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Multiple β-defensin isoforms identified in early developmental stages of the teleost *Paralichthys olivaceus*

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ABSTRACT

The β-defensin-like gene and its cloned isoforms (fBDI-1 to -5) were identified in an expressed sequence tag (EST) library from the early developmental stages of the olive flounder, *Paralichthys olivaceus*. The fBDI cDNA clones show identical amino acid sequences in 24 residues of the signal peptide and 38 residues of the mature peptide; however, the propiece region varies in sequence and length, from 5 to 15 amino acid residues. The predicted molecular weight of the mature peptide is 3.83 kDa, and its predicted isoelectric point is 4.1, showing anionic properties. The genomic organisation of the isoforms was analysed using bacterial artificial chromosome (BAC) DNA containing the fBDI gene. Southern blotting and sequence analyses of fBDI BAC DNA confirmed that the fBDI isoforms cluster at the same locus and exhibit the conserved gene organisation reported for other fish defensin genes. The fBDI mRNA was expressed constitutively in early developmental stages after hatching, and pathogen challenge induced fBDI expression in the head kidney of juvenile fish. We also produced a recombinant fBDI peptide (smfBD) using the expression plasmid pET32 and examined its bioactivity toward *Escherichia coli*.

1. Introduction

The immune system of vertebrates is composed of two major subdivisions, the innate immune system and the adaptive immune system. Innate immunity provides the first line of defense against external pathogens, and the adaptive immune system acts as a second line of defense and also affords protection against re-exposure to the same pathogen. Newly hatched fish larvae require defensive factors to resist pathogen attacks before their other defense mechanisms are fully developed. Although the time of appearance of specific immune parameters and adaptive defense competence varies greatly between fish species, the adaptive immune system develops late in marine species, which therefore depend on innate defenses for the first 2–3 months after hatching [1–4]. Additionally, for ectothermic vertebrates such as teleost fish, the innate immune system is more important because the adaptive immune defenses take a considerable time to respond and are very temperature-dependent [5].

A major mechanism of the innate immune system is the release of antimicrobial peptides. Antimicrobial peptides act very quickly and many exert their effect by disrupting the integrity of bacterial cell membranes and/or affecting intracellular targets. Defensins are small, cationic, amphipathic, cysteine-rich antimicrobial peptides found in plants, insects, and vertebrates, including fish. In vertebrates, three different defensin subfamilies exist (α-, β-, and θ-defensins), which differ in terms of disulfide bridge pairing and positioning of their six conserved cysteine residues [6]. In α-defensins, three disulfide bonds are formed by the linkage of C1–C6, C2–C4, and C3–C5, whilst in β-defensins, six cysteines are linked in the pattern C1–C5, C2–C4, and C3–C6. Moreover, θ-defensin, a cyclic peptide also containing three pairs of disulfide bonds, is only found in nonhuman primates, such as the rhesus macaque [7], and is believed to have arisen from peptide splicing of two hemi-α-defensins [8]. Additionally, neither α- nor θ-defensins have been found in phylogenetically earlier vertebrates, such as birds and fish, suggesting that all defensin subfamilies must have evolved from an ancestral β-defensin gene by duplication and diversification [9]. Most antimicrobial peptides, including defensins, are encoded by multigene families. In a recent comparative genomic study, multiple β-defensins were identified in some vertebrates, including 39 in humans, 37 in chimpanzees, 43 in dogs, 52 in mice, and 43 in rats [10,11]. The human genome has five β-defensin clusters, and the rodent and dog genomes have four different clusters[12]. In chicken, at least 14 β-defensin genes are located in a single ~86.0-kb β-defensin cluster on chromosome 3q3.5–q3.7 [13,14]. In fish, paralogue β-defensin sequences from higher vertebrates were

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recently identified in zebrafish (*Danio rerio*), fugu (*Takifugu rubripes*), tetraodon (*Tetraodon nigroviridis*) [15], rainbow trout (*Oncorhynchus mykiss*) [16], and medaka (*Oryzias latipes*) [17]. The β-defensins in zebrafish, fugu, and tetraodon were identified via a computational search strategy and were revealed to exist as multiple forms on the same or different chromosomes. Zebrafish β-defensin-1 and -2, termed zfDB-1 and -2, respectively, are located on chromosome 21, and β-defensin-3 (termed zfDB-3) is on chromosome 15 [15]. In rainbow trout and medaka, a single type of β-defensin gene was identified through expressed sequence tag (EST) analysis, and recombinant β-defensins have shown antiviral and antibacterial activities in rainbow trout and medaka, respectively, *in vitro* [16,17].

In this study, we identified a novel anionic β-defensin-like gene (fBDI) and its isoforms in early developmental stages of the olive flounder, *Paralichthys olivaceus*. Although fBDI and its isoforms were expressed constitutively during the larval period (between 1 day post-hatching (dph) and 35 dph), fBDIs in juvenile fish were induced by pathogen challenge. Moreover, we also assessed the biological activity of the recombinant peptide smfBDI against *Escherichia coli*. Our results suggest that inducible and constitutive fBDI expression may represent a key antimicrobial innate defense during early developmental stages in flounder.

2. Materials and methods

2.1. Fish

P. olivaceus larvae were obtained by induced spawning from brood stock at the Genetic and Breeding Research Center (GBRC) of the National Fisheries Research & Development Institute (NFRDI) on Geoje Island in South Korea. The different stages selected were 1, 3, 7, 14, 21, 28, and 35 dph. From the 3rd to the 10th day, larvae were fed with rotifers, *Brachionus plicatilis*. From the 11th to the 14th day, larvae were co-fed with rotifers and *Artemia nauplii*. From the 15th to the 19th day, larvae were fed with *A. nauplii* alone. From the 20th to the 28th day, larvae were co-fed with *A. nauplii* and a commercial diet, and from the 29th day onward, larvae were fed with a commercial diet alone. Five grams of larvae samples collected at each time point were rinsed twice with phosphate-buffered saline (PBS), frozen in liquid nitrogen, and kept at −80 °C until use.

The induction of flounder β-defensin expression was examined in the head kidney using RT-PCR following artificial bacterial infection with *Edwardsiella tarda* (strain KFE isolated from flounder in South Korea). For the artificial infection, healthy juvenile flounders (mean weight, 100 g) obtained from the GBRC were used. The fish were anaesthetised with MS-222 (3-aminobenzoic acid ethyl ester; Sigma) and infected with *E. tarda* by intraperitoneal injection of a sub-lethal dose (1.2×10^6 cells) suspended in PBS buffer. Tissues were collected from three fish at 0, 1, 3, 6, 12, 24, and 72 h post-injection, and frozen at −80 °C for RNA extraction.

2.2. RNA isolation, cDNA library construction, and EST analysis

Total RNA was immediately extracted using Trizol reagent according to the manufacturer's protocol (Invitrogen). Then, mRNA was purified from total RNA of each sample according to the manufacturer's instructions (Promega). A cDNA library was constructed from the mRNA mixture using 1 μg of each pooled sample from animals at 1, 3, 7, 14, 21, 28, and 35 dph using the ZAP-cDNA synthesis kit and ZAP-cDNA GigapackIII Gold cloning kit (Stratagene). Randomly selected cDNA clones were sequenced using a T3 primer with an ABI Prism 3130XL Genetic Analyzer and ABI sequence analysis software. BLAST analysis of all EST sequences revealed that six ESTs (FLDS-4-F10, FLDS-4-F11, FLDS-4-C12, FLDS-9-13-E02, FLDS-10-72-H09, and FLDS-14-46-F06) were homologous to β-defensin-1 in rainbow trout (*O. mykiss*; GenBank accession no. ABR68250). The EST clones were then selected for further cloning of the fBDI gene from olive flounder.

2.3. Bioinformatic analysis

Multiple alignments were performed with ClustalX [18] and refined using GENETYX version 8.0 (SDC Software Development, Japan). The putative signal peptide was predicted using the SignalP 3.0 Server (http://www.cbs.dtu.dk/services/SignalP/). A computer search for putative *cis*-elements was performed using the Transcription Element Search System (TESS) program (http://www.cbil.upenn.edu/cgi-bin/tess).

2.4. BAC clone screening

The flounder BAC library was constructed as described previously, with modifications [19]. In brief, high molecular weight DNA was isolated from the sperm of 10 brood stocks supplied by the GBRC of NFRDI. The dissociated sperm cells were embedded in agarose and digested with proteinase K, and the DNA was partially digested with *Eco*RI. The resultant fragments were ligated into the pCC1BAC vector (Epicentre). The average insert size of genomic fragments was approximately 100 kb. The BAC library of 76,800 clones was estimated to contain approximately 7.6 Gbp, which is more than 8.6-fold coverage of the 880 Mb of the *P. olivaceus* genome [20]. To screen a target gene from the BAC library, we constructed three levels of BAC DNA pools in accordance with a BAC pooling protocol [19].

The flounder genomic BAC library was screened for fBDI using the BAC pooling system with PCR primers specific for the fBDI open reading frame (ORF). The oligonucleotide sequences used in this study are given in Table 1. PCR-based BAC library screening was carried out as previously reported [19]. The obtained fBDI genomic BAC clones were purified and used for Southern blotting and genomic organisation analyses of fBDI.

2.5. Southern blot analysis

Approximately 5 μg of BAC DNA was digested with the restriction endonucleases *Bam*HI, *Hind*III, *Pst*I, and *Sal*I and separated on a 1.0% agarose gel by pulsed field electrophoresis using the CHEF Mapper (BioRad). The DNA was capillary-transferred to a piece of Hybond-N+ membrane (Amersham) with 20 × SSC using a standard protocol [21]. The DNA was UV-crosslinked to the membranes, which were then hybridised at 42 °C for 12 h in DIG Easy Hyb hybridisation solution (Roche) containing digoxigenin (DIG)-labeled DNA probe. The probe was labeled with a PCR DIG Probes Synthesis kit (Roche) using primers specific for the fBDI gene.

Table 1
Oligonucleotide primers used in this study.

Primer name	Sequence (5′–3′)	Purpose
fBDI-ORF-F	ATGTCTCGTTATCGTGTGGCT	BAC library screening
fBDI-ORF-R	TTGGCTGAATTATGGTTTGGT	BAC library screening
fBDI-RT-F	ACGCAGTTTCAGACCCAACA	Real-time PCR
fBDI-RT-R	CGGAACAGCCAAGAGCTCCA	Real-time PCR
smfBDI-F[a]	gggccatggCTATGTCTTGTTATCGTGTGGCTG	Recombinant peptide expression
smfBDI-R[a]	gggcagctgTTATGGTTTGGTTACGCAACATAC	Recombinant peptide expression
18S rRNA-F	ATGGCCGTTCTTAGTTCCTG	RT-PCR of internal control
18S rRNA-R	CCACGCTGATCCAGTCAGT	RT-PCR of internal control

[a] smfBDI-F and -R contain sites for restriction enzyme *Nco*I and *Sal*I (underlined), respectively.