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Molecular characterization and functional analysis of two distinct liver-expressed antimicrobial peptide 2 (LEAP-2) genes in large yellow croaker (*Larimichthys crocea*)

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ABSTRACT

Liver-expressed antimicrobial peptide 2 (LEAP-2) plays a vital role in the host innate immune system. In the present study, two LEAP-2 genes (LcLEAP-2A and LcLEAP-2C) from large yellow croaker (*Larimichthys crocea*) were cloned, both of which consist of 3 exons and 2 introns. The LcLEAP-2A transcripts were expressed in a wide range of tissues, with the highest mRNA levels found in the liver and intestine, while LcLEAP-2C transcripts showed obvious lower mRNA levels in all tested tissues compared to LcLEAP-2A. Upon infection by *Vibrio alginolyticus*, LcLEAP-2A transcripts were significantly up-regulated in liver, trunk kidney, spleen, head kidney, and gill, but down-regulated in intestine. In addition, significant up-regulation of LcLEAP-2C transcripts were also detected in all tissues tested, including intestine. The LcLEAP-2A and LcLEAP-2C mature peptides were chemically synthesized and found to exhibit selective antimicrobial activity *in vitro* against various species of bacteria. LcLEAP-2C, but not LcLEAP-2A, had antimicrobial activity against *V. alginolyticus*. Moreover, LcLEAP-2C treatment at low concentrations was evaluated and found to improve survival rate in *V. alginolyticus*-infected large yellow croaker, resulting in a decrease in bacterial load and expression of inflammatory cytokines. These results suggest that LcLEAP-2 isoforms play an important role in innate immunity by killing bacteria and inhibiting early inflammatory response in large yellow croaker.

1. Introduction

The large yellow croaker (*Larimichthys crocea*) is a very economically important species of marine cultured fish [1]. However, recent rapid growth of the large yellow croaker farming industry has led to increasingly severe outbreaks of infectious diseases caused by marine microorganisms such as *Vibrio alginolyticus*, resulting in great economic losses [2–4]. Although a variety of effective first-line drug treatments have recently been developed to control marine bacteria, these drugs often negatively affect many organisms, including fish and humans [5,6]. Moreover, the increasing number of antibiotic-resistant pathogenic microorganisms in the aquatic environment create a challenge for development and use of antibiotic strategies to control fish diseases [7,8]. Thus, there is a strong need for effective and environmentally friendly commercial therapeutics against marine bacteria. In this regard, considerable attention has been paid to antimicrobial polypeptides (AMPs), a class of peptides known as nature's defense against pathogens. Now, several AMPs have been found in large yellow croaker. Hepcidin is expressed mainly in kidney and shows broad spectrum of antimicrobial activity, especially more effective to marine Vibrio [9,10]. Piscidin, a typically gill-expressed peptide, plays an important role against external protozoan parasite [11]. AMP-W3, a peptide from the gastrointestinal tract, shows a broad spectrum of antimicrobial activity against bacteria and fungi [12].

AMPs play a vital role in the innate immune system as antimicrobial agents by disrupting the membrane integrity of invading microbes [13–15]. Since the aquatic environment provides considerable exposure to various pathogens, fish possess a very large number of AMPs against a broad spectrum of pathogens [16–18]. LEAP-2, first isolated from human in 2003, is an important blood-derived peptide that exhibits antimicrobial activity [19,20]. Structure analysis showed that the human LEAP-2 protein contains a core structure with two disulfide bonds, which are not essential for the bactericidal activity [21]. Recently, LEAP-2 sequences have been cloned and reported in some teleosts, including grass carp (*Ctenopharyngodon idella*) [22], channel catfish (*Ictalurus punctatus*)

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[23], blue catfish (*Ictalurus furcatus*) [23], rainbow trout (*Oncorhynchus mykiss*) [24], Japanese flounder (*Paralichthys olivaceus*) [25], common carp (*Cyprinus carpio*) [26] and blunt snout bream (*Megalobrama amblycephala*) [27].

The tissue expression pattern of LEAP-2 can vary in different fish species. For example, LEAP-2A and LEAP-2C transcripts are only detected in the liver of rainbow trout [24], while LEAP-2 transcripts in channel catfish are considerably lower in liver than in other tissues [23]. The common carp LEAP-2A expression levels in liver are lower than in several other tissues [26]. Some studies show that LEAP-2 expression is closely related to fish immune response against pathogenic organisms. In rainbow trout infected with *Aeromonas salmonicida*, LEAP-2 transcripts in the intestine and liver are induced [28]. Upon infection by *Aeromonas hydrophila*, LEAP-2 expression in grass carp is significantly up-regulated in liver, gill, skin, muscle, spleen, blood, head kidney, heart, and intestine, but down-regulated in trunk kidney and brain [22]. It has been reported that the human LEAP-2 possesses antimicrobial activity correlating with membrane affinity [29]. Furthermore, LEAP-2 is not involved in the physiological response of epithelia to iron, nor is it mitogenic for epithelial cells or chemotactic for monocytes [20]. Moreover, human LEAP-2 has also been found to bind with plasmid DNA [29]. However, the role of LEAP-2 in regulating the fish innate immunity against bacterial infection still remains obscure.

In this study, two LEAP-2 genes were identified in the large yellow croaker and named LcLEAP-2A and LcLEAP-2C. The mRNA expression of these sequences in different tissues after bacterial challenge was studied, and the antimicrobial activity of peptides was examined *in vitro* and *in vivo*.

2. Materials and methods

2.1. Fish rearing

Large yellow croaker, weighting 65–70 g, were obtained from a commercial farm in Ningbo, China. Fish were kept in seawater tanks at 25–27 °C in a recirculating system with filtered sea water. After acclimatizing for one week, the fish were used in experiments as described below. All experiments were performed according to the Experimental Animal Management Law of China and approved by the Animal Ethics Committee of Ningbo University.

2.2. Bacterial challenge

Overnight cultures of *V. alginolyticus* ATCC 17749 were diluted 1:100 in Tryptic Soy Broth Medium (TSB) (Sigma, Shanghai, China), grown at 28 °C with shaking, and harvested in the logarithmic phase of growth. Cells were washed once in sterile phosphate buffer solution (PBS), resuspended, and then diluted to the appropriate concentration in sterile PBS. The final concentration of bacteria was confirmed by plating serial dilutions on solid media. The 50% lethal dose (LD_{50}) of *V. alginolyticus* was determined to be about 5×10^6 CFU/fish in this study. Large yellow croaker were infected with *V. alginolyticus* (5×10^6 CFU/fish) by intraperitoneal (ip) injection with PBS as a control. The liver, spleen, head kidney, trunk kidney, intestine, and gill were collected at 0, 4, 8, 12, and 24 h post-injection (hpi) as previously reported [30], and preserved at −80 °C until examined.

2.3. Determination of LcLEAP-2A and LcLEAP-2C cDNA and genomic sequences

The cDNA sequences of the LcLEAP-2A and LcLEAP-2C genes were determined by using a liver transcriptome analysis of large yellow croaker in combination with RACE [31]. The authenticity of LcLEAP-2A and LcLEAP-2C cDNA was confirmed by reverse transcription polymerase chain reaction (RT-PCR) amplification, followed by cloning and sequencing. The genomic DNA of large yellow croaker was isolated from liver tissue with a DNA Extraction Kit (TaKaRa, Dalian, China), dissolved in TE buffer, and stored at −80 °C before use. To amplify the genomic DNA sequence of LcLEAP-2A and LcLEAP-2C, long-distance PCR amplification was carried out using LA Taq DNA polymerase (TaKaRa) as previously described [31]. Based on the cDNA sequences of LcLEAP-2A and LcLEAP-2C, primers were designed to amplify the genomic sequences (Table 1). Thermal cycling conditions for PCR were as follows: 94 °C for 10 min, 30 cycles of 94 °C for 30 s, 58 °C for 30 s, and 72 °C for 2 min, and a final extension at 72 °C for 10 min. The PCR product was cloned into the pMD19-T Simple Vector (TaKaRa) and sequenced (Invitrogen, Shanghai, China).

2.4. Sequence analysis

LEAP-2 sequence used for multiple alignment and phylogenetic analysis were listed in Table 2. The similarity between the obtained sequences with other known sequences was analyzed using BLAST search (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The cleavage site of signal peptides was predicted by the SignalP4.1 program (http://www.cbs.dtu.dk/services/SignalP/). Multiple sequence alignment and phylogenetic and molecular evolutionary analyses were conducted using MEGA version 5 [32].

2.5. Real-time quantitative PCR (RT-qPCR)

To determine the tissue expression patterns of LcLEAP-2A and LcLEAP-2C, liver, trunk kidney, spleen, head kidney, intestine, and gill were collected separately from three healthy fish for RNA extraction. To examine the variation in mRNA expression of LcLEAP-2A and LcLEAP-2C in tissues following stimulation with *V. alginolyticus* fish were sacrificed and tissues were harvested at 4, 8, 12, and 24 hpi. To identify the change in mRNA expression of LcTNF-α and LcIL-1β in tissues as a result of *V. alginolyticus* infection followed by treatment with chemically synthesized LcLEAP-2A and LcLEAP-2C mature peptides (GL Biochem, Shanghai, China), fish were sacrificed and tissues were harvested at 4, 8, 12 or 24 h after LcLEAP-2A and LcLEAP-2C treatment.

RT-qPCR was carried out as previously described [30]. Total RNA for cDNA synthesis was extracted from large yellow croaker tissues by using RNAiso reagents (TaKaRa). Gene-specific primers were designed based on the cloned cDNA fragments of LcLEAP-2A, LcLEAP-2C, LcTNF-α, and LcIL-1β (Table 1). As an internal PCR

Table 1
Oligonucleotide primers used in this work.

Gene	Primer	Sequence (5′→3′)	Accession number
LcLEAP-2A	LEAP-2AgF	GGTCCGTTGGTCCAACAAC	KJ024787
	LEAP-2AgR	CTAGTAGTTCACGGTCTCTGA	
	LEAP-2AF	TGAGGAGGATAGCTCGGATG	
	LEAP-2AR	GTTCACGGTCTCTGAGGTGG	
LcLEAP-2C	LEAP-2CgF	CTGCCGGTGCCTGAAGAC	KJ024789
	LEAP-2CgR	TCAGCTGGAGATCCAGAAAG	
	LEAP-2CF	GGTGCCTGAAGACCAGAATG	
	LEAP-2CR	CAGTATTTTGTGCCGCACTC	
LcIL-1β	IL-1βF	ATCTGGCAAGGATCAGCTCA	DQ306711
	IL-1βR	ACCAGTTGTTGTAGGGGACG	
LcTNF-α	TNF-αF	TGGAGTGGAAGAACGGTCAA	EF070393
	TNF-αR	GAGAGGTGTGAGGCGTTTTC	
Lcβ-actin	β-actinF	GATGTGGATCAGCAAGCAGG	GU584189
	β-actinR	GAGCTGAAGTTGTTGGGTGT	