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Short communication

# A homolog of Kunitz-type serine protease inhibitor from rock bream, *Oplegnathus fasciatus*: Molecular insights and transcriptional modulation in response to microbial and PAMP stimulation, and tissue injury

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ABSTRACT

Serine proteases and their inhibitors play vital roles in diverse biological processes. In this study, we identified and characterized cDNA coding for a Kunitz-type serine protease inhibitor (SPI), which we designated as RbKSPI, in a commercially important species, rock bream. The full-length cDNA sequence of *RbKSPI* consisted of 2452 bp with an open reading frame (ORF) of 1521 bp encoding a polypeptide of 507 amino acid (aa) residues. In the RbKSPI protein, MANEC, PKD, LDLa, and two Kunitz domains responsible for various functions were identified as characteristic features. Homology analysis revealed that RbKSPI shared the highest identity with the Kunitz homolog in *Takifugu rubripes* (77.6%). Phylogenetic analysis indicated that RbKSPI clusters with other teleostean KSPIs. In tissue-specific expression analysis, *RbKSPI* transcripts were detected in all the tested tissues, with the highest expression in gill tissue, followed by kidney and intestine. The mRNA expression of *RbKSPI* significantly increased in blood cells upon stimulation with two strains of bacteria (*Edwardsiella tarda* and *Streptococcus iniae*) and two pathogen-associated molecular patterns (PAMPs; LPS and poly I:C). Meanwhile, down-regulated expression of *RbKSPI* was observed in response to tissue injury. Collectively, these results suggest that the *RbKSPI* may be involved in essential immune defense against microbial pathogens and in the wound-healing process.



## 1. Introduction

Rock bream (*Oplegnathus fasciatus*) is a commercially important fish species in the South Korean mariculture industry owing to its high market price and demand. However, regular outbreaks of various diseases such as Edwardsiellosis, Streptococcicosis, and iridoviral disease have caused significant loss to fish production [1]. Therefore, an understanding of host-pathogen relations and responses associated with the various pathological/environmental stresses might be beneficial to the management of fish farming practices. Hence, identification and characterization of the key genes responsible for many biological aspects may provide novel insights into fish immunity. Recently, much research has begun to explore the immunomodulatory potential of various protease inhibitors.

Serine protease inhibitors (SPIs) are widely distributed in all living organisms and play vital roles in many biological processes such as blood coagulation, inflammation, tissue injury, the complement system, activation of prophenoloxidase, and immune defense in vertebrates and invertebrates [2–8]. In general, SPI activities are regulated by controlling the excessive proteolytic activity of the corresponding serine protease (SP). SPIs are categorized into several families based on structural and functional characteristics [9]. Some families of SPIs, known as Kazal, Kunitz,

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and serpin, have been well documented in various organisms [8–10].

The family of KSPIs has been abundantly identified in many organisms, including animals, plants, and microbes. According to the MEROPS database, various subfamilies of KSPIs have been identified, and the KSPIs of animals and plants were grouped into the I2 and I3A families of protease inhibitors, respectively (http://merops.sanger.ac.uk/index.shtml). At least three subfamilies of KSPIs have been characterized in vertebrates, and they are involved in various anti-inflammatory processes [7]. Generally, KSPIs are characterized by one or more Kunitz domains. Each Kunitz domain is composed of around sixty amino acid (aa) residues and stabilized by three conserved disulfide bridges [11]. KSPIs have been identified with one, two, three, or even more Kunitz domains [12].

Several KSPIs have been isolated from venomous and blood-sucking animals such as snakes, ticks, sea anemones, scorpions, and spiders [13–19]. A few studies have demonstrated the immune-defensive ability of KSPIs, particularly in some invertebrates [20,21]. However, no reports have characterized KSPIs in fish, although genomic information for some species is available in the NCBI (National Center for Biotechnology Information) database. In the present study on rock bream, we identified and characterized a novel SPI protein that contains two Kunitz domains. In addition, transcriptional regulation of this protein in response to the application of bacterial, viral, and immune stimulants and the protein's possible involvement in the wound-healing process were investigated.

## 2. Materials and methods

### 2.1. Construction of an mRNA sequence database and identification of rock bream KSPI cDNA

An mRNA sequence database was constructed for the rock bream using a Roche 454 Genome Sequencer FLX System (GS-FLX™) as described in a previous report [22]. A single sequence, homologous to other KSPIs, was selected from the rock bream database by means of homology screening using the Basic Local Alignment Search Tool (BLAST) in the NCBI.

### 2.2. Bioinformatics analysis

The identified *RbKSPI* gene was analyzed by DNAssist (version 2.2) to determine the open reading frame (ORF) and aa sequence. The NCBI BLASTp tool was employed to analyze the deduced aa sequence of RbKSPI and to identify homologous sequences in other species. Several tools from the Expert Protein Analysis System (ExPASy) [23] such as the SignalP 4.1 server for canonical signal peptides, the NetNGlyc 1.0 server for possible N-glycosylation sites, and the simple modular architecture research tool (SMART) for domain architecture were used. Multiple alignment analysis was performed using the ClustalW program. To determine the molecular distance among KSPI orthologs, a phylogenetic tree was constructed using the Neighbor-Joining (NJ) method [24] in MEGA software, version 6.0 [25]. The two Kunitz domains identified in RbKSPI were individually submitted to the SWISS-MODEL server [26] for protein structure homology analysis. The analogous models were derived using human KSPI (PDB ID: 1YC0 and 4ISN, respectively) as templates and visualized by PyMOL molecular graphic software version 1.3.

### 2.3. Animal rearing, immune challenges, and tissue-injury experiments

Healthy rock bream (average weight of 50 g) were acclimatized in a flat-bottomed tank (400 L) with sand-filtered seawater (salinity 34 ± 1 ppt, pH 7.6 ± 0.5) and continuous aeration at 24 ± 1 °C. All fish were reared in the tank for one week prior to the challenge experiments. To investigate the spatial distribution of *RbKSPI* mRNA, three healthy fish were randomly sampled, and nine different tissues (gills, kidney, intestine, liver, skin, heart, head kidney, spleen, and muscle) were removed by dissection after blood (~1 mL) had been collected with a sterile syringe.

For the immune challenge experiments, two bacterial strains (*Edwardsiella tarda* and *Streptococcus iniae*), rock bream iridovirus (RBIV), and two immunostimulants (LPS and Poly I:C) were injected as previously described [27]. Briefly, 100 μL of *E. tarda* ($5 \times 10^3$ CFU/μL), *S. iniae* ($1 \times 10^5$ CFU/μL), LPS (1.25 μg/μL of *Escherichia coli* 055:B5; Sigma) and Poly I:C (1.5 μg/μL; Sigma–Aldrich) suspended in phosphate-buffered saline (PBS) were injected intraperitoneally. Meanwhile, the RBIV ($10^2$ $TCID_{50}$/fish) suspended in PBS was injected intramuscularly. The same volume (100 μL) of PBS was injected into fish in a different group, which served as the negative control. Three fish, chosen randomly from each group, were sacrificed at different time intervals (3, 6, 12, 24, and 48 h), and blood cells and gill tissue were isolated. The injury experiment was conducted as previously described by Umasuthan et al. [28], and blood samples were collected at different time points (3, 6, 12, 24, and 48 h). All harvested tissues were snap-frozen in liquid nitrogen and stored at −80 °C.

### 2.4. Quantification of mRNA expression of RbKSPI

The total RNA extraction and first-strand cDNA synthesis were conducted on isolated tissues as previously reported [22]. To determine the expression level of *RbKSPI* mRNA, we performed SYBR Green quantitative real-time PCR (qPCR) on a TP800 Thermal Cycler Dice™ Real-Time System (TaKaRa) following MIQE guidelines [29]. Gene-specific primers were used to amplify the fragments of *RbKSPI* (Forward: CAACGCCTGCTATGGCTCAGAAA and Reverse: AGTCCTCGCACTTCTGCTCATCT) and rock bream *β-actin* (Forward: TCATCACCATCGGCAATGAGAGGT and Reverse: TGATGCTGTTGTAGGTGGTCTCGT), which was selected for use as an internal reference gene (Accession No. FJ975145). All qPCR was carried out in triplicate in a 20-μL reaction mixture containing 4 μL of cDNA template, 10 μL of 2 × SYBR® *Premix Ex Taq*™ (TaKaRa, Japan), 0.5 μL of each primer (10 pmol/μL) and 5 μL of $dH_2O$. The PCR profile included one cycle of 95 °C for 10 s, followed by 40 cycles of 95 °C for 5 s, 58 °C for 10 s, and 72 °C for 20 s. An additional cycle of 95 °C for 15 s, 60 °C for 30 s, and 95 °C for 15 s was performed to assess the specificity of the amplified product at the end of each reaction. The Livak method [30] was used to analyze the relative mRNA expression level of *RbKSPI*. All data were represented as mean ± standard deviation (SD), and the quantity of *RbKSPI* mRNA was relative to that of the *β-actin* mRNA. Significant differences between the challenge and control groups were analyzed with GraphPad (GraphPad Software, Inc.) statistical software, and the *P* value was set as $< 0.05$.

## 3. Results and discussion

### 3.1. Molecular characteristics of RbKSPI

Based on the EST analysis, a full-length cDNA sequence of rock bream *RbKSPI* (2452 bp) was identified and submitted to GenBank (Accession No. KP793739). The predicted coding sequence (CDS) of 1524 bp encoded a protein of 507 aa with a calculated molecular mass of 56,236 and theoretical isoelectric point (pI) of 5.7. A putative signal sequence was identified by the SignalP program at the N-terminus of RbKSPI, and the cleavage site was located between