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Molecular characterization of the liver-expressed antimicrobial peptide 2 (LEAP-2) in a teleost fish, *Plecoglossus altivelis*: Antimicrobial activity and molecular mechanism

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ABSTRACT

Liver-expressed antimicrobial peptide 2 (LEAP-2) is widespread in fish and plays an important role in the host's innate immune system. In this study, full-length cDNA for LEAP-2 (PaLEAP-2) gene was cloned and sequenced from ayu, *Plecoglossus altivelis*. PaLEAP-2 mRNA was detected in a wide range of tissues, with the highest level of transcripts found in the liver. Upon induction by *Vibrio anguillarum*, its expression significantly increased in the liver, kidney, spleen, gill, and heart, but decreased in the intestine. The PaLEAP-2 mature peptide was chemically synthesized; it exhibited selective antimicrobial activity against various bacteria *in vitro*. PaLEAP-2 at high concentration reduced the bacterial load and improved the survival rate of *V. anguillarum*-infected ayu. Moreover, it inhibited the expression of mRNAs for *TNF-α* and *IL-1β* in *V. anguillarum*-infected ayu, both at high and low concentrations. PaLEAP-2 induced hydrolysis of the pET-22b plasmid DNA and bacterium genomic DNA. These results suggest that PaLEAP-2 plays a role in ayu immune responses against bacterial infection.

1. Introduction

Ayu, *Plecoglossus altivelis*, is an economically important freshwater fish cultured in Asian countries, including Japan, China, and Korea. Over the past 10 years, ayu farming has rapidly increased in China, and diseases caused by *Vibrio anguillarum* have become widespread in this fish (Li et al., 2009). A variety of antibiotics have been used extensively for controlling bacteria in aquatic animals (Kümmerer, 2009). However, these drugs often accumulate in high levels in aquatic organisms such as fish and eventually affect human health (Costanzo et al., 2005; Hahn and Schulz, 2007). Moreover, the increasing numbers of antibiotic-resistant bacteria in the aquatic ecosystems are a challenge in the use of antibiotic strategies in the control of fish diseases (Gao et al., 2012). Antimicrobial peptides (AMPs), which are mostly positively charged molecules with short amino acid chains, defend the host against pathogens by either direct antimicrobial action or a broad range of immunomodulatory functions (Jenssen et al., 2006; Steckbeck et al., 2014).

Liver-expressed antimicrobial peptide (LEAP) is cysteine-rich peptides. Two forms of LEAP were isolated from human blood, named LEAP-1 and LEAP-2 (Krause et al., 2000, 2003). LEAP-1 is also termed hepcidin, has four pairs of disulfide bonds, and is predominantly expressed in the human liver (Krause et al., 2000). The antimicrobial activities of LEAP-1 have been identified against bacteria, fungi, viruses and protozoans *in vivo* and *in vitro* (Marguti, 2012; Rajanbabu and Chen, 2011; Shike et al., 2002). Furthermore, LEAP-1 has been reported to play an important role in the maintaining normal iron homeostasis (Ganz, 2005; Nemeth et al., 2004; Nicolas et al., 2002). The expression of LEAP-1 mRNA was found to be induced by viruses (Zhou et al., 2011), lipopolysaccharides (Masso-Silva et al., 2011) and bacteria (Cuesta et al., 2008). LEAP-2 is the human blood-derived peptide with antimicrobial activity that is predominantly expressed in the liver (Krause et al., 2003); it has four highly conserved cysteine residues that form two pairs of disulfide bonds (Henriques et al., 2010; Hocquellet et al., 2010; Howard et al., 2010). Some studies have demonstrated that LEAP-2 has selective antimicrobial activity against bacteria and fungi (Hocquellet et al., 2010; Li et al., 2014; Liu et al., 2010). Like other cationic AMPs, LEAP-2 disrupts the physical integrity of bacterial membranes (Townes et al., 2009), and human LEAP-2 binds

Abbreviations: hpi, hours post infection; LEAP-2, liver-expressed antimicrobial peptide 2; MIC, minimum inhibitory concentration; RT-qPCR, real-time quantitative PCR.

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the plasmid DNA (Hocquellet et al., 2010). Moreover, it has been reported that hepatitis C virus and human immunodeficiency virus infections were associated with decreased expression of LEAP-2 in the colonic tissue, which suggested that LEAP-2 may play an important role in immune regulation (Shata et al., 2013).

LEAP-2 sequences have been cloned and reported in several teleosts: rainbow trout (*Oncorhynchus mykiss*) (Zhang et al., 2004), channel catfish (*Ictalurus punctatus*) (Bao et al., 2006), blue catfish (*Ictalurus furcatus*) (Bao et al., 2006), grass carp (*Ctenopharyngodon idella*) (Liu et al., 2010), blunt snout bream (*Megalobrama amblycephala*) (Liang et al., 2013), common carp (*Cyprinus carpio*) (Yang et al., 2014), yellow catfish (*Pelteobagrus fulvidraco*) (Ren et al., 2014), large yellow croaker (*Larimichthys crocea*) (Li et al., 2014) and miiuy croaker (*Miichthys miiuy*) (Liu et al., 2014). In fish, LEAP-2 plays an important role in the first-line defense against bacterial invasion within the innate immune system. The mRNA expression of this peptide can be dramatically increased by bacterial challenge (Liu et al., 2014; Ren et al., 2014; Yang et al., 2014; Zhang et al., 2004). LEAP-2 transcription was significantly up-regulated in the skin of the common carp (Yang et al., 2014); in the in intestine of rainbow trout (Zhang et al., 2004); in the liver of grass carp (Liu et al., 2010). These differences of LEAP-2 induction patterns indicate that its expressions of various species in response to bacterial challenge may be species-specific in fish. LEAP-2 isoforms have been reported in several fish species (Li et al., 2014; Yang et al., 2014), and they present a different antimicrobial spectrum against gram-positive, gram-negative bacteria and fungi (Li et al., 2014).

In the present study, the full-length cDNA of *LEAP-2* was identified in ayu, and its mRNA expression after bacterial challenge was studied in different tissues. The antimicrobial activity of PaLEAP-2 was examined *in vitro* and *in vivo*, and its activity in hydrolyzing DNA was measured.

2. Materials and methods

2.1. Determination of ayu LEAP-2 (PaLEAP-2) cDNA

Only one type of PaLEAP-2 cDNA had been identified by high-throughput transcriptome analysis. The cDNA sequence of the PaLEAP-2 gene was subsequently obtained using the transcriptome analysis of ayu in combination with rapid amplification of cDNA ends (RACE). 5′-RACE was performed using the 5′-RACE kit (Smart RACE cDNA amplification kit; Clontech, TaKaRa, Dalian, China). The first polymerase chain reaction (PCR) was performed with the 5′-RACE outer primer (Supplemental Table 1), and the resulting PCR products were used as a template for the second PCR using 5′-RACE inner primer (Supplemental Table 1). The sequence obtained by RACE was identical to that obtained in the transcriptome analysis. The authenticity of PaLEAP-2 cDNA was confirmed by reverse transcription (RT)-PCR amplification, followed by cloning and sequencing. Multiple alignments were analyzed using ClustalW (http://clustalw.ddbj.nig.ac.jp/). Phylogenetic and molecular evolutionary analyses were conducted using MEGA version 5 (Tamura et al., 2011). Related LEAP-2 sequences are listed in Supplemental Table 2.

2.2. Fish rearing

Ayu, weighing 20–25 g, were obtained from a commercial farm in Fuxi, Ninghai County, Ningbo City, China. Fish were kept in water tanks at 20–22 °C with a recirculating system with filtered water as previously described (Lu et al., 2015). The fish were fed with pelleted dry food once a day and acclimatized to laboratory conditions for 2 weeks before experiments. All experiments were performed according to the Experimental Animal Management Law of China and approved by the Animal Ethics Committee of Ningbo University.

2.3. Bacterial challenge

V. anguillarum challenge was performed as described previously (Lu et al., 2015). Overnight cultures of *V. anguillarum* were diluted 1:100 in a 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, resuspended, and adjusted to a final concentration of 1.2×10^5 colony-forming units (CFU)/mL in sterile saline. To obtain dead *V. anguillarum*, liquid cultures were heat inactivated at 60 °C for 1 h before harvesting the bacteria. Ayu were challenged by intraperitoneal (ip) injection of viable or dead *V. anguillarum* at a dose of 1.2×10^4 CFU, and saline was used as control. Ayu tissues (the brain, gills, heart, kidney, liver, and spleen) were collected at 4, 8, 12, and 24 h post infection (hpi), frozen in liquid nitrogen, and preserved at 80 °C until further examination.

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

To determine the tissue expression patterns of PaLEAP-2, the liver, kidney, spleen, intestine, heart, and gill tissues were collected separately from three healthy fish for RNA extraction. To examine the variation in PaLEAP-2 mRNA expression following stimulation with viable or dead *V. anguillarum*, fish were sacrificed and tissues were harvested at 4, 8, 12, and 24 hpi. To examine the changes in *PaTNF-α* and *PaIL-1β* mRNA expression after viable or dead *V. anguillarum* infection followed by treatment with chemically synthesized PaLEAP-2 mature peptides (GL Biochem, Shanghai, China), the fish were sacrificed and tissues were harvested at 4, 8, 12, or 24 h after PaLEAP-2 treatment.

RT-qPCR was carried out as previously described (Lu et al., 2015). Total RNA for cDNA synthesis was extracted from ayu tissues using RNAiso reagents (TaKaRa). Gene-specific primers were designed on the basis of sequenced cDNA fragments of *PaLEAP-2*, *PaTNF-α*, *PaIL-1β*, and *Paβ-actin* (Supplemental Table 1). RT-qPCR was performed using a SYBR premix Ex Taq (Perfect Real Time) (TaKaRa). The reaction mixture was incubated for 5 min at 95 °C, followed by 35 amplification cycles of 30 s at 95 °C, 30 s at 60 °C, and 30 s at 72 °C in an RT-Cycler™ real-time fluorescence quantitative PCR thermocycler (CapitalBio, Beijing, China). Cycle threshold (Ct) values of PaLEAP-2 for all samples were normalized to *Paβ-actin* using the ΔCt method. For each group, tissue samples were taken from three fish.

2.5. Antimicrobial activity in vitro

The mature peptides of PaLEAP-2 were chemically synthesized to over 95% purity (GL Biochem) containing two disulfide bonds (Cys63 with Cys74, Cys69 with Cys79). Antimicrobial activities of the synthetic PaLEAP-2 were determined against a panel of microorganisms including *Vibrio vulnificus*, *Pseudomonas putida*, *Escherichia coli* DH5α, *Edwardsiella tarda*, *Vibrio alginolyticus*, *Vibrio parahaemolyticus*, *V. anguillarum*, and *Pseudomonas aeruginosa*. A microdilution assay was used to determine the minimal inhibitory concentration (MIC) of various agents as previously described, with some modifications (Chang et al., 2006; Li et al., 2014). The MIC was determined as the lowest concentration of synthesized mature peptides that inhibited the growth of the tested microorganism. Briefly, serial dilutions of the peptides were made to 100, 50, 25, 12.5, 6.25, 3.125, and 1.563 μg/mL final concentrations in 96-well microtiter plates. An aliquot of 80 μL of each solution was added to the corresponding well of a 96-well plate. The bacteria were incubated to mid-logarithmic phase and diluted in suitable media to give final