Fish & Shellfish Immunology 47 (2015) 743–750

Contents lists available at ScienceDirect

Fish & Shellfish Immunology

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

Full length article

Cathepsin S, but not cathepsin L, participates in the MHC class II-associated invariant chain processing in large yellow croaker (*Larimichthys crocea*)

Qiuhua Li [a, b, c], Jingqun Ao [b, c, *], Yinnan Mu [b, c], Zhijun Yang [b, c], Ting Li [b, c], Xin Zhang [b, c], Xinhua Chen [b, c]

[a] School of Marine Sciences, Ningbo University, Ningbo 315211, China
[b] Key Laboratory of Marine Biogenetic Resources, Third Institute of Oceanography, State Oceanic Administration, Xiamen 361005, China
[c] South China Sea Bio-Resource Exploration and Utilization Collaborative Innovation Center, Xiamen 361005, China

ARTICLE INFO

Article history:
Received 27 July 2015
Received in revised form
5 October 2015
Accepted 9 October 2015
Available online 22 October 2015

Keywords:
Large yellow croaker (*Larimichthys crocea*)
Cathepsin S
Cathepsin L
Invariant chain processing
Antigen presentation

ABSTRACT

Two cysteine proteases, cathepsin S (CatS) and cathepsin L (CatL), have been identified as the key enzymes involved in the processing of invariant chain (Ii chain) in mammals. However, little is known about the roles of fish cathepsins in the Ii chain processing. In this study, large yellow croaker cathepsin S (LycCatS) and L (LycCatL) were identified and characterized. Based on the sequence comparison and phylogenetic analysis, both LycCatS and LycCatL are highly conserved to their counterparts in teleost. These two cathepsins were constitutively expressed in all tissues and immune-related cells tested, although at different levels. Both recombinant LycCatS (rLycCatS) and LycCatL (rLycCatL) possess the typical cysteine protease activity. Like other mammalian endopeptidase cathepsins, rLycCatS and rLycCatL could be autocatalytically activated to remove propeptides and release active mature peptides. On the other hand, the autocatalytic activation of rLycCatL could be inhibited by recombinant large yellow croaker Ii chain (rLyc-TR-Ii), but the autocatalytic activation of rLycCatS was not affected by rLyc-TR-Ii. Furthermore, the activated rLycCatS can efficiently process rLyc-TR-Ii in a stepwise manner *in vitro*, while the activated rLycCatL can not. These data indicate that cathepsin S may be the main cathepsin involved in the Ii chain processing in bony fish.

1. Introduction

Cysteine cathepsins are a group of enzymes belonging to the papain family of cysteine proteases [1], all of which share a conserved catalytic site formed by cysteine, histidine, and asparagine residues [2]. Based on structural and cleavage site differences, these papain-like cysteine proteases are classified into two distinct groups, endopeptidase cathepsin (cathepsin F, L, K, S and V) and exopeptidase cathepsin (cathepsin B, C, H and X) [3,4]. Two mouse endopeptidase cathepsins, cathepsin S (CatS) and L (CatL), were found to be differentially expressed in different types of antigen presenting cells (APCs), including B cells, macrophages, dendritic cells, specialized thymic and intestinal epithelium cells [5]. Like other mammalian cathepsins, they are synthesized as an inactive pre-proenzyme with signal peptide, propeptide and mature peptide [6,7]. In the acidic environment of lysosomes or late endosomes, their propeptide is removed by autocatalytic processing or by other proteases [7–9]. In mammalian specific immune defense system, it has been demonstrated that cathepsins played an important role in MHC class II-mediated antigen processing and presentation [5,10,11].

MHC class II molecules are constitutively expressed on the surface of professional antigen-presenting cells, which present proteolytic fragments of self or foreign protein antigens to the $CD4^+$ T helper cells [7,12,13]. MHC class II molecules assemble to form a heterodimer in the endoplasmic reticulum where they then form MHC class II-Ii complexes with the Ii chain [12]. The Ii chain can stabilize the nascent MHC class II heterodimers, target the complexes to the endosome and prevent the peptides being loaded onto MHC class II with CLIP (class II-associated invariant chain peptide) [14]. Therefore, endosomal MHC class II-Ii complexes can only become competent for antigenic peptide loading after Ii chain

* Corresponding author. 184 Daxue Road, Xiamen, Fujian Province 361005, China.
E-mail address: ajingqun@tio.org.cn (J. Ao).

http://dx.doi.org/10.1016/j.fsi.2015.10.009
1050-4648/

processing. Ii chain is cleaved sequentially to discrete intermediates known as p22, p10 and CLIP fragments (22, 10 and 3 kDa Ii chain intermediate, respectively) [15]. Cathepsin B (CatB) and cathepsin D (CatD), the most abundant cysteine protease and aspartyl protease, were primarily suggested as the enzymes responsible for the processing of Ii chain intermediates [16,17]. However, the studies on CatB- and CatD-deficient mice indicated that these two enzymes were dispensable for MHC class II antigen presentation [18,19]. Recent research showed that only cathepsin B and H in a cell-free antigen-processing system were sufficient for successful processing and editing of influenza HA1 epitopes [20]. On the other hand, researches using protease inhibitors and gene-knockout mice demonstrated a significant role for CatS and CatL in the late stage of Ii chain processing. In CatS-deficient mice, Ii chain p10 intermediate was strongly accumulated and the presentation of majority of exogenous antigens was diminished in B cells and dendritic cells [21]. In CatL-deficient mice, impaired late-stage Ii chain processing in thymic epithelial cells (TECs) and a marked decrease in $CD4^+$ T cell numbers were observed [22]. However, it has been demonstrated that in human thymic epithelial cells CatS was the only cysteine protease to efficiently process the p10 fragment, while CatL and CatV were shown not to participate in Ii-p10 processing [23]. These data indicate that CatS is required for late stages of Ii chain processing in APCs, yet CatL may exert different functions in Ii chain processing in different species.

So far, both CatS and CatL have been identified and characterized in several teleost, such as *Oncorhynchus mykiss* [24], *Senegalese sole* [25], *Cyprinus carpio* [26], *Hypophthalmichthys molitrix* [27], *Lutjanus argentimaculatus* [6], and *Oplegnathus fasciatus* [28]. The mRNA level of *L. argentimaculatus* cathepsin S was significantly increased in head kidney macrophages following *Bacillus subtilis* challenge [6]. The expression levels of *O. fasciatus* cathepsin B and L were also up-regulated in liver or blood cells after LPS or bacterial induction [28], indicating that they may play important roles in teleost immunity. Recently, CatS from *L. argentimaculatus* and CatB from *Larimichthys crocea* were demonstrated to be able to cleave the Ii chain *in vitro* [6,29]. However, the details on how fish cathepsins process the Ii chain processing in antigen presentation remain poorly understood.

In this study, cathepsin S (LycCatS) and cathepsin L (LycCatL) were identified and characterized from large yellow croaker (*L. crocea*), an economically important marine fish in China. The recombinant LycCatS and LycCatL expressed in *Pichia pastoris* possess the typical cysteine proteinase activity, and can be autocatalytically activated *in vitro*. The autocatalytic activation of rLycCatL could be inhibited by rLyc-TR-Ii, but that of rLycCatS was not affected. Furthermore, the activated rLycCatS, but not activated rLycCatL, could process the rLyc-TR-Ii *in vitro*, indicating that cathepsin S may be the main cathepsin involved in the Ii chain processing in bony fish.

2. Materials and methods

2.1. cDNA cloning and sequence analysis of LycCatS and LycCatL

The complete cDNA sequences of *LycCatS* and *LycCatL* were obtained from the transcriptome analysis of *Aeromonas hydrophila* infected large yellow croaker spleen [30]. Then PCR was performed to validate the sequences by the primer sets of CatS-F/CatS-R and CatL-F/CatL-R (Table 1). The resulted fragments were then sequenced and assembled.

The protein sequences were analyzed using BLAST program (http://www.ncbi.nlm.nih.gov/BLAST) and the SMART online program (http://smart.embl-heidelberg.de). The protein family signature was identified by InterPro software (http://www.ebi.ac.uk/Tools/InterProScan/). Signal peptides were predicted using the SignalP 3.0 Server (http://www.cbs.dtu.dk/services/SignalP/). The potential *N*-glycosylation site was predicted by NetNGlyc 1.0 Server (http://www.cbs.dtu.dk/services/NetNGlyc/). The potential *O*-glycosylation site was predicted by NetOGlyc 4.0 Server (http://www.cbs.dtu.dk/services/NetOGlyc/). Phylogenetic tree was constructed based on amino acid sequences with the Molecular Evolution Genetics Analysis (MEGA) software version 4.0 using the neighbor-joining method [31].

2.2. Expression profile analysis of LycCatS and LycCatL in different tissues and immune-related cells

To analyze the expression profiles of *LycCatS* and *LycCatL* in large yellow croaker tissues, fish (mean weight 100 g) were obtained from a mari-culture farm at Lianjiang, Fujian, China. Tissue sampling and cDNA synthesis were performed as described before [29].

To check the expression of LycCatS and LycCatL in the immune-related cells, macrophages was isolated from head kidney and maintained according to Li et al. [32]. Lymphocytes were separated from blood by density gradient centrifugation using percoll (GE Healthcare Life Sciences, USA) as described previously [33]. Briefly, blood was collected from the caudal vein sinus of the large yellow croakers using a 23-gauge needle. Then it was diluted with PBS containing 25 U/mL of heparin sodium at a ratio of 1:5, and was centrifuged at 4 °C, 650 × g for 30 min after being loaded onto 34%/51% percoll density gradients. The band located at the gradient interface was collected, and cell pellet was suspended in L-15 medium and centrifuged twice to remove percoll. Finally, cells were resuspended in L-15 medium containing 0.1 g/L $NaHCO_3$, 2.38 g/L HEPES (Amresco, USA), 0.33 g/L glucose, 50 μM 2-mercaptoethanol, 0.1% fetal bovine serum (FBS, Gibco, USA), 200 IU/mL penicillin, and 200 μg/mL streptomycin. After incubated at 22 °C for 5 h, adherent cells were removed and suspended lymphocytes were collected. LYCK head kidney cell line was established and maintained in our laboratory [34]. Total RNA was extracted from isolated cells and transcribed into first strand cDNA according to the method described previously [34].

Real-time PCR was performed using the cDNA from tissues or isolated cells with primer sets consisting of CatS-F1/CatS-R1 and CatL-F1/CatL-R1, respectively (Table 1). *β-actin* was amplified as an internal control with a primer set of Actin-F1 and Actin-R1 (Table 1). The PCR was run at 95 °C for 3 min, and then 40 cycles of 5 s at 95 °C, 15 s at 60 °C and 20 s at 72 °C.

2.3. Expression, purification and protease activity assay of recombinant LycCatS and LycCatL

The gene fragment encoding the residues 27–336 of *LycCatS* was amplified with the primer set of rCatS-F1 and rCatS-R1 (Table 1). The gene fragment encoding the residues 17–335 of *LycCatL* was amplified with the primer set of rCatL-F1 and rCatL-R1 (Table 1). The amplified fragments were cloned into the *P. pastoris* expression vector pPICZαA (Invitrogen) to construct the recombinant expression plasmids, pPICZαA-*LycCatS* and pPICZαA-*LycCatL*, respectively. The engineered *P. pastoris* SMD1168 strains were then produced, identified and induced for the expression of recombinant LycCatS and LycCatL (rLycCatS and rLycCatL) by methanol according to the manufacturer's instructions (EasySelect™ Pichia Expression Kit, Invitrogen). After 72 h of induction, culture supernatant was collected, subjected to 12% SDS-PAGE and western-blot analysis. The recombinant proteins were then purified by Ni Sepharose™ 6 Fast Flow (GE healthcare, UK) according to the manufacturer's protocol and quantitated by spectrophotometry (Nanodrop 1000 spectrophotometer, Thermo Fisher Scientific, USA).