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Identification, expression and bioactivity of *Paramisgurnus dabryanus* β-defensin that might be involved in immune defense against bacterial infection

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ABSTRACT

β-defensins are a large family of multi-disulfide-bonded peptides with broad-spectrum antimicrobial activities that contribute to innate host defense in many organisms, but little information is available about β-defensins produced by freshwater fish lacking scales. We therefore cloned and identified a β-defensin gene from Chinese loach (*Paramisgurnus dabryanus*) by designing degenerate primers and using thermal asymmetric interlaced PCR. This gene is the first defensin gene ever identified in a non-scaled freshwater fish. Annotation of the protein domain architecture showed that the putative Chinese loach β-defensin contains the signature motif of six conserved cysteines within the mature peptide, an aspect similar to β-defensins of other marine fish. We also used quantitative real-time PCR to investigate the expression pattern of the Chinese loach β-defensin gene, mRNA of which could be observed in various tissues. After challenge with the pathogenic bacterium *Aeromonas hydrophila*, β-defensin expression was induced in the eye, gill, skin, and spleen of the adult loach. The bioactivity of the recombinant *P. dabryanus* β-defensin was examined against pathogenic bacteria, and the results suggest that this class 2 β-defensin has potential applications for treatment of bacterial infections.

1. Introduction

Defensins are among the most studied antimicrobial peptides and are important mediators of the innate immune response. Defensins are small peptides (30–45 amino acid residues) that act as the first line of host defense against many bacteria, fungi, and viruses. They are mainly expressed in skin and mucosal epithelia where they kill pathogens by disrupting microbial membranes, thereby preventing pathogen colonization [1]. Defensins are also involved in endotoxin neutralization, leukocyte chemotaxis, immunomodulation, angiogenesis, iron metabolism, and wound repair [2–5].

Defensins are usually rich in positively charged amino acids like arginine and lysine and possess common characteristics including 3–4 disulfide bonds. Based on the position of the cysteines in the amino acid sequence and topology of the disulfide bonds, defensins are classified into three subfamilies, α, β, and θ. θ-defensins form a loop structure and have only been found in leukocytes of non-human primates [2]. Among the three defensins, β-defensins (BDs) are the primitive members in vertebrates; BDs evolved by duplication, positive selection, and chromosomal translocation to yield the α and θ forms that are found exclusively in mammals [6,7]. Most studies have focused on human BDs, which are mainly expressed in epithelial cells of different tissues. The BDs contain a triple antiparallel β-sheet stabilized by three disulfide bridges; an α-helix comprises the N-terminal region [8,9].

Compared with studies in mammals, fish BDs have been studied at only a rudimentary level. The first BD genes in fish were identified via mining of the genome databases for zebrafish (*Danio rerio*), tetraodon (*Tetraodon nigroviridis*), and fugu (*Takifugu rubripes*) [10]. BD genes in rainbow trout, medaka (*Oryzias latipes*), gilthead seabream (*Sparus aurata*), olive flounder (*Paralichthys olivaceus*), and orange-spotted grouper (*Epinephelus coioides*) have been identified through analysis of expressed sequence tags (ESTs), and recombinant BDs have been shown to have antiviral and antibacterial activities [11–16]. Interestingly, a BD gene was found to be predominantly expressed in pituitary and testis in grouper, suggesting a pivotal role for BDs in reproduction and endocrine regulation in addition to innate host defense [17].

The Chinese loach *Paramisgurnus dabryanus* (Cypriniformes: Cobitidae) is an important freshwater fish that lacks scales and is distributed across a wide area of Eurasia from Russia to China and

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Taiwan. It has been regarded as "Ginseng in water" and thus has great economic value; indeed parts of this loach have been included in traditional Chinese medicines for many years to treat inflammation, carbuncles, hepatitis, osteomyelitis, and for recovery from disabilities [18]. Because fish live in microbe-rich environments, their skin is at particularly high risk of infection as it is constantly exposed to water-borne microbes; thus the skin mucus provides fish with the primary line of defense against those pathogens. The skin of teleost fish is composed of five layers. The mucus layer covers the epidermis, and the epidermis is a stratified squamous epithelium composed of three cell layers: the superficial layer composed of flattened squamous cells, the intermediate layer composed of squamous and cuboidal cells, and the basal layer composed of columnar epithelial cells covering the basement membrane. The basement membrane separates the epidermis and the dermis. The scales are dermis structures and consequently are covered by the epidermis [19]. Because the Chinese loach lacks scales—an aspect of freshwater fish that is thought to underlie greater sensitive to water-borne pathogens—we considered this loach to be an excellent model in which to investigate the innate immune response.

Few studies have been performed on BDs from freshwater fish. Because there are no genome projects available to identify BD genes via database mining or EST analysis, we cloned and identified a BD gene from Chinese loach by designing degenerate primers based on an alignment of known BD amino acid sequences from marine fish. The observed antimicrobial activity of our recombinant *P. dabryanus* BD (pdBD) suggests that this new class 2 BD (BD-2) may have potential applications in fighting bacterial infections.

2. Materials and methods

2.1. Loach specimens, tissues and cells

Adult loach specimens were obtained from a local farm in Beijing, China. Fish were reared in a constant flow-through circulating aerated freshwater system at 27 °C with a 12:12 h light/dark photoperiod and fed twice daily. Fish were anesthetized with tricaine methanesulfonate (MS222, 120 mg/l, Sigma, USA), and tissue fragments of skin, brain, gill, kidney, liver, spleen, ovary, eye, heart, hindgut, and muscle were dissected and immediately frozen in liquid nitrogen, after which the samples were stored at −70 °C or used immediately for RNA isolation. For transfection studies, human embryonic kidney 293T (HEK293T) cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 100 μg/ml penicillin, and 100 μg/ml streptomycin. Cells were incubated at 37 °C in an atmosphere of 5% CO_2 in air.

2.2. Extraction of total RNA, cDNA synthesis, and genomic DNA isolation

Total RNA was extracted from above-mentioned samples using the SV Total RNA Isolation System (Promega, USA). RNA was quantified by measuring absorbance at 260 nm and 280 nm; RNA samples having a ratio of A260:A280 >1.8 were used for further experiments. RNA integrity was assessed by electrophoresis in 1.2% agarose gels. The ratio of 28S:18S rRNA signal intensity was always higher than 2.0. The First Strand cDNA Synthesis kit ReverTra Ace-α (TOYOBO, Japan) was used for first-strand cDNA synthesis. The resulting cDNA was stored at −20 °C. Genomic DNA was isolated using the CTAB method as described [20] and subsequently purified with a gel extraction kit (Omega, USA).

2.3. Design of degenerate primers and cloning of the full-length BD gene (pdBD) from P. dabryanus

Genomic DNA and total RNA were isolated and purified from *P. dabryanus* infected by *Aeromonas hydrophila* as described above. Two degenerate primers (DF-DP-F, DF-DP-R, Table 1) were designed based on an alignment of BD peptides from *Oncorhynchus mykiss* (GenBank: CBB12547.1), *Siniperca chuatsi* (GenBank: ACO88907.1), *O. latipes* (GenBank: ACG55699.1), and *E. coioides* (GenBank: AFA41485.1); the sequences of two regions (WTCGYRGLC and GCPRRYRCC), both located in the mature peptide, were used to design the primers. Thermal asymmetric interlaced PCR (TAIL-PCR) was applied to amplify the 5′ flanking regions by genome walking (gene walking kit, TAKARA, Japan) using the upstream specific primers DF-TAIL-SP1-3 with both cDNA and genomic DNA as template. The downstream specific primers DF-3′RACE-OUTER/INNER were designed to amplify the 3′ untranslated region by rapid amplification of cDNA ends (RACE) PCR (TAKARA 3′full RACE kit). Primers DF-YZ-F and DF-YZ-R for amplification of the predicted full-length *pdBD* were designed and tested with genomic DNA and cDNA to analyze the organization of *pdBD* and to confirm that the contiguous sequences obtained by genome walking and 3′RACE were from single transcripts. The PCR products were cloned into pEASY-T3 (TransGen, China) for sequencing and BLAST analysis.

2.4. Bioinformatic analysis

The translated pdBD protein sequence was subjected to phylogenetic analysis along with 33 complete homologenes available from GenBank; sequences were aligned using ClustalW according to Multiple Sequence Alignment (http://align.genome.jp/). Intron

Table 1
Primers designed for cloning full-length *pdBD* as well as for real-time PCR.

Name	Sequence (5′–3′)	Application
EF1α-F	ACAGCAAGAACGACCCACC	Real-time PCR
EF1α-R	AAAGCGACCAAGAGGAGGAT	Real-time PCR
DF-RQ-F	TATGACACAGAAATACAGGGATGGAC	Real-time PCR
DF-RQ-R	CTTCATAAGCAAGCGTTTCTCCATAATGG	Real-time PCR
DF-pcDNA-F	TGGATCCACCATGAGAGTACTGAGACTGCTTGTTAT	Expression
DF-pcDNA-R	TGAATTCTTAATGATGATGATGATGATGAAATCGCAAAGCACAGCACC	Expression
DF-DP-F	TGGACATGTGGCTAYMGNGGNYTNTG	Cloning
DF-DP-R	ACAGCATCTGTATCKTCKNGGRCANCC	Cloning
DF-YZ-F	ATGAGAGTACTGAGACTGCTTGTTAT	Cloning
DF-YZ-R	TTAAAATCGCAAAGCACAGCACCT	Cloning
DF-TAIL-SP1	CCAACTACATATTAGCCGGCCATTCCAC	TAIL-PCR
DF-TAIL-SP2	CAGTCACATGGTCTTCATAAGCAAGCG	TAIL-PCR
DF-TAIL-SP3	CTGTATCTTCGAGGGCATCCATGATAACCG	TAIL-PCR
DF-3′RACE-O	TGGCTACCGTGGGTTCTGC	3′RACE
DF-3′RACE-I	GGTGCCCCCGACGATACAGATGCTGT	3′RACE