish Shellfish Immunol. 61 (2017) 16–23.
[29] H.J. Han, D.Y. Kim, W.S. Kim, C.S. Kim, S.J. Jung, M.J. Oh, et al., A typical *Aeromonas salmonicida* infection in the black rockfish, *Sebastes schlegeli Hilgendorf*, in Korea, J. Fish Dis. 34 (2011) 47–55.
[30] M. Zhang, L. Sun, The tissue factor pathway inhibitor 1 of *Sciaenops ocellatus* possesses antimicrobial activity and is involved in the immune response against bacterial infection, Dev. Comp. Immunol. 35 (2011) 247–252.
[31] X. Fu, N. Li, Y. Lai, X. Luo, Y. Wang, C. Shi, et al., A novel fish cell line derived from the brain of Chinese perch *Siniperca chuatsi*: development and characterization, J. Fish Biol. 86 (2015) 32–45.
[32] X.P. Zhao, Y.H. Hu, Y. Liu, J.J. Wang, G.H. Wang, R.J. Wang, A high-mobility group box 1 that binds to DNA, enhances pro-inflammatory activity, and acts as an anti-infection molecule in black rockfish, *Sebastes schlegelii*, Fish Shellfish Immunol. 56 (2016) 402–409.
[33] L. Ma, W. Wang, C. Liu, H. Yu, Z. Wang, X. Wang, et al., Selection of reference genes for reverse transcription quantitative real-time PCR normalization in black rockfish (*Sebastes schlegeli*), Mar. Genomics 11 (2013) 67–73.
[34] M. Zhang, Z.Z. Xiao, L. Sun, Suppressor of cytokine signaling 3 inhibits head kidney macrophage activation and cytokine expression in *Scophthalmus maximus*, Dev. Comp. Immunol. 35 (2011) 174–181.
[35] W. Zhang, W. Liang, C. Li, Inhibition of marine Vibrio sp. by pyoverdine from *Pseudomonas aeruginosa* PA1, J. Hazard Mater. 302 (2016) 217–224.
[36] F. Lecaille, J. Kaleta, D. Br€omme, Human and parasitic papain-like cysteine proteases: their role in physiology and pathology and recent developments in inhibitor design, Chem. Rev. 102 (2002) 4459e4488.
[37] F. Aranishi, H. Ogata, K. Hara, K. Osatomi, T. Ishihara, Purification and characterization of cathepsin L from hepatopancreas of carp *Cyprinus carpio*, Comp. Biochem. Physiol. B118 (1997) 531–537.
[38] M.S. Heu, H.R. Kim, D.M. Cho, J.S. Godber, J.H. Pyeun, Purification and characterization of cathepsin L-like enzyme from the muscle of anchovy, *Engraulis japonica*, Comp. Biochem. Physiol. B118 (1997) 523–529.
[39] M. Perišić Nanut, J. Sabotič, A. Jewett, J. Kos, Cysteine cathepsins as regulators of the cytotoxicity of NK and T cells, Front. Immunol. 2 (2014) 616.
[40] B. Turk, D. Turk, V. Turk, Lysosomal cysteine proteases: more than scavengers, Biochim. Biophys. Acta 1477 (2000) 98–111.
[41] X. Cai, C. Gao, H. Song, N. Yang, Q. Fu, F. Tan, et al., Characterization, expression profiling and functional characterization of cathepsin Z (CTSZ) in turbot (*Scophthalmus maximus L.*), Fish Shellfish Immunol. 84 (2019) 599–608.
[42] S. Wei, S. Wang, M. Yang, Y. Huang, J. Wei, X. Huang, et al., Characterization of cathepsin C from orange-spotted grouper, *Epinephelus coioides* involved in SGIV infection, Fish Shellfish Immunol. 84 (2019) 423–433.