Developmental and Comparative Immunology 38 (2012) 495–504

Contents lists available at SciVerse ScienceDirect

Developmental and Comparative Immunology

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

Genomic characterization and expression profiles upon bacterial infection of a novel cystatin B homologue from disk abalone (*Haliotis discus discus*)

H.K.A. Premachandra [a], Qiang Wan [a], Don Anushka Sandaruwan Elvitigala [a], Mahanama De Zoysa [b], Cheol Young Choi [c], Ilson Whang [a,*], Jehee Lee [a,d,*]

[a] Department of Marine Life Sciences, School of Marine Biomedical Science, Jeju National University, Jeju Special Self-Governing Province 690-756, Republic of Korea
[b] Laboratory of Aquatic Animal Diseases, College of Veterinary Medicine, Chungnam National University, Yuseong-gu, Daejeon 305-764, Republic of Korea
[c] Division of Marine Environment and Bioscience, Korea Maritime University, Busan 606-791, Republic of Korea
[d] Marine and Environmental Institute, Jeju National University, Jeju Special Self-Governing Province 690-814, Republic of Korea

ARTICLE INFO

Article history:
Received 2 May 2012
Revised 13 June 2012
Accepted 28 June 2012
Available online 7 August 2012

Keywords:
Cysteine protease inhibitors
Cystatin B
Disk abalone
Bacterial challenge
Papain inhibition
Transcriptional analysis

ABSTRACT

Cystatins are a large family of cysteine proteinase inhibitors which are involved in diverse biological and pathological processes. In the present study, we identified a gene related to cystatin superfamily, AbCyt B, from disk abalone *Haliotis discus discus* by expressed sequence tag (EST) analysis and BAC library screening. The complete cDNA sequence of AbCyt B is comprised of 1967 nucleotides with a 306 bp open reading frame (ORF) encoding for 101 amino acids. The amino acid sequence consists of a single cystatin-like domain, which has a cysteine proteinase inhibitor signature, a conserved Gly in N-terminal region, QVVAG motif and a variant of PW motif. No signal peptide, disulfide bonds or carbohydrate side chains were identified. Analysis of deduced amino acid sequence revealed that AbCyt B shares up to 44.7% identity and 65.7% similarity with the cystatin B genes from other organisms. The genomic sequence of AbCyt B is approximately 8.4 Kb, consisting of three exons and two introns. Phylogenetic tree analysis showed that AbCyt B was closely related to the cystatin B from pacific oyster (*Crassostrea gigas*) under the family 1.Functional analysis of recombinant AbCyt B protein exhibited inhibitory activity against the papain, with almost 84% inhibition at a concentration of 3.5 μmol/L. In tissue expression analysis, AbCyt B transcripts were expressed abundantly in the hemocyte, gill, mantle, and digestive tract, while weakly in muscle, testis, and hepatopancreas. After the immune challenge with *Vibrio parahemolyticus*, the AbCyt B showed significant ($P < 0.05$) up-regulation of relative mRNA expression in gill and hemocytes at 24 and 6 h of post infection, respectively. These results collectively suggest that AbCyst B is a potent inhibitor of cysteine proteinases and is also potentially involved in immune responses against invading bacterial pathogens in abalone.

1. Introduction

Cysteine proteases are widespread in all living organisms, such as mammals, birds, fish, insects, plant and protozoa, and are involved in diverse biological processes (Chapman et al., 1997; Rzychon et al., 2004). Cysteine proteases play key roles in antigen presentation, apoptosis, protein processing, as well as several pathological conditions like cancer progression, inflammation and neuro-degeneration (Chapman et al., 1997; Kopitar-Jerala, 2006). A number of studies have reported that cysteine proteinases produced by bacteria and parasites play a critical role as a virulence factor in development of many diseases (Mottram et al., 2004; Rudenskaya and Pupov, 2008; Takahashi et al., 1994). The proteolytic activity of these proteases can be inhibited by proteinase inhibitors such as cystatins. Cystatins are a large family of natural tight-binding reversible inhibitors of C1 family cysteine proteinases. Like other cysteine proteinases, cystatins have been found in diverse organisms. Many cysteine proteinases including the plant-derived papain, and the mammalian cathepsin, B, H and L can interact with cystatins (Barrett, 1987;Turk and Bode, 1991). Based on the structural differences, the cystatin superfamily can be categorized into four families. Family 1 cystatins, also known as stefins, are comprised of cystatin A and B, characterized by low molecular weight (~11 kDa) and a single cystatin-like domain structure. This group of cytoplasmic proteins is composed of ~100 amino acid residues that lack of disulfide bond, signal

* Corresponding authors. Address: Marine Molecular Genetics Lab, Department of Marine Life Sciences, College of Ocean Science, Jeju National University, 66 Jejudaehakno, Ara-Dong, Jeju 690-756, Republic of Korea. Tel.: +82 64 754 3472; fax: +82 64 756 3493 (J. Lee).
E-mail addresses: ilsonwhang@hanmail.net (I. Whang), jehee@jejunu.ac.kr (J. Lee).

0145-305X/$ - see front matter © 2012 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.dci.2012.06.010

peptide, and carbohydrate side chain (Barrett et al., 1986; Turk and Bode, 1991). Cystatins C, D, E, S, and SN secretory inhibitors in family 2, known by the superfamily name cystatin, possess molecular weight of ~13 kDa and contain a single cystatin-like domain, two intra-molecular disulfide bonds, whereas lack of carbohydrates (Cornwall and Hsia, 2003; Li et al., 2010). Family 3 cystatins (kininogens) exhibit high structural complexity with more than one cystatin-like domain, which are large multifunctional glycoproteins with inhibitory activities (Kellermann et al., 1987). Another family, designated as family 4, was reported recently and consists of cystatins specially from nematodes (Khaznadji et al., 2005; Li et al., 2010). All cystatins contain a papain-binding site that binds to the catalytic site of papain-like proteinases, and inhibits them reversibly. This site is created by several conserved regions of the protein, including a glycine in the N-terminal region, a central QxVxG motif in first hairpin loop of the protein, and a less conserved PW motif in the second hairpin loop in the C terminal region (Björk and Ylinenjärvi, 1989; Margis et al., 1998; Rzychon et al., 2004).

Cystatins are involved in both biological and pathological processes which cysteine proteinases participate in, including protein homeostasis, inflammatory responses, antigen processing, metastasis, immune responses, and cathepsin dependent apoptosis (Abrahamson et al., 2003; Kopitar-Jerala, 2006; Lefebvre et al., 2008; Shah and Bano, 2009; Synnes, 1998). Among cystatins, cystatin B is an intracellular cysteine proteinase inhibitor which is a tightly-binding reversible inhibitor of cathepsin B, H, and L through forming a dimer stabilized by non-covalent forces to inhibit papain and cathepsin B, H, and L (Anastasi et al., 1983; Cimerman et al., 2001; Lefebvre et al., 2008). Cystatin B was also thought to play a role in protection against the proteinases leaking from lysosomes, and in biological defense system against invaders (Lefebvre et al., 2004; Li et al., 2010; Xiao et al., 2010). Defects in cystatin B cause progressive myoclonic epilepsy type 1 (EPM1), which is an autosomal recessive disorder characterized by severe, stimulus-sensitive myoclonus, and tonic–clonic seizures in human (Pennacchio et al., 1996; Riccio et al., 2005).

Although many evidences have been reported from vertebrates and some invertebrates, the function of cystatin B in mollusk species is still poorly understood. Disk abalone (*Haliotis discus discus*) is a valuable marine gastropod species. However, significant mortality is present in disk abalone aquaculture due to many reasons, including pathogens, stressful environments, pollutants, and disease outbreaks. To investigate the role of cystatin B-like proteins in disk abalones, the present study was carried out at the molecular level. In the present study, we identified a gene encoding for cystatin B from a previously constructed cDNA library of disk abalone *H. discus discus*, and functionally analyzed the papain inhibitory activity of recombinant protein. Furthermore, to understand the role in innate immune response, the temporal mRNA expression analysis was employed after bacterial infection for the first time in mollusks species.

2. Materials and methods

2.1. Experimental animals

Healthy disk abalones were purchased from 'Youngsoo' commercial abalone farm in Jeju Island, Republic of Korea. They were maintained as 40 animals per tank in flat bottomed tanks (250 L) with sand-filtered aerated seawater at a salinity of 34‰ at 18 ± 1 °C during the experimental period at Marine and Environmental Research Institute of Jeju National University. Abalones were acclimatized for 7 days before the experiment, and they were fed with fresh sea weed, *Undaria pinnatifida*, during the acclimatization period.

2.2. Bacterial artificial chromosome (BAC) library construction and screening

A BAC library of *H. discus discus* was constructed by Lucigen® Co. (Middleton, Wisconsin) using randomly sheared genomic DNA from gill tissue of disk abalone. Around 92,160 clones, possessing an average insert size of 120 Kb were arrayed in 240 of 384-well microtiter plates. Screening of the BAC-library was carried out with a PCR-based method (TaKaRa Bio, USA) following the manufacturer's instructions using gene-specific primers (Table 1). The identified clones were isolated from the corresponding wells and confirmed by colony PCR with gene-specific primers. After confirmation, BAC DNA from positive clone was isolated and purified using QIAGEN Large-Construct Kit, following the manufacture's protocol and was subjected to sequencing by Roche (454) Genome Sequencer FLX(GS-FLX™) system (Macrogen Inc. Korea).

2.3. Identification and sequence characterization of AbCyt B in DNA and protein level

Full length cDNA sequence of abalone cystatin B (AbCyt B) was identified by analyzing the previously constructed abalone expressed sequence tag (EST) database sequences (Munasinghe et al., 2006). BLAST (http://www.blast.ncbi.nlm.nih.gov/Blast.cgi) analysis indicated that one of the ESTs was homologous to previously identified cystatin Bs. This sequence was selected to design the BAC library screening primers and qRT-PCR primers used in the study (Table 1). The identified BAC clone sequence was used to analyze the genomic sequence.

The nucleotide and deduced amino acid sequences were analyzed using respective blastx and blastp programs at National Center for Biotechnology Information (NCBI). To determine the domains and conserved regions in AbCyt B, ExPASy PROSITE (http://www.prosite.expasy.org/) and motif scan (http://www.hits.isb-sib.ch/cgi-bin/PFSCAN) programs were used. The identity and similarity percentages of AbCyt B were detected using EMBOSS pairwise alignment algorithms (http://www.ebi.ac.uk/Tools/psa) at amino acid level.

2.4. Multiple sequence alignment, phylogenetic analysis and computer simulation modeling

The amino acid sequences obtained from BLAST analysis were used for the multiple alignment and phylogenetic tree construction. The amino acid sequences were aligned using ClustalW2 Multiple Sequence Alignment software with the default settings (http://www.ebi.ac.uk/Tools/msa/clustalw2). The phylogenetic tree was created using the neighbor-joining algorithm within MEGA (Molecular Evolutionary Genetic Analysis) software package (version 5.05).Bootstrap values were calculated with 1000 replications to estimate the robustness of internal branches. In order to

Table 1
List of oligonucleotides used in the study.

Name	Primer sequence (5′–3′)	Purpose
Ab-cytb F1	GTGGTGGTGCAAC CGAAGTGAAAT	Real-time PCR amplification, BAC screening
Ab-cytb R2	TGCTACCACTTGTGA ACGGAAGGA	Real-time PCR amplification, BAC screening
Ab-cytb F3	GAGAGAgaattcATGTGTGG TGGTGCAACCGAAG	Forward primer to ORF amplification, EcoRI in pMAL c2X
Ab-cytb R4	GAGAGActgcagCTACTTGGCATC AAAATAATCCAGATCTGATGCA	Reverse primer to ORF amplification, PstI in pMAL c2X
Ab-Rib F5	TCACCAACAAGGACATCATTTGTC	Real-time PCR amplification
Ab-Rib R6	CAGGAGGAGTCCAGTGCAGTATG	Real-time PCR amplification