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Transcriptional profiling, molecular cloning, and functional analysis of C1 inhibitor, the main regulator of the complement system in black rockfish, *Sebastes schlegelii*

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ABSTRACT

C1-inhibitor (C1inh) plays a crucial role in assuring homeostasis and is the central regulator of the complement activation involved in immunity and inflammation. A C1-inhibitor gene from *Sebastes schlegelii* was identified and designated as *SsC1inh*. The identified genomic DNA and cDNA sequences were 6837 bp and 2161 bp, respectively. The genomic DNA possessed 11 exons, interrupted by 10 introns. The amino acid sequence possessed two immunoglobulin-like domains and a serpin domain. Multiple sequence alignment revealed that the serpin domain of SsC1inh was highly conserved among analyzed species where the two immunoglobulin-like domains showed divergence. The distinctiveness of teleost C1inh from other homologs was indicated by the phylogenetic analysis, genomic DNA organization, and their extended N-terminal amino acid sequences. Under normal physiological conditions, *SsC1inh* mRNA was most expressed in the liver, followed by the gills. The involvement of SsC1inh in homeostasis was demonstrated by modulated transcription profiles in the liver and spleen upon pathogenic stress by different immune stimulants. The protease inhibitory potential of recombinant SsC1inh (rSsC1inh) and the potentiation effect of heparin on rSsC1inh was demonstrated against C1esterase and thrombin. For the first time, the anti-protease activity of the teleost C1inh against its natural substrates C1r and C1s was proved in this study. The protease assay conducted with recombinant black rockfish C1r and C1s proteins in the presence or absence of rSsC1inh showed that the activities of both proteases were significantly diminished by rSsC1inh. Taken together, results from the present study indicate that SsC1inh actively plays a significant role in maintaining homeostasis in the immune system of black rock fish.

1. Introduction

The complement system plays a significant role in eradicating pathogenic infections as a central component of innate immunity, and also acts as a bridge between the innate and adaptive immune systems [1,2]. Complement activation is an efficient mode of clearing invading pathogens. At the same time, the role of inappropriate complement activation in pathogenesis has been demonstrated using both knockout [3,4] and transgenic [5,6] animal models. As uncontrolled activation may cause tissue damage leading to serious pathological conditions arising from bio-incompatibility, it is crucial to limit this activity through tight regulation in assuring homeostasis [7]. For this purpose, the complement system consists of an array of soluble plasma proteins and receptors that inhibit complement activation, named regulators of complement activation (RCA) [7]. The complement system can be activated by three pathways, termed the classical, alternative, and lectin pathways. Activation of the classical pathway is initiated by antibody binding to the corresponding antigen [8]. The alternative pathway is triggered by microbial surfaces and a variety of complex polysaccharides leading to spontaneous hydrolysis of the putative thioester bond in complement component 3 (C3) [9,10]. The lectin pathway is activated by binding of mannan-binding lectins or ficolins to pathogen associated molecular patterns (PAMP) present on the surface of microorganisms in an antibody independent manner [11]. Complement reactions proceed in a sequential manner through the proteolytic cleavage of a series of inactive protease zymogens that are linked and

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activated as a cascade. This leads to the generation of active products that mediate various biological activities, such as inflammation and vascular permeability, through their interaction with specific cellular receptors and other serum proteins [12].

The complement component 1 inhibitor (C1inh) is a protease inhibitor of the serpin family, which contorts the active site of target proteinases, making them inactive by the conformational change actuated in the serpin upon peptide bond cleavage [13]. It is one of the major regulators of the classical complement pathway, controlling vascular permeability and suppressing inflammation by inactivating C1r and C1s proteases. It inhibits the activated form of these initial proteases through the classical complement pathway by forming a stable complex, and is the only inhibitor to act on these proteases [14]. Additionally, C1inh regulates the lectin pathway by inhibiting mannan-binding lectin-associated serine proteases (MASPs) [15]. It also controls contact activation by regulating plasma kallikrein and activated factor XII, an intrinsic coagulation system, by inhibiting activated factor XI and fibrinolytic proteases such as plasmin and tissue plasminogen activator [16]. The therapeutic capacity of C1inh against sepsis, vascular leak syndrome, acute myocardial infarction, endotoxin shock, and hereditary angioedema (HAE) has been reported [7,16]. Characterization has been conducted in a few mammals, including mice and human [17,18] and in some fish species, such as Nile tilapia, rock bream, and large yellow croakers [19–21]. In this study, for the first time we have proved the functional inhibitory potential of the teleostean C1-inhibitor on its direct substrates C1r and C1s in black rockfish.

Black rockfish (*Sebastes schlegelii*) is one of the important aquaculture fish species in the Republic of Korea. In recent years, it has been reported that the aquaculture industry faces severe production loss due to pathogenic bacterial infections [22–24]. Therefore, understanding the molecular mechanisms in immunity could be an appropriate way to find a remedy for pathogenic attack. The present study discusses the identification, *in silico* and functional characterization of C1inh from black rockfish (*SsC1inh*) at the genomic, transcriptomic, and proteomic levels. Protease inhibition by recombinant SsC1inh (rSsC1inh) was exhibited by a protease assay using black rockfish recombinant proteins C1r (rSsC1r) and C1s (rSsC1s) as substrates, to delineate that the C1inh in black rockfish is functionally active and involved in the immunity mechanism of black rock fish.

2. Materials and methods

2.1. Identification of SsC1inh gDNA

The rock fish genomic DNA library was constructed using the *de novo* genome assembly method. Briefly, sequencing libraries were made using the mate-pair and illumina paired-end library preparation protocols (Illumina, San Diego, CA, USA), and were subjected to size selection for illumina MiSeq and NextSeq sequencing. Using the PacBio manufacture protocols (Pacific Biosciences, CA, USA) the sequencing libraries were prepared to obtain long non-fragmented DNA sequences. Finally, the curated transcripts and genes from the consensus gene model were subjected to functional annotation. The genomic DNA sequence of *SsC1inh* (MG551291) was picked out from the rock fish genomic DNA library using the Basic Local Alignment Search Tool (BLAST) algorithm (https://blast.ncbi.nlm.nih.gov/Blast.cgi).

2.2. Identification of SsC1inh cDNA from transcriptome database

The cDNA sequence of rockfish C1inh (MG551291) was identified from our previously constructed rockfish transcriptome database [25] using the BLAST algorithm (https://blast.ncbi.nlm.nih.gov/Blast.cgi), and was termed *SsC1inh*.

2.3. In silico characterization of SsC1inh

DNAssist (version 2.2) was used to predict the open reading frame (ORF) and the encoded amino acid sequence. Homologous protein sequences for SsC1inh were identified using BLAST. Pairwise sequence alignment and multiple sequence alignment with other organisms were conducted using EMBOSS Needle (https://www.ebi.ac.uk/Tools/psa/emboss_needle/) and the ClustalW multiple alignment application of BioEdit sequence alignment editor software, respectively. The SignalP program (http://www.cbs.dtu.dk/services/SignalP/) was applied to check for the presence of signal peptides. Using the ExPASy ProtParam tool (http://web.expasy.org/protparam), the physical and chemical parameters of SsC1inh were predicted. The DISULFIND server (http://disulfind.disi.unitn.it/) was used to assess the occurrence of possible cysteine disulfide bonds. The tertiary structure of the SsC1inh serpin domain was generated with the SWISS-MODEL online tool (https://swissmodel.expasy.org/), using the crystal structure of the latent human C1-inhibitor serpin domain (PDB ID: 2oay.1) as a template. The most suitable model was chosen based on the confidence score (C-score), which estimates the quality of predicted models, and the structure was further developed using PyMOL molecular graphic software version 1.7.4. Pairwise structural comparison was carried out with the DALI protein structure comparison server (http://ekhidna2.biocenter.helsinki.fi/dali/index.html#tabs-2). A phylogenetic tree was constructed using the MEGA 6.0 package (http://www.141megasoftware.net/). To deduce the confidence value for the phylogenetic analysis, bootstrap trials were replicated 5000 times. The exon-intron structure was constructed using the gene mapper tool version 2.5 by analyzing the genomic DNA and complementary DNA sequences.

2.4. Cloning, recombinant expression, and purification of the putative SsC1inh protein

A pair of sequence-specific forward and reverse primers, containing adapter nucleotide sequences with EcoRI and HindIII restriction recognition sites, respectively, at their 5′ ends, were designed (Table 1) to amplify the sequence encoding the complete SsC1inh mature peptide for cloning into the pMAL-c5X expression vector (New England Biolabs, Ipswich, MA, USA). To amplify the coding sequence, PCR was carried out in a 50 μL reaction mixture with the designed primers and ExTaq polymerase (TaKaRa, Japan) using cDNA isolated from the liver as a template. PCR conditions were set with initial denaturation at 94 °C for 3 min, 35 cycles of amplification at 94 °C for 30 s, 60 °C for 30 s, and 72 °C for 1 min, with a final extension at 72 °C for 5 min. The PCR product was resolved on 1.5% agarose gel and an AccuPrep® gel

Table 1
Primers used in this study.

Name	Sequence (5′–3′)	Description
SsC1inh-F	GGTGAGGGTGCCGATTCTCTATCA	qPCR screening forward primer
SsC1inh-R	TGTAAAGACTGCTGTCACCCGAGAG	qPCR screening reverse primer
SsEF1α-F	AACCTGACCACTGAGGTGAAGTCTG	qPCR screening forward primer
SsEF1α-R	TCCTTGACGGACACGTTCTTGATGTT	qPCR screening reverse primer
SsC1inh-CF	GAGAGAgaattcGTAAATCTCCAGGTGGTACCTGGTTCCA	Cloning forward primer
SsC1inh-CR	GAGAGAaagcttTCATGGCTCGGTCACTCTGCC	Cloning reverse primer