

Molecular Characterization and Expression Pattern of a Liver-Expressed Antimicrobial Peptide 2 (LEAP-2) Gene in Yellow Catfish (*Pelteobagrus fulvidraco*)

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Abstract

Liver-expressed antimicrobial peptide 2 (LEAP-2) is a member of antimicrobial peptides, which acts as an important component of the host innate immune system and plays a crucial role in host defense against microbial invasion. In this study, a LEAP-2 gene (P_fLEAP-2) was amplified from the liver of yellow catfish (*Pelteobagrus fulvidraco*) by reverse transcription PCR. The length of P_fLEAP-2 open reading frame was 282 bp, which encode 94 amino acids (aa) including a 28-aa signal peptide, a 21-aa prodomain, and a 45-aa mature peptide. The results of tissue distribution showed that P_fLEAP-2 mRNA was mainly expressed in the gill, skin, stomach, and head kidney. The significant up-regulation of P_fLEAP-2 in liver after 4 hours of challenge of *Edwardsiella tarda* suggested P_fLEAP-2 might play an important role in the early immune defense of the yellow catfish against bacterial pathogen.

Keywords: *Pelteobagrus fulvidraco*; LEAP-2; Expression pattern; Bacterial challenge

Introduction

Antimicrobial peptides (AMPs) are essential components of the host nonspecific defense system in organisms, which act as antimicrobial agents by disrupting the membrane integrity of invading pathogens [1-4]. Because of scarce drug-resistance to microbes, AMPs are considered as promising therapeutics against antibiotic-resistant bacterial strains [5,6]. Liver-expressed antimicrobial peptide 2 (LEAP-2) is the second blood-derived peptide from human that is predominantly expressed in the liver and exhibits antimicrobial activity [7]. All the members of the LEAP-2 family have a highly conserved motif containing four cysteine residues to form two disulphide bonds which makes it to exhibit distinct amino acid sequence, secondary structure and expression pattern from other peptides [7]. The characteristics of LEAP-2 have been reported in human, monkey, pig, mouse, cow, and chicken [8-12]. To date, LEAP-2 genes have been reported only in limited fish species including channel catfish [10], grass carp [11], rainbow trout [12], and blunt snout bream [13]. However, the molecular characteristics of LEAP-2 and its functions in innate host defense in fish are still unclear.

Yellow catfish *Pelteobagrus fulvidraco* is an important cultured fish species in China. Despite of the importance in aquaculture, there is little information on the immunological mechanism in this fish. Actually, the problem of diseases mainly caused by pathogenic bacteria such as *Edw. ictaluri* and *Edw. tarda* is more and more serious in yellow catfish and causes serious losses [14]. In present study, we isolated and characterized a LEAP-2 gene designated P_fLEAP-2 from yellow catfish *P. fulvidraco*. Tissue-specific expression pattern of P_fLEAP-2 was tested by quantitative real-time PCR. Expression profile of P_fLEAP-2 in liver after bacterial challenge was also analyzed. In addition, antimicrobial activity of recombinant P_fLEAP-2 mature peptides was discussed.

Materials and Methods

Fish cultivation and sample collection

Yellow catfish with body mass about 200 g were obtained from a commercial fish farm in Shaoxing City, Zhejiang Province, China. All fish were healthy and acclimatized for 14 days in a circulating water

system with temperature at 22-23°C and dissolved oxygen above 5 mg/L. The experimental fishes were euthanized with tricaine methane sulfonate (MS222) at a concentration of 100 mg per liter before sample collection. Samples were frozen in liquid nitrogen and stored at -80°C until analysis.

Cloning of P_fLEAP-2 cDNA

Total RNA was extracted from the liver of yellow catfish using TRIzol Reagent (Invitrogen, Carlsbad, USA) according to the manufacturer protocol, and quantified by measuring absorbance at 260 nm with a Nano Drop 2000 spectrophotometer (Thermo Scientific, Wilmington, USA). Gene-specific primers for RT-PCR, LEAP-2F and LEAP-2R, were designed based on P_fLEAP-2 sequences of channel catfish *Ictalurus punctatus* (GenBank accession no. AY845141) and blue catfish *I. furcatus* (GenBank accession no. AY845142). The first-strand cDNA was synthesized with the M-MLV Reverse Transcriptase Rnase H Minus, Point Mutant (Promega, Madison, USA) in a reaction mixture of 20 µl containing 4 µl×5 reaction mix, 200 ng total RNA, 1 µl primer of Oligo(dT)12-18 (20 µM), 2 µl SuperScript III RT/Platinum Taq polymerase mix, 1 µl dNTP. The reactions were completed in a thermocycler at 42°C for 60 min. The first strand of cDNA was used as the template to amplify the fragment using the gene-specific primers LEAP-2F (5'-TCTAGAAAAGAGCAACATCATTTCAGCAG-3') and LEAP-2R (5'-GAATTCTTAGGAAATTATAGGTTGGCTAAAGG-3'). The amplification conditions were: 94°C for 2 min, then 30 cycles of 94°C for 45 s, 55°C for 45 s, 72°C for 60 s, followed by a final extension step of 10 min at 72°C. Amplified products were analyzed on a 1.2%

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agarose gel stained. PCR products were isolated from gel by using a Gel Extraction Kit (Promega) before they were ligated with pGEM-T vector and transformed into DH5 α competent cell of *Escherichia coli* (TaKaRa, Dalian, China). Ten positive clones were selected randomly and sequenced.

Bioinformatics analysis

Determinations of the gene and amino acid sequence were performed using the Expert Protein Analysis System (ExPASy; <http://au.expasy.org>). The signal peptide was predicted using the SignalP program (<http://www.cbs.dtu.dk/services/SignalP/>). Domain prediction was performed using the simple modular architecture research tool (SMART; <http://smart.embl-heidelberg.de/>). Needle program (<http://www.ebi.ac.uk/Tools/emboss/align/>) was used to calculate identities among nucleotide sequences of fish LEAP-2. The deduced amino acid sequence of PfLEAP-2 was aligned with these of the other fish LEAP-2 using Cluster W 1.81 [15] (Table 1). Phylogenetic tree was constructed with MEGA 5 program [16] by the Neighbor-Joining method [17]. The evolutionary distance was measured as the proportion of difference (p-distance). The confidence in the phylogenetic tree branch topology was accessed with bootstrap using 10, 000 replicates.

Tissue-specific expression pattern of PfLEAP-2 by quantitative real-time PCR

Tissue-specific expression pattern of PfLEAP-2 in 11 tissues of five yellow catfish including the brain, liver, hind kidney, head kidney, spleen, intestine, heart, gill, muscle, skin, and stomach were analyzed by quantitative real-time PCR (qRT-PCR) using the iQTM5 apparatus (BioRad, Hercules, USA). The expression levels of PfLEAP-2 mRNA in liver tissues of infected yellow catfish at different stages were also analyzed by qRT-PCR. cDNA from total RNA was synthesized as described in section 2.2. The gene-specific primers (RT-HcAmyF: 5'-TCGGCATAACCATCACCCAT-3'; RT-HcAmyR: 5'-GATTGATTACCAGATCAGGGAAAGAT-3') for qRT-PCR were designed basing on the cDNA sequence of PfLEAP-2. β -actin of yellow catfish (GenBank accession no. EU161066) was selected as the endogenous reference gene for calibration and amplified with primers actin-F (5'-CCGCACTGGTTGTTGACAACG-3') and actin-R (5'-TGCCAATGGTGATGACCTGTCC-3'). qRT-PCR was performed on an iQTM5 Real Time PCR Detection System (Bio-Rad, Hercules, USA) with the SYBR[®] Premix Ex Taq[™] PCR kit (TaKaRa) according to the manufacturer protocol. The reaction was performed in the following program: denaturation at 95°C for 10 s, 40 cycles of 95°C for 5 s, 60°C for 30 s, and 72°C for 20 s, followed by the determination

of the melting curve, which was carried out through 0.5°C increments from 55°C to 95°C. All samples were performed in triplicate with a standard curve of serially diluted synthetic RNA. The relative mRNA level was determined by the 2-($\Delta\Delta$ Ct) method [18] using β -actin as internal control and liver as calibrator tissue.

Expression profile of PfLEAP-2 after bacterium challenge

The bacterium *Edw. tarda* was cultured for 24 hours at 28°C in shaken bottles with LB medium. The culture was centrifuged at 5000 rpm for 15 minutes at 4°C. The pellet was re-suspended in sterile PBS and the cell density was correlated to a dose of 107 cells per mL count using serial dilution and spread plate techniques. Yellow catfish were divided randomly into two groups (20 fish per group) and cultured under the same conditions. Fish of treatment group was injected with *Edw. tarda* 0.1 mL per individual, while fish of control group with 0.1 mL sterile PBS. The sample of liver from five fish of two groups were isolated at 4 h, 24 h, 3 d, and 6 d post-injection respectively and stored at -80°C until analysis. The expression profile of PfLEAP-2 mRNA was tested with the qRT-PCR method as described in section 2.4.

Fusion expression, purification of mature peptide

The cDNA fragment of PfLEAP-2 mature peptide was amplified using the primers of Mp-LEAP2F (5'-GAATTCATGGCCCGTATGA-3') and Mp-LEAP2R (5'-CTACTCGAG TTAGGAAATTAT-3') designed on the sequence of PfLEAP-2 cDNA sequence, and were ligated to the vector pGEX-4T-1 (TransGen Biotech, Beijing, China). The recombinant plasmids, pGEX-m-leap was transformed into DH5 α competent cells of *E. coli* (TaKaRa). The forward positive clones were screened by PCR with vector primer M13F primer (5'-CACGACGTTGTAAAACGAC-3') and Mp-LEAP2R, and further confirmed by sequencing. The positive recombinant plasmid, pGEX-m-leap was isolated by Axy Prep[™] Plasmid Miniprep Kit (Axygen Biosciences, Union City, USA), transferred into *E. coli* BL21 (DE3) plysS Competent Cells (Promega), and subsequently incubated in LB medium. The *E. coli* BL21 (DE3) plysS inserted empty pGEX-4T-1 vector was also cultured in LB medium as negative control. The expression of recombinant protein was induced for 5 h in LB medium with 1.0 mM Isopropyl β -D-1-thiogalactopyranoside (IPTG) at 28°C. Recombinant protein was purified by affinity chromatography using a Glutathione Sepharose 4B column (GE Healthcare, Little Chalfont, UK). Purified products were assessed on 15% SDS-PAGE gel. Half of the fusion proteins were digested with thrombin on column before elution with PBS, whereas the remaining proteins were eluted from the column with glutathione. Purification and elution were performed according to the instruction manual. Purified products were assessed with western-blotting performed as described in [19].

Antimicrobial activity assay

The antimicrobial activity was determined by microdilution susceptibility assays as described in [20]. The bacteria *Edw. tarda* were cultured overnight in LB medium at 28°C. The suspension used for each assay was adjusted to 1 \times 10⁵ CFU/mL in LB medium. Recombinant mature peptide of LEAP-2 was added into bacteria suspension with final concentrations of (0, 20, 40, 60 μ g/mL). All assays were performed in triplicate. The suspensions were incubated at 28°C in a shaker at 200 rpm. The concentrations of bacteria suspension were determined with ATB expression densimat (Merieux, France) every 2 hours until the absorption values of control group (0 μ L/mL) at OD 630 reached 0.6. Growth curve was performed to calculate inhibition level. Meanwhile, 30 μ L of bacteria suspensions contained recombinant mature peptides

Species	GenBank Accession no.	Identities of protein (%)	Identities of mature peptide (%)
<i>Ictalurus furcatus</i>	AAX45792	89.4	97.8
<i>Ictalurus punctatus</i>	AAX45791	87.2	97.8
<i>Ctenopharyngodon idella</i>	ACR54299	72.8	88.9
<i>Danio rerio</i>	NP_001122249	68.5	86.7
<i>Oncorhynchus mykiss</i>	AAS49157	60.2	84.4
<i>Larimichthys crocea</i>	AFC90192	54.3	71.1
<i>Paralichthys olivaceus</i>	ACB97648	50.5	73.3
<i>Oreochromis niloticus</i>	XP_003457771	50.0	73.3
<i>Takifugu rubripes</i>	XP_003966912	50.0	73.3
<i>Oryzias latipes</i>	XP_004080006	42.4	63.2

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Table 1: The identities of amino acid sequences between PfLEAP-2 and other fish LEAP-2 from GenBank Database.

was spread on LB plate. Colonies were counted following overnight incubation at 28°C.

Statistical analysis

The differences of mRNA expression levels in tissues and at different stages after challenge with *Edw. tarda* were analyzed with one-way ANOVA, and further comparisons were performed with Tukey's HSD test using the SPSS software (version 19.0). Significance level was set at $P < 0.05$.

Results

cDNA and protein sequences of PfLEAP-2

The 285-bp cDNA sequence cloned in present study contained the full open reading frame (ORF) of yellow fish PfLEAP-2, which was deposited in GenBank with accession no. GQ228478. The deduced PfLEAP-2 protein comprised of 94 amino acids and contained a putative signal peptide of 28 residues, a prodomain peptide of 21 residues, and a mature peptide of 45 residues. The mature peptide had 4 conserved cysteine residues, namely Cys70, Cys76, Cys81, and Cys86 (Figure 1). The conserved cleavage site of RMAR was also identified in the mature peptide. Sequence identities of PfLEAP-2 to LEAP-2 from other fishes ranged between 42.4-89.4% and 63.2-97.8% for the entire proteins and mature peptides, respectively (Table 1 and Figure 1).

Phylogenetic analysis

The neighbor-joining tree based on the amino acid sequences of LEAP-2 showed that the PfLEAP-2 had closest relationship with *Ictalurus punctatus* which are other species from Siluriformes (Figure 1). Some LEAP-2 from same species, such as *Oryzias latipes*, *Maylandia zebra* and *Oncorhynchus mykiss* were clustered into different clades (Figure 2)

Tissue distribution of PfLEAP-2 mRNA in yellow catfish

The tissue-specific expression pattern of PfLEAP-2 in yellow

catfish was examined by qRT-PCR using β -actin as an internal control. PfLEAP-2 transcripts were abundant in the gill, detectable in the skin, stomach, and head kidney, but low in the muscle, spleen, liver, hind kidney, intestine, heart and brain (Figure 3).

Expression pattern of PfLEAP-2 gene in the liver after *Edw. tarda* challenge

The change of PfLEAP-2 transcripts after the pathogenic bacteria stimulation was performed by qRT-PCR. As shown in Figure 4, the expression of PfLEAP-2 mRNA in liver was up-regulated rapidly after infection time of 4 h ($P < 0.05$), but then returned to the control level afterwards (Figure 4).

Expression of recombinant PfLEAP-2 mature peptide and Western blot analysis

SDS-PAGE analysis showed that the recombinant PfLEAP-2 mature peptide expressed a 31 kD fusion protein (Figure 5). The content of recombinant protein in *E. coli* was increased in a time-dependent manner. Western-blotting analysis indicated that the 31 kDa fusion protein were cleaved into two parts by thrombin, including GST proteins with a molecular weight of 26.4 kDa and mature peptides with a molecular weight of 4.6 kDa (Figure 6).

Antimicrobial activity of recombinant PfLEAP-2 mature peptide

The results of antimicrobial assay showed that the purified recombinant PfLEAP-2 mature peptide exhibited no activity against *Edw. tarda* under the protein concentrations of 20 to 60 μ g/mL (Figure 7).

Discussion

As an important component of antimicrobial peptides in innate immune system, LEAP-2 has been identified in some fish species [21]. In this study, we reported a novel LEAP-2, named PfLEAP-2 from the

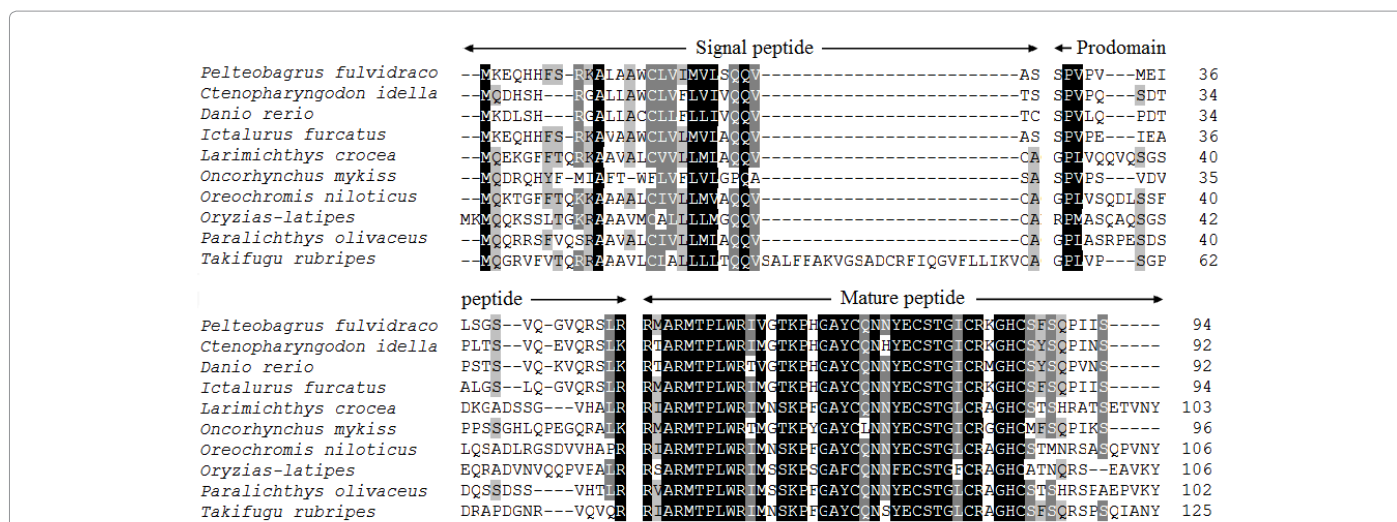


Figure 1: Multiple alignment of the amino acid sequences of PfLEAP-2 from yellow catfish and other fish generated with the Clustal W 1.81. The GenBank accession numbers of the aligned sequences are yellow catfish (*Pelteobagrus fulvidraco*, ACT33044), grass carp (*Ctenopharyngodon idella*, FJ390414), zebrafish (*Danio rerio*, AAI62807), channel catfish (*Ictalurus punctatus*, AAX45792), large yellow croaker (*Larimichthys crocea*), rainbow trout (*Oncorhynchus mykiss*, AAR11766), Nile tilapia (*Oreochromis niloticus*), Japanese medaka (*Oryzias latipes*), and Japanese flounder (*Paralichthys olivaceus*, ACB97648). Signal peptide, prodomain and mature peptides are also indicated. Asterisks denote the conserved cysteine residues in mature peptides

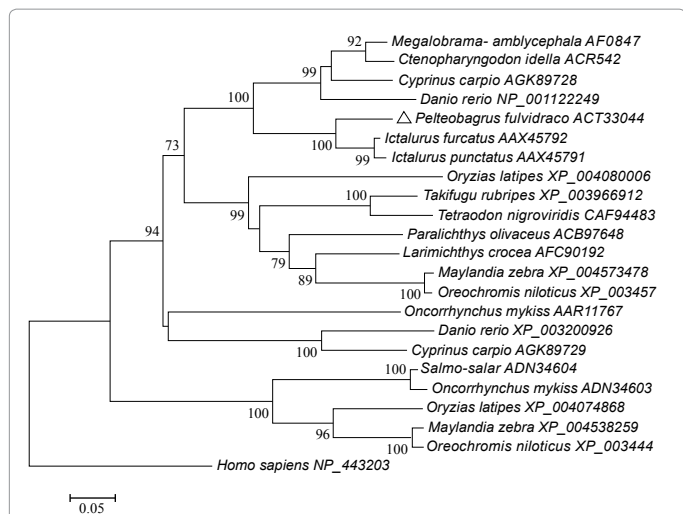


Figure 2: Neighbor-Joining tree based on entire amino acid sequences of fish LEAP-2. The evolutionary distances are computed using the p-distance method. The LEAP-2 from human is treated as outgroup. The percentages of replicate trees in which the associated taxa cluster together in the bootstrap test (10,000 replicates) have shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The GenBank accession numbers of LEAP-2 have shown next to each species.

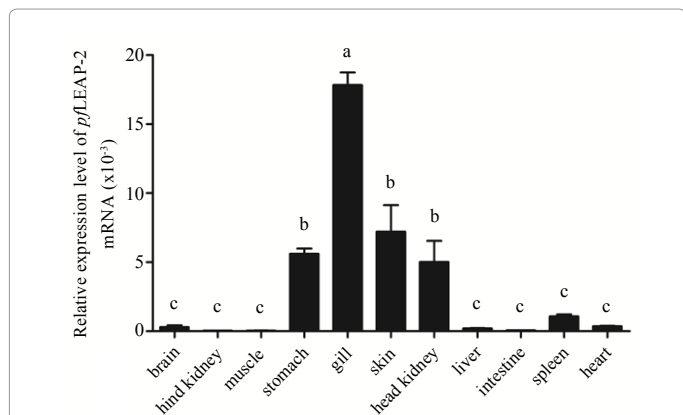


Figure 3: Expression of PLEAP-2 gene analyzed by qRT-PCR in tissues of yellow catfish with β -actin as a reference gene. The liver was used as a calibrator tissue. Each bar was mean \pm SEM from three independent samples. Different letters mean values that are significantly difference ($P < 0.05$) by one-way ANOVA followed by Tukey's HSD test.

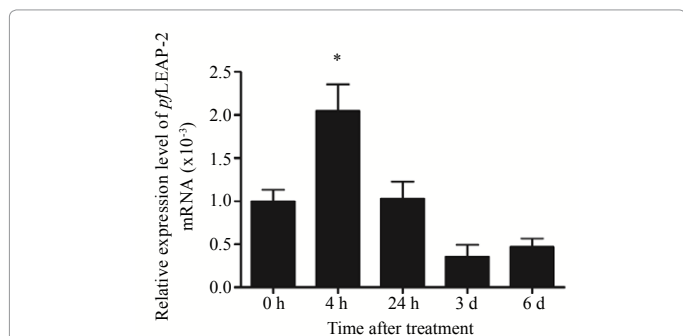


Figure 4: The change of PLEAP-2 transcripts in the liver after challenge with *Edw. tarda* by qRT-PCR. Significant difference was indicated with the asterisk by one-way ANOVA followed by Tukey's HSD test ($*P < 0.05$).

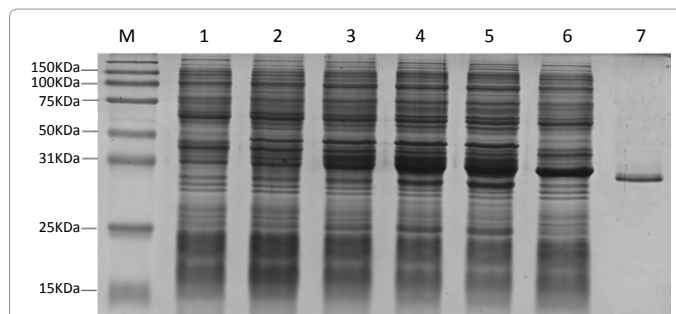


Figure 5: Expression and purification of recombinant PLEAP-2 fusion peptide by SDS-PAGE electrophoresis. lane 1, PGEX-1 without IPTG induction; lane 2, pGEX-m-LEAP without IPTG induction; lane 3, pGEX-m-LEAP induction for 2 h; lane 4, pGEX-m-LEAP induction for 4 h; lane 5, pGEX-m-leap induction for 5 h; lane 6, PGEX-1 induction for 5 h; lane 7, purified recombinant PLEAP-2 fusion peptide.

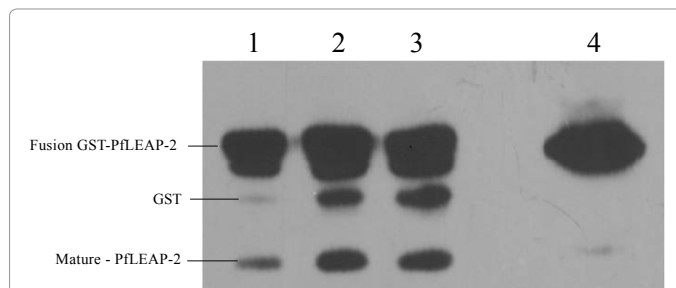


Figure 6: Western-blotting analysis of recombinant PLEAP-2 Protein. Thrombin cleaving products of recombinant PLEAP-2 Protein include the PLEAP-2 mature peptide and GST. Lanes: 1: purified mature LEAP-2 induction for 1 h; lane 2, purified mature LEAP-2 induction for 4 h, lane 3, purified mature LEAP-2 induction for 5 h; lane 4, purified fusion GST-LEAP-2 induction for 5h.

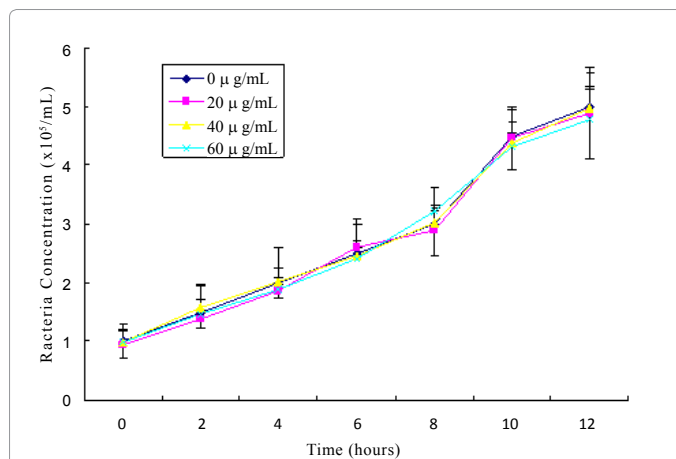


Figure 7: Time course of antimicrobial activities of recombinant PLEAP-2 mature peptide against *Edw. tarda* which was cultured in LB medium with different concentrations of recombinant PLEAP-2 mature peptide.

yellow catfish *P. fulvidraco*. Compared with other LEAP-2 proteins, the PLEAP-2, especially its mature peptide, was conserved in vertebrates. Phylogenetic analysis showed that the teleost LEAP-2 were divided into two clades, and PLEAP-2 was properly placed into the subclade of Siluriformes. However, two LEAP-2 isoforms from same species such as *O. latipes* in Cypriniformes, *M. zebra* in Perciformes and *O. mykiss* in Salmoniformes were clustered into different clades (Figure 2). Such

overlapping distribution of LEAP-2 in phylogenetic tree suggests that gene duplication of LEAP-2 occurred in teleosts [10].

In vertebrate, the liver produces two cysteine-containing molecules LEAP-1 (hepcidin) and LEAP-2 [22]. Beside of liver, the transcripts of LEAP-2 mRNA was also detected abundantly in the intestine of rainbow *O. mykiss* and trout blunt snout bream *M. amblycephala* [12,13]. In this study, the transcripts of PfLEAP-2 mRNA were predominantly expressed in the gill, skin and stomach but low in the liver. Similar result of tissues distribution of LEAP-2 was also detected in channel catfish [10]. Gill, stomach, and skin serve as the first barrier against infection in catfish [23]. The higher expression levels of PfLEAP-2 mRNA in these tissue indicated that PfLEAP-2 may play a role during early period of immune defense against pathogens.

Edwardsiella sp. of the family Enterobacteriaceae, such as *Edw. tarda* and *Edw. ictaluri* were the important causative agent, and have caused serious disease problems in catfish culture industries [14, 24-26], and reduced enteric septicaemia of catfish (ESC). To date, both *Edw. tarda* and *Edw. ictaluri* were isolated from the diseased yellow catfish, and caused "red head" disease [27] and "hole -in-the-head" disease [26]. In this study, the up-regulation of PfLEAP-2 at 4h after the challenge of *Edw. tarda* (Figure 4) suggests that this gene react rapidly to the pathogen invasion and may play an important role in the early immune defense of the yellow catfish against bacterial pathogen. Similar induction pattern was also reported in the spleen of channel catfish in which LEAP-2 was significantly induced at 4 h after challenge of *Edw. ictaluri* [10]. However, in another report [28], LEAP2 was not significantly different from that of control fish at all-time points after *Edw. ictaluri* infection.

Recombinant LEAP-2 obtained by in vitro expression has been proven to exhibit dose-dependent and selective antimicrobial activity against bacterial pathogen in human, bird, and fish [7,11,29]. In this study, the recombinant PfLEAP-2 has been successfully expressed in *E. coli* and purified. The PfLEAP-2 mature peptide cleaved by thrombin is verified by Western-blotting analysis. However, the purified recombinant PfLEAP-2 mature peptides displayed no antimicrobial activity against *Edw. tarda* even at the terminal concentration of 60 ug/mL (Figure 7). This result indicated that the antimicrobial activity of PfLEAP-2 against pathogen may also selective. The role of PfLEAP-2 in the immune defense of yellow catfish is needed to be further studied.

Acknowledgements

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