Fish & Shellfish Immunology xxx (2016) 1–11

Contents lists available at ScienceDirect

Fish & Shellfish Immunology

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

Full length article

Molecular cloning and functional characterization of cathepsin B from the sea cucumber *Apostichopus japonicus*

Huahui Chen [a], Miao Lv [a], Zhimeng Lv [a], Chenghua Li [a, *], Wei Xu [b], Weiwei Zhang [a], Xuelin Zhao [a], Xuemei Duan [a], Chunhua Jin [a]

[a] School of Marine Sciences, Ningbo University, PR China
[b] Louisiana State University, Agricultural Center, USA

ARTICLE INFO

Article history:
Received 28 September 2016
Received in revised form
8 November 2016
Accepted 12 November 2016
Available online xxx

Keywords:
Apostichopus japonicus
Cathepsin B
Gene expression
Recombinant protein
Cell apoptosis

ABSTRACT

Cathepsin B (CTSB), a member of lysosomal cysteine protease, is involved in multiple levels of physiological and biological processes, and also plays crucial roles in host immune defense against pathogen infection in vertebrates. However, the function of CTSB within the innate immune system of invertebrates, particularly in marine echinoderms, has been poorly documented. In this study, the immune function of CTSB in *Apostichopus japonicus* (designated as *AjCTSB*), a commercially important and disease vulnerable aquaculture specie, was investigated by integrated molecular and protein approaches. A 2153 bp cDNA representing the full-length of *AjCTSB* was cloned via overlapping ESTs and RACE fragments. *AjCTSB* contained an open reading frame of 999 bp encoding a secreted protein of 332 amino acid residues with a predicted molecular mass of 36.8 kDa. The deduced amino acid of *AjCTSB* shared a typical activity center containing three conserved amino acid residues (Cys^{108}, His^{277} and Asn^{297}). Phylogenetic tree analysis also supported that *AjCTSB* was a new member of CTSB family with clustering firstly with invertebrate CTSBs. Quantitative real time PCR analysis revealed that *AjCTSB* was ubiquitously expressed in all examined tissues with the highest levels in intestine. The *Vibrio splendidus* challenged sea cucumber and LPS-exposed coelomocytes could both significantly boost the expression of *AjCTSB*. Moreover, the purified recombinant *AjCTSB* exhibited dose-dependent CTSB activities at the concentration ranged from 0 to 0.24 μg μL^{-1}. Further functional analysis indicated that coelomocytes apoptosis was significantly inhibited by 0.16-fold *in vivo* and the apoptosis execution *Ajcaspase 3* was extremely reduced in *Apostichopus japonicus* coelomocytes treated with specific *AjCTSB* siRNA. Collectively, all these results suggested that *AjCTSB* was an important immune factor and might be served as apoptosis enhancers in pathogen challenged sea cucumber.

1. Introduction

As a group of hydrolytic enzymes, lysosomal proteases were found ubiquitously expressed in most vertebrate and invertebrate species, which has major functions including protein degradation, antigen processing, protein precursor processing, and cell apoptotic processing [1]. Cathepsins, a group of intracellular hydrolytic proteases, were a class of lysosomal proteases with endopeptidases activities [2]. To date, three major types of cathepsins have been identified based on their targeting amino acid residues in their active sites, including serine protease (cathepsin A and G), aspartic protease (cathepsin D and E) and cysteine protease (cathepsin B, C, F, H, K, L, O, S, W and Z) [3]. Their functions have been intensively investigated in cathepsin-deficient mice [4–6]. One of the notable functions of cathepsins is the involvement in multiple immune response pathways [7–9].

As one of the major types of cathepsins, cyteine proteases, which are members of the papain family and belong to the C1 peptidase family, are ubiquitously produced in almost all organisms and responsible for intracellular and extracellular protein degradation and turnover via catalyzing protein hydrolysis [10,11]. In addition, they are also be acting important regulators and signaling molecules in different levels of biological processes, such as antigen processing, hormone activation and inflammatory responses [12,13]. Different than other cysteine proteases, cathepsin B (CTSB)

* Corresponding author. 818 Fenghua Road, Ningbo University, Ningbo, Zhejiang Province 315211, PR China.
E-mail address: lichenghua@nbu.edu.cn (C. Li).

http://dx.doi.org/10.1016/j.fsi.2016.11.033
1050-4648/

was found to have double enzymatic activities of exopeptidase and endopeptidase [14]. In structure, CTSB is composed of two spherical domains with a cysteine residue C^{25} and a histidine residue H^{159} [15]. It exhibits peptidyl-dipeptidase, carboxypeptidase or endopeptidase activities depending on pH as well as substrate, such as the exopeptidase activity function mainly through two sequential histidines within the characteristic “occluding loop” at the “rear” of the cleft of substrate-binding pocket [16]. CTSB has been found to participate in cellular process during many disorder and diseases, such as immunological disorders [17], inflammatory response [18], apoptosis [19], Alzheimer's disease [20] and cancer [21]. Originally identified from rat [22], CTSB has been found and studied in a number of vertebrate and invertebrate species. All the previous studies on CTSB suggested that this molecule is structurally and functionally conserved across species [23–25]. Recently, increasing efforts were made in understanding the role of CTSB in the host defense systems of aquatic species, including orange-spotted grouper with a stimulation of Singapore grouper iridovirus (SGIV) [26],and UV-inactivated grass carp hemorrhage virus and LPS challenged flounder [27]. Despite the good amount of work on CTSB, actual functions of CTSB within the innate immune system of invertebrates remain largely unknown.

Considering the conservation of CTSB in the evolution, we are particularly interested in understanding the role of CTSB in echinoderma species since the immune systems of vertebrates and echinoderma species may be developed from common ancestor of the deuterostomes (PMID:10426433). Here we use sea cucumber *A. japonicus* as a model to investigate the role played by CTSB in echinoderm innate immunity. It is also noteworthy that *A. japonicus* is also an economically important aquaculture species in China with an annual production of around 200,969 tons in 2015 [28]. Recent outbreaks of infectious diseases, such as skin ulceration syndrome (SUS) severely impacted the production of *A. japonicus* [29]. *Vibrio splendidus* was believed to be the major cause of *A. japonicus* SUS [30]. And infection of *V. spendidus* leads to anorexia, shaking head and mouth tumidity at the early stage, flowed by tissue atrophy and skin ulceration in *A. japonicus* [31]. Degradation of infected tissues in organisms is primarily achieved by proteases as a self-protection mechanism to prevent the healthy tissue from further infections. CTSB has demonstrated the abilities in many organisms to cleave the basement membrane and degrade extracellular matrix proteins, such as collagen IV, fibronectin, elastin, *etc* [32–34]. However, the connection between SUS and CTSB activation in *A. japonicus* has not been studied. Here, we fully characterized the DNA and protein sequences of CTSB in *A. japonicus* (designated as *AjCTSB* and r*AjCTSB*). The spatial and time-course expression profiles of *AjCTSB* along the progress of *V. splendidus* challenge and LPS exposed were also tested *in vivo* and *in vitro* with *A. japonicus* individuals and coelomocytes primary culture model, respectively. Functional analyses of *AjCTSB* showed a close relationship between *AjCTSB* and cell apoptosis in *A. japonicus*. These findings highlighted the positive roles of *AjCTSB* in echinoderm host defense system and provided an important reference in the study of lysosomal proteases along the evolution of organisms. The fully characterized *AjCTSB* gene can be also used as a potential target in the development of therapeutic strategy for SUS in production of *A. japonicus*.

2. Materials and methods

2.1. Experimental animals, pathogenic microorganisms and challenge experiment

Sixty healthy adult sea cucumbers (120 ± 13 g) were collected from Dalian Pacific Aquaculture Company (Dalian, China) and acclimatized in 30 L aerated natural seawater (28‰ salinity at 16 °C) for three days. *Vibrio splendidus* strain, which was previously isolated from *A. japonicus* diagnosed with SUS in our laboratory, was inoculated into 2216 E liquid medium at 28 °C with shaking at 220 rpm until reaching an optical density to 1.0 at 600 nm wave length. The culture was collected by centrifuge at 5000 rpm for 10 min and re-suspended in filtered seawater for subsequent challenge experiments.

For immune challenged experiment, one tank with five individuals served as the control, and the other five tanks contained live *V. splendidus* at a final concentration of approximately 1.0×10^7 CFU mL^{-1}. The infection dose and sampling points were determined by immune gene expression analysis. The coelomocytes from five individuals in the control group was denoted as 0 h, and the challenged groups were obtained at 6 h, 24 h, 48 h, 72 h and 96 h, respectively. Sea cucumbers were dissected by sterilized scissors on ice and the coelomic fluids were filtered through a 300 Mesh Cell Cribble to remove large tissues debris. The coelomocytes were harvested by centrifuging at 800×*g* for 5 min.

For spatial expression analysis, five tissues of sea cucumber including coelomocytes, tentacle, muscle, respiratory trees and intestine were collected from uninfected healthy individuals using sterilized scissors and tweezers. These tissues were ground into powder in liquid nitrogen using a mortar and a pestle. We performed 5 replicates in experimental group as well as the control group, and all samples were stored at −80 °C for RNA extraction and cDNA synthesis.

2.2. Cloning and analysis of the full-length cDNA of AjCTSB

Total RNA was extracted using RNAiso plus (Takara, Japan) according to the supplied protocol. The quality and quantity of RNA of each sample was determined by Nanodrop 2000 assay. All extracted RNA samples with A260/A280 ratio greater than 1.7 were used for cDNA synthesis. First strand cDNA was generated by Primescript™ II 1st cDNA Synthesis Kit (Takara, Japan) following the manufacture's protocol (1 μg total RNA per 10 μL reaction). Two gene specific primers, namely, P1: 5′-TGGCACAGATTCAAATGGAG-3'; P2:5′-GGGAGATAGCGGATTCTT-3′ were designed based on the corresponding expressed sequence tags (ESTs) in our finished transcriptions data [35] for 3′ RACE. PCR products were purified and ligated into the pMD18-T simple vector (Takara, Japan). After transformed into the competent *Escherichia coli* DH5α cells (Takara, Japan), three positive clones for each product were sequenced at Invitrogen (Shanghai, China).

The cDNA sequence of *AjCTSB* was analyzed using the BLAST algorithm at the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/blast), and the deduced amino acid sequence was analyzed using the Expert Protein Analysis System (http://www.expasy.org/). The molecular mass (MM) and theoretical isoelectric point (pI) of *AjCTSB* were calculated by the ProtParam tool (http://www.expasy.ch/tools/protparam.html). The putative signal peptide cleavage site was identified using the SignalP 4.1 Server (http://www.cbs.dtu.dk/services/SignalP/). Domain in these amino acid sequences were detected using the simple modular architecture research tool (SMART) program (http://www.smart.emblheidelbergde/) and multiple alignments analysis of each protein were performed using the Clustal Omega Multiple Alignment program (http://www.ebi.ac.uk/clustalw/). A neighbor-joining phylogenetic tree was constructed based on different types of cathepsins using Mega 5.0 program with 1000 bootstraps.

2.3. Spatial expression analysis of AjCTSB mRNA by qPCR

The tissue distribution analysis of *AjCTSB* gene was conducted in five healthy sea cucumber tissues by qPCR, including muscle, tentacle,

Please cite this article in press as: H. Chen, et al., Molecular cloning and functional characterization of cathepsin B from the sea cucumber *Apostichopus japonicus*, Fish & Shellfish Immunology (2016), http://dx.doi.org/10.1016/j.fsi.2016.11.033