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Caspase 3 from rock bream (*Oplegnathus fasciatus*): Genomic characterization and transcriptional profiling upon bacterial and viral inductions

Don Anushka Sandaruwan Elvitigala [a,1], Ilson Whang [a,1], H.K.A. Premachandra [a], Navaneethaiyer Umasuthan [a], Myung-Joo Oh [b], Sung-Ju Jung [b], Sang-Yeob Yeo [c], Bong-Soo Lim [d], Jeong-Ho Lee [e], Hae-Chul Park [f,**], Jehee Lee [a,d,*]

[a] *Department of Marine Life Sciences, School of Marine Biomedical Sciences, Jeju National University, Jeju Self-Governing Province 690-756, Republic of Korea*
[b] *Department of Aqualife Medicine, Chonnam National University, Chonnam 550-749, Republic of Korea*
[c] *Department of Biotechnology, Division of Applied Chemistry & Biotechnology, Hanbat National University, Daejeon 305-719, Republic of Korea*
[d] *Marine and Environmental Institute, Jeju National University, Jeju Special Self-Governing Province 690-814, Republic of Korea*
[e] *Genetics & Breeding Research Center, National Fisheries Research & Development Institute, Geoje 656-842, Republic of Korea*
[f] *Graduate School of Medicine, Korea University, Ansan, Gyeonggido 425-707, Republic of Korea*

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ABSTRACT

Caspase 3 is a prominent mediator of apoptosis and participates in the cell death signaling cascade. In this study, caspase 3 was identified (*Rbcasp3*) and characterized from rock bream (*Oplegnathus fasciatus*). The full-length cDNA of *Rbcasp3* is 2683 bp and contains an open reading frame of 849 bp, which encodes a 283 amino acid protein with a calculated molecular mass of 31.2 kDa and isoelectric point of 6.31. The amino acid sequence resembles the conventional caspase 3 domain architecture, including crucial amino acid residues in the catalytic site and binding pocket. The genomic length of *Rbcasp3* is 7529 bp, and encompasses six exons interrupted by five introns. Phylogenetic analysis affirmed that Rbcasp3 represents a complex group in fish that has been shaped by gene duplication and diversification. Many putative transcription factor binding sites were identified in the predicted promoter region of Rbcasp3, including immune factor- and cancer signal-inducible sites. Rbcasp3, excluding the pro-domain, was expressed in *Escherichia coli*. The recombinant protein showed a detectable activity against the mammalian caspase 3/7-specific substrate DEVD-*p*NA, indicating a functional role in physiology. Quantitative real time PCR assay detected Rbcasp3 expression in all examined tissues, but with high abundance in blood, liver and brain. Transcriptional profiling of rock bream liver tissue revealed that challenge with lipopolysaccharides (LPS) caused prolonged up-regulation of *Rbcasp3* mRNA whereas, *Edwardsiella tarda* (*E. tarda*) stimulated a late-phase significant transcriptional response. Rock bream iridovirus (RBIV) up-regulated Rbcasp3 transcription significantly at late-phase, however polyinosinic-polycytidylic acid (poly(I:C)) induced Rbcasp3 significantly at early-phase. Our findings suggest that Rbcasp3 functions as a cysteine-aspartate-specific protease and contributes to immune responses against bacterial and viral infections.

1. Introduction

Apoptosis, one of the biochemically classified programmed cell death types, is a highly regulated process in multicellular organisms that is triggered by external and internal stimuli [1–3]. Apoptosis can play a significant role in cellular immunity, acting as an immune response to infections, especially those related to viruses [4,5]. The primary regulators of apoptosis are the host-encoded caspases [6–8]. Caspases are an evolutionarily conserved family of **c**ysteine-**a**spartic **s**pecific **p**rote**ase**s responsible for a diverse array of cellular functions, the well-recognized of which are apoptosis and inflammation. In pre-apoptotic cells, caspases

* Corresponding author. 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.
** Corresponding author. Graduate School of Medicine, Korean University, Ansan, Gyeonggido 425-707, Republic of Korea. Tel.: +82 31 412 6712; fax: +82 31 412 6729.
E-mail addresses: hcpark67@korea.ac.kr (H.-C. Park), jehee@jejunu.ac.kr, jeheedaum@hanmail.net (J. Lee).
[1] These authors contributed equally to this work.

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exist as inactive pro-enzymes (zymogenes) [9], which mainly consist of three distinct domains: a pro-domain, followed by a large subunit and a small subunit. The latter two subunits are connected by a linker region, which itself is flanked by aspartic acid residues [10].

Caspases can be self-activated or be activated by upstream-caspase proteases in death cascade that cleaves conserved aspartic acids in the C terminal region [11]. To date, 11 human caspases have been identified and functionally categorized into two groups; inflammatory caspases and apoptotic caspases. The latter has been further divided into initiators and effectors [8]. The effector caspases (caspase 1, 3, 4, 5, 6, 7, and 11) are activated by the self-activated initiator caspases, which function in the upstream of the apoptotic signaling pathway [12,13]. Caspases and caspase-like enzymes have also been identified in non-metazoans, such as plants, fungi, and prokaryotes [14]. Caspases are regulated at several stages, such as at the transcriptional and post-transcriptional levels [1]. Moreover, enzymatic activity of caspases can be inhibited by members of a conserved family of proteins known as inhibitor of apoptosis (IAP) factors [15].

Caspase 3, one of the effector caspases, is involved in executing the cell death signaling cascade of intrinsic and extrinsic apoptotic pathways, following its activation by caspase 8 and caspase 9, respectively [16]. Activated caspase 3 mediates many of the characteristic morphological alterations of apoptosis, such as breakdown of several cytoskeletal proteins, cleavage of polyadenosine dipeptide ribose polymerase (PARP) and degradation of the inhibitor of caspase-activated DNAses (ICADs), resulting in the release of CAD to cleave cell DNA and ultimately directing the cell toward death [7].

Caspase 3 has been identified and characterized in several teleost fish species; Studies of caspase 3 homologs in European sea bass (*Dicentrarchus labrax)*, zebrafish (*Danio rerio*), large yellow croaker (*Pseudosciaena crocea*), and Atlantic salmon (*Salmo salar*) have revealed an immune-related functions in these fishes [17–20]. Furthermore, two isoforms of caspase 3 (A and B) have been identified in Medaka (*Oryzias latipes*) [21] and Atlantic salmon [20].

Rock bream is one of the most economically important marine fish species in South Korea, which domiciliates in the coastal areas of the Pacific and Indian Ocean. In recent years, the mariculture sources of rock bream have experienced an alarming increase in prevalence and virulence of pathogenic infections, which have resulted in considerable economic losses [22,23]. Therefore, it is important to gain a detailed understanding of the unknown genetic and immunological mechanisms against pathogens in rock bream, in order to launch effective disease control interventions and disease-tolerant species by genetic breeding. In this study, we discovered and characterized the rock bream caspase 3 (*Rbcasp3*) at transcriptional and genomic levels. We determined the basal tissue distribution and transcriptional response in liver tissue to immune challenges with lipopolysaccharide (LPS), *Edwardsiella tarda*, rock bream iridovirus (RBIV), and polyinosinic-polycytidylic acid (poly (I:C)). We not only demonstrated that Rbcasp3 harbors immune-related hydrolytic activity using recombinant protein, but also determined that apoptosis represents an immune responsive process in rock bream.

2. Materials and methods

2.1. Identification of full-length cDNA sequence of Rbcasp3

Using the Basic Local Alignment Tool (BLAST) algorithm (http://www.ncbi.nlm.nih.gov/BLAST), full-length cDNA sequence of caspase 3 (contig number-07658) in -rock bream was identified from a previously established cDNA sequence data base [24].

2.2. Rbcasp3 genomic BAC library construction and PCR screening

Using rock bream genomic DNA, a random sheared bacterial artificial chromosome (BAC) library was custom constructed (Lucigen, USA). The library was screened by PCR in order to identify the clone containing the full-length Rbcasp3 gene using a sequence specific primer pair Rbcasp3-qF and Rbcasp3-qR (Table 1), designed according to the identified *Rbcasp3* cDNA sequence. The identified BAC clone was sequenced by GS-FLX™ system (Life Sciences, USA).

2.3. In silico analysis of rock bream caspase 3 DNA and protein sequences

The orthologous sequences of *Rbcasp3* were compared by the BLAST search program. Pairwise sequence alignment (http://www.Ebi.ac.uk/Tools/emboss/align) and multiple sequence alignment (http://www.Ebi.ac.uk/Tools/clustalw2) were performed using the ClustalW2 program. The phylogenetic relationship of *Rbcasp3* was determined using the Neighbor-Joining method and Molecular Evolutionary Genetics Analysis (MEGA) software version 4 [25]. Prediction of protein domains was carried out using the ExPASy-prosite data base (http://prosite.expasy.org) and the MotifScan scanning algorithm (http://myhits.isb-sib.ch/cgi-bin/motif_scan). Some properties of Rbcasp3 were determined by ExPASy Prot-Param tool (http://web.expasy.org/protparam).

Genomic sequence of *Rbcasp3* obtained from the BAC clone was used to identify the exon–intron structure and predict the promoter region along with potential transcriptional factor binding sites. The transcription initiation site (TIS) was predicted using the online neural network promoter prediction tool from Berkeley Drosophila Genome Project [26]. Potential cis acting elements located ~1 Kb upstream of the TIS were detected using TFSEARCH ver.1.3 and Alibaba 2.1 software. Furthermore, the tertiary structure of Rbcasp3 pro-enzyme was modeled based on the ab-initio protein prediction strategy, using the online server I-TASSER [27,28]. Subsequently, the three dimensional (3D) image was generated utilizing RasMol 2.7.5.2 software.

Table 1
Primers used in this study. F and R refer to forward and reverse primers, respectively. The lowercase letters indicate restriction enzyme sites introduced for cloning.

Name	Purpose	Sequence (5′ → 3′)
Rbcasp3-F	ORF amplification (without pro- domain)	GAGAGAgaattcGCCAAGCCCAGCTCCCACAG
Rbcasp3-R	ORF amplification (without pro-domain)	GAGAGActgcagTCAAGGAGAAAAATACATCTCTTTGGTCAGCATTG
Rbcasp3-qF	qRT-PCR primer	TGAGGGTGTGTTCTTTGGTACGGA
Rbcasp3-qR	qRT-PCR primer	TTCCCACTAGTGACTTGCAGCGAT
Rb-β-actin-F	qRT PCR internal reference	TCATCACCATCGGCAATGAGAGGT
Rb-β-actin-R	qRT PCR internal reference	TGATGCTGTTGTAGGTGGTCTCGT