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Identification and characterization of a β-defensin gene involved in the immune defense response of channel catfish, *Ictalurus punctatus*

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ABSTRACT

Antimicrobial peptides are small peptides that play important roles in a host's innate immune response. As an important antimicrobial peptide, β-defensin widely distribute in mammals, insects and plants with broad-spectrum antimicrobial activity. In this study, the β-defensin gene of the channel catfish, *Ictalurus punctatus*, was cloned, sequenced, and subjected to a bioinformatic analysis. The β-defensin gene of the channel catfish contains three exons and two introns, and encodes a precursor peptide consisting of two domains: a signal peptide of 24 amino acid residues and a mature peptide of 43 amino acid residues. The mature peptide is estimated to have a molecular mass of 7.1 kDa and a theoretical isoelectric point of 8.21. Channel catfish β-defensin (ccBD) has six conserved cysteine residues, forming three disulfide bridges at C1–C5, C2–C4, and C3–C6, and a β-sheet in the predicted three-dimensional structure. A phylogenetic analysis suggests that ccBD belongs to the type 1 β-defensins. Real-time quantitative PCR showed that channel catfish β-defensin transcripts are constitutively expressed in various tissues in healthy fish, with highest expression in the skin. The expression of *ccBD in vivo* increased significantly in the head kidney (2.9-fold), gill (2.2-fold), and skin (6.6-fold) at 48 h after bacterial (*Edwardsiella ictaluri*) challenge. *In vitro*, lipopolysaccharide (LPS), a bacterial mimic, induced significant changes in *ccBD* expression in leukocytes from the spleen (3.4-fold) and head kidney (3.9-fold) 24 h after stimulation. Chemically synthesized ccBD displayed marked inhibitory activity against a broad range of bacteria. These results suggest that ccBD is involved in the innate antibacterial defenses of the channel catfish.

1. Introduction

Antimicrobial peptides are crucial components of the innate immune system, potently increasing an animal's resistance to infection by pathogens. So far, more than 2600 antimicrobial peptides have been identified, with diverse sequences and structures, occurring in bacteria, fungi, plants, insects, fish, amphibians, birds, and mammals (Wang et al., 2016). More than 100 fish antimicrobial peptides have been isolated, expressed, and their antimicrobial activities analyzed. Antimicrobial peptides not only have broad antimicrobial activities, but also have other key biological properties, such as their participation in immunomodulation through the chemotaxis of the host immune cells and as potential bridges between the innate and adaptive immune systems (Hancock et al., 2016; Masso-Silva and Diamond, 2014).

Defensins, important members of the antimicrobial peptides, are rich in cysteines, and contribute to the host's defenses against bacterial, fungal, and viral infections (Brogden, 2005). Defensins can be divided into three classes based on the mode of disulfide bond formation between their cysteine residues: α-, β-, and θ-defensins. Among these, β-defensins are the ancestral defensins in the vertebrates, and were first isolated from bovine tracheal neutrophils and epithelial cells (Diamond et al., 1991). These peptides contain 35–50 amino acid residues, with disulfide bonds between cysteine 1 (C1)–C5, C2–C4 and C3–C6. Their characteristics have been investigated in fish (Zou et al., 2007), reptiles (Dalla Valle et al., 2012), birds (Van Dijk et al., 2008), and mammals (Lehrer and Ganz, 2002), and they are potentially expressed in all extant vertebrates. In humans, the β-defensins are widely expressed throughout the

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body in epithelial cells, macrophages, monocytes, and dendritic cells (DCs) (Hazlett and Wu, 2011; Semple and Dorin, 2012).

Unlike the mammalian β-defensins, fish β-defensins have only been studied to a rudimentary stage. To date, the β-defensin genes have been profiled in 14 kinds of fish (Masso-Silva and Diamond, 2014). Recent studies have focused on the antimicrobial activities and expression levels of the β-defensins in fish. The tilapia β-defensin gene showed increased expression after the fish was stimulated used bacteria and peptides potent antibacterial activity against *Streptococcus agalactiae* (Dong et al., 2015). The expression of β-defensin in the rainbow trout was significantly upregulated after stimulation with *Yersinia ruckeri in vivo* (Casadei et al., 2009; Falco et al., 2008). In atlantic cod, the expression of β-defensin was increased after challenged by *Vibrio anguillarum*, and recombinant peptides revealed antibacterial activity against *Planococcus citreus* and *Micrococcus luteus* (Ruangsri et al., 2012). The recombinant β-defensin in flounder was also displayed antibacterial activity against *Escherichia coli* (Bohye et al., 2010).

With application, the overexpression was showed antivirus abilities on recombinant β-defensin cell in orange-spotted grouper, suggested potential clinic value (Guo et al., 2012). Injecting synthetic NK-lysin, one of antimicrobial peptides to challenged tongue sole by *Vibrio anguillarum* was appeared significant decrease of bacteria quantity (Zhang et al., 2014). Antimicrobial peptides constitute potential new drugs, and have become a research hotspot. The channel catfish is an important commercial fish in China, but the health of channel catfish aquaculture is threatened by bacterial diseases (Geng et al., 2014, 2010; Wang et al., 2015a). Understanding the antimicrobial peptides of the channel catfish will contribute to the health of the aquaculture industry. Therefore, in this study, we cloned the gene encoding β-defensin (*ccBD*) in the channel catfish, *Ictalurus punctatus*, and analyzed the characteristics of the gene sequence and the derived amino acid sequence. The differential expression of the β-defensin gene was analyzed in different tissues. The antibacterial activity of the synthetic ccBD polypeptide was investigated. To understand the effects of bacterial infection on β-defensin expression, channel catfish were exposed to the Gram-negative bacterial pathogen *Edwardsiella ictaluri*, and leukocytes were challenged with a synthetic lipopolysaccharide (LPS), a gram-negative bacterial mimic.

2. Materials and methods

2.1. Experimental animals

Healthy cultured channel catfish (bodyweight: 608.5 ± 25.3 g; length: 34.2 ± 7.8 cm) were used in the tissue distribution study, and smaller fish (bodyweight: 62.5 ± 7.3 g; length: 13.4 ± 2.1 cm) were used in the challenge experiment. Both sets of fish were purchased from a local fishery (Sichuan, China). The fish were maintained under laboratory conditions (temperature: 23 ± 1 °C; pH 7.3 ± 0.2) and kept in tanks (60 × 50 × 40 cm^3) for an acclimation period of 1 week. During that time, the fish were fed a commercial feed, with a daily ration equivalent to 2% of their total biomass. All the experimental fish handling procedures were approved by the Animal Care and Use Committee of Sichuan Agricultural University.

2.2. RNA extraction and cDNA synthesis

Total RNA was extracted from the various fish tissues with RNAiso Plus (Takara, Dalian, China), according to the instructions of the manufacturer. The RNA concentration and purity were determined by RNA electrophoresis and the absorbance ratio (A_{260}/A_{280}) in a NanoDrop 2000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). Each RNA with an absorbance ratio of 1.8–2.0 was used for cDNA synthesis. cDNA was synthesized with the PrimeScript™ RT Reagent Kit with gDNA Eraser (Perfect Real Time; TaKaRa), according to the manufacturer's protocol.

2.3. cDNA cloning of ccBD

To isolate the cDNA of *ccBD*, a homology search in channel catfish (taxid: 7998) Transcriptome Shotgun Assembly (TSA) Database of NCBI (http://blast.ncbi.nlm.nih.gov/Blast.cgi) was performed with the zebrafish β-defensin 1 sequence (CAJ57442). A number of putative channel catfish β-defensin TSA sequences were obtained (GenBank accession nos: JT417488.1, JT349607.1, GELA01019385.1, GELA01022641.1, and GEHJ01127439.1). Based on these sequences, we identified a putative channel catfish β-defensin sequence containing a complete open reading frame (ORF). The primer pairs *Ip*β-defensin-F and *Ip*β-defensin-R were designed (Table 1), and cDNA generated from the RNA extracted from the spleens of fish stimulated with *Edwardsiella ictaluri* was used as the PCR template. The cycling parameters were 94 °C for 5 min, followed by 35 cycles at 72 °C for 30 s, 55 °C for 30 s, and 72 °C for 1 min, with a final extension at 72 °C for 5 min. The PCR fragment was subjected to electrophoresis on a 2.0% agarose gel. The PCR product was extracted with the TaKaRa MiniBEST Agarose Gel DNA Extraction Kit (TaKaRa), and then cloned into the pMD19-T vector (TaKaRa). *Escherichia coli* DH5α cells were transformed with the plasmid, which was sequenced by Chengdu Qingke Biotech Corp. (Sichuan, China).

2.4. In silico analysis

The potential open reading frame (ORF) and deduced amino acid sequence of ccBD were analyzed with the EditSeq software (Lasergene, Madison, WI). The *ccBD* gene structure and location (synteny analysis) were analyzed with BLAST at http://blast.ncbi.nlm.nih.gov/Blast.cgi and http://www.genomicus.biologie.ens.fr/ (Qi et al., 2015). The deduced amino acid sequence was analyzed at the ExPASy Molecular Biology server (http://www.expasy.org/tools/) and the signal peptide was predicted at the SignalP 4.1 server (http://www.cbs.dtu.dk/service/SignalP). A three-dimensional (3D) structural homology model was predicted at the CPHmodels online 3.2 server (http://www.cbs.dtu.dk/services/CPHmodels/) and visualized with the PyMOL Molecular Graphics System software (http://www.pymol.org/) (Bandyopadhyay et al., 2014). A multiple-sequence alignment including the deduced amino acid sequences was constructed with Mobyle @Pasteur (http://mobyle.pasteur.fr/) and a phylogenetic tree was constructed with the neighbor-joining (NJ) algorithm with the MEGA7 software (http://www.megasoftware.net) (Dong et al., 2015).

Table 1
Oligonucleotide primers used for cloning and expression analysis.

Primer name (forward and reverse)	Primer sequence (5′ → 3′)	Primers used
*Ip*β-defensin-F	CAAGCATGAAACCTCAGTGTACACT	Cloning
*Ip*β-defensin-R	TCAGCAGTCAAAGGAAATATGACAC	Cloning
β-defensin-F	ACTTCTGGTCCTGCTGGTCATCTTA	qPCR
β-defensin-R	AGACATGCACCTTGTCTGCAAACTC	qPCR
EF-1α-F	GTTGAAATGGTTCCTGGCAA	qPCR
EF-1α-R	TCAACACTCTTGATGACACCAAC	qPCR
18S rRNA-F	GGACACGGAAAGGATTGACAGA	qPCR
18S rRNA-R	GAGGAGTCTCGTTCGTTATCGG	qPCR