Fish and Shellfish Immunology 81 (2018) 21–28

Contents lists available at ScienceDirect

Fish and Shellfish Immunology

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

Full length article

Characterization, expression signatures and microbial binding analysis of cathepsin A in turbot, *Scophthalmus maximus* L.(*Sm*CTSA)

Qiang Fu[a,1], Ning Yang[a,1], Chengbin Gao[a], Mengyu Tian[a], Shun Zhou[a], Xingjiang Mu[b], Fanyue Sun[c], Chao Li[a,*]

[a] Marine Science and Engineering College, Qingdao Agricultural University, Qingdao, 266109, China
[b] Key Laboratory of Exploration and Utilization of Aquatic Genetic Resources, Ministry of Education, College of Fisheries and Life Science, Shanghai Ocean University, Shanghai, 201306, China
[c] Center for Regenerative Medicine and Skeletal Development, Department of Reconstructive Sciences, University of Connecticut Health Center, Farmington, CT, 06030, USA

ARTICLE INFO

Keywords:
ctsa
Mucosal immunity
Turbot
Microbial ligand binding
Innate immunity

ABSTRACT

Mucosal immune system is one of the most vital components in the innate immunity and constitutes the first line of host defense against bacterial infections, especially for the teleost, which live in the pathogen-rich aquatic environment. Cathepsins, a superfamily of hydrolytic enzymes produced and enclosed within lysosomes, play multiple roles at physiological and pathological states. In this regard, we sought here to identify Cathepsin A in turbot (*Sm*CTSA), characterize its mucosal expression patterns following *Vibrio anguillarum* and *Streptococcus iniae* infections in mucosal tissues, and explore its binding ability with three microbial ligands for the first time. The *Sm*CTSA was 2631 bp long containing a 1422 bp open reading frame (ORF) that encoded 473 amino acids. Phylogenetic analysis revealed that *Sm*CTSA showed the closest relationship to half-smooth tongue sole (*Cynoglossus semilaevis*). In addition, *Sm*CTSA was ubiquitously expressed in all examined healthy tissues, with high expression levels in head kidney (HK) and intestine, while the lowest expression level in blood. Moreover, *Sm*CTSA was significantly differentially expressed at least two timepoints in each mucosal tissue, suggesting its potential important roles in innate immune responses of turbot. Finally, in vitro assays showed that recombinant *Sm*CTSA bound Lipopolysaccharide (LPS) with high affinity, and lipoteichoic acid (LTA) and peptidoglycan (PGN) with relatively low affinity. This study provides valuable data for understanding the roles of *ctsa* in the host defense against bacterial infections.

1. Introduction

The Cathepsin protein family is composed of lysosomal proteolytic enzymes that play an important role in maintaining homeostasis in organisms. In particular, these proteases are involved in intracellular protein degradation/turn over [1], hormone maturation [2], antigen processing [3] and immune responses [4]. Most of lysosomal cysteine cathepsins belong to the C1 peptidase family, which are also known as the papain family [5]. On the one hand, cathepsins can be divided into three groups: cysteine proteases (cathepsins B, C, F, H, K, L, O, S, W and X or Z), aspartic proteases (cathepsins D and E) and serine proteases (cathepsins A and G), based on conserved amino acid motifs [6]. On the other hand, based on the tissue distribution, cathepsins B, C, F, H, L, O and Z compose the ubiquitously expressed group, and the cathepsins K, W, and X, are cell- or tissue-specific expressed [7].

Cathepsin A (*ctsa*) was firstly reported in bovine spleen [8]. It is classified into the exopeptidase category and belongs to the papain family of serine protease [9]. *Ctsa* was reported to have both catalytic and protective functions [10]. In detail, *ctsa* has three distinctive hydrolytic activities for carboxypeptidase [11–13], deamidase and esterase [13,14], suggesting that extralysosomal *ctsa* may be involved in regulation of peptide hormones [15]. Besides the enzymic functions, *ctsa* also plays significant roles in protecting lysosomal β-galactosidase and neuraminidase from intralysosomal proteolysis by forming a macromolecular complex [16,17]. Furthermore, defects in the enzymatic activity of *ctsa* have been reported to cause galactosialidosis, a lysosomal storage disorder in human [16,18]. In addition, *ctsa* was also reported to exert roles in chaperone-mediated autophagy and cellular oxidative stress response [19,20]. Even though *ctsa* is a multifunctional protease, which plays vital and multiple roles especially in the

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

https://doi.org/10.1016/j.fsi.2018.07.002
Received 11 May 2018; Received in revised form 28 June 2018; Accepted 3 July 2018
Available online 04 July 2018

lysosome, few studies have reported the characterization of *ctsa* in teleost species, let alone its participations in responses to bacterial infections in teleost species.

Turbot (*Scophthalmus maximus*) is one of the most extensively maricultured species in China, but its sustainable production is constantly threatened by severe disease outbreaks. In particular, the diseases caused by *Vibrio anguillarum*, *Streptococcus iniae*, *Vibrio vulnificus*, *E. tarda*, etc., cause major economic losses to turbot industry. In order to develop diseases control and prevention strategies, many efforts have been made to identify the immune genes in turbot, as well as their immune expression patterns following bacterial infections [21–27]. Moreover, fish species depend more heavily on mucosal barriers than their terrestrial counterparts, living in the pathogen-rich aquatic environment, as their mucosal surfaces are continuously interacting with a broad range of commensals and primary pathogens [28]. The mucosal surfaces constitute the first immune barrier of fish host defense, thus a better understanding of mucosal barriers is vital for aquaculture research to improve vaccine delivery via immersion or feeding [29]. In this study, we identified and characterized a *ctsa* homolog from turbot (*Sm*CTSA). In addition, we explored its expression patterns both in healthy tissues and following different bacterial infections in mucosal tissues. Moreover, we purified the recombinant *Sm*CTSA (r*Sm*CTSA) protein and explored its binding ability with microbial ligands. To our best knowledge, the present study reported the characterization, the mucosal expression patterns of *ctsa* after bacterial infections, and the binding ability of recombinant protein in turbot for the first time.

2. Materials and methods

2.1. Sequence identification

Sequences of *ctsa* gene from other vertebrate species including channel catfish (*Ictalurus punctatus*), zebrafish (*Danio rerio*), medaka (*Oryzias latipes*), Atlantic salmon (*Salmo salar*), fugu (*Takifugu rubripes*), pike (*Esox lucius*), half-smooth tongue sole (*Cynoglossus semilaevis*), tilapia (*Oreochromis niloticus*) and large yellow croaker (*Larimichthys crocea*) were used as queries to search against our transcriptome database using TBLASTn program with a cutoff E-value of $1e^{-10}$ [30]. After that, Clustal Omega (http://www.ebi.ac.uk/Tools/msa/clustalo/) was used to eliminate duplicates in the initial retrieved pool of sequences and generate a set of unique sequences for further analysis. Then, the coding sequence were predicted using ORF (opening reading frames) finder (http://www.ncbi.nlm.nih.gov/gorf/gorf.html), which were further validated by BLAST against NCBI non-redundant (nr) protein database. The coding sequences were validated by the sequencing with the primers Sm-CTSA-ORF F/R (Table 1). The characteristic functional domains were identified by the simple modular architecture research tool (SMART; http://smart.embl-heidelberg.de/). Signal peptide sequences were further analyzed using the web-based SignalP 4.1 Server (http://www.cbs.dtu.dk/services/SignalP/) with the settings 'eukaryotes', default D-cutoff value and 'SignalP-TM' applied.

Table 1
Primers used in this study.

Primer	Sequence (5′- 3′)
CDS clone	
Sm-CTSA-ORF F	5′ ATGCAGGCGGCGGTGGT 3′
Sm-CTSA-ORF R	5′ TCAGTAAGGTTGCCCCTTGATAAAT 3′
qRT-PCR	
Sm-CTSA F	5′ CTCACTCACCTGGTCACTGT 3′
Sm-CTSA R	5′ TCAGGCCTTCGAGTGTGAAT 3′
18s RNA F	5′ ATGGCCGTTCTTAGTTGGTG 3′
18s RNA R	5′ CTCAATCTCGTGTGGCTGAA 3′
Protein expression	
Sm-CTSA-Pr F	5′ GCGGGCGACGAGGTGAC 3′
Sm-CTSA-Pr R	5′ TCAGTAAGGTTGCCCCTTGATAAAT 3′

2.2. Sequence structure analysis

The ExPASy's Prot Param server (http://web.expasy.org/protparam/) [31] and PROSITE server (http://prosite.expasy.org/) were used to characterize the physiochemical properties of *Sm*CTSA, including theoretical isoelectric point (pI), molecular weight, N-glycosylation sites, molecular formula, total number of positive and negative residues, instability index, aliphatic index and grand average hydropathicity (GRAVY). Proteins are recognized as stable with instability index smaller than 40. The aliphatic index of a protein is regarded as a positive factor for the increase of thermostability of globular proteins and is mainly defined as the relative volume occupied by aliphatic side chains (alanine, valine, isoleucine and leucine). The GRAVY score is calculated as the sum of hydropathy values of all the amino acids, divided by the number of residues in the sequence. MatGAT program was used to calculate the percentages of similarity and identity of turbot and other organisms *ctsa* gene [32]. The secondary structures were browsed on the Pole Bioinformatique Lyonnais (PBIL) server (https://prabi.ibcp.fr/htm/index.php). The presumed 3D protein structural model was established using PHYRE2 Protein Fold Recognition Server (Phyre2 server) (http://www.sbg.bio.ic.ac.uk/phyre2/html/page.cgi?id=index) [33].

2.3. Phylogenetic analysis

The unrooted phylogenetic tree was built using amino acid sequences of *Sm*CTSA and other organisms, with inclusion of human (*Homo sapiens*), mouse (*Mus musculus*), chicken (*Gallus gallus*), turtle (*Chelonia mydas*), frog (*Xenopus tropicalis*), channel catfish (*I. punctatus*), zebrafish (*D. rerio*), medaka (*O. latipes*), Atlantic salmon (*S. salar*), fugu (*T. rubripes*), pike (*E. lucius*), half-smooth tongue sole (*C. semilaevis*), tilapia (*O. niloticus*) and large yellow croaker (*L. crocea*). ClustalW2 program was used to performed the multiple protein sequence alignment [34]. The neighbor-joining method within the Molecular Evolutionary Genetics Analysis (MEGA 6) package was utilized to conduct the phylogenetic and molecular evolutionary analyses [35]. Data were analyzed using Poisson correction, and gaps were removed by complete deletion. The topological stability of the neighbor-joining tree was evaluated by 1000 bootstrapping replications.

2.4. Syntenic analysis

Syntenic analysis was conducted to better support the orthologies for *Sm*CTSA, basing on the comparison of the neighboring genes of *Sm*CTSA with stickleback (*Gasterosteus aculeatus*), human, mouse, pig (*Sus scrofa*) and chicken. Briefly, the protein sequences of neighbor genes of *Sm*CTSA were predicted from the turbot scaffold by FGENESH program [36]. The identified protein sequences were annotated against NCBI non-redundant (nr) database and UniProt Knowledgebase (UniProtKB) by BLASTP. The conserved syntenic pattern of *ctsa* gene in other species was determined in Ensembl database and Genomicus [37].

2.5. Bacterial challenge and sample collection

In order to investigate the immune roles of *Sm*CTSA in the host defense against bacterial infections, the Gram-negative bacteria *V. anguillarum* and the Gram-positive bacteria *S. iniae* were selected to conduct the bath challenge. Turbot fingerlings were obtained from turbot hatchery (Haiyang, Shandong, China) with average weight 15.6 g and length 5.5 cm. Prior to experimental challenge, the fish were acclimated in the laboratory in a flow-through system for at least one week. After a pre-challenge, the bacteria were re-isolated from symptomatic fish and biochemically confirmed before cultured. During challenge, symptomatic fish were confirmed to be infected with *V. anguillarum* and *S. iniae*, respectively. During the experiments, the fish were immersed in