Fish and Shellfish Immunology 79 (2018) 363–369

Contents lists available at ScienceDirect

Fish and Shellfish Immunology

journal homepage: www.elsevier.com/locate/fsi

Short communication

Evolution, expression, and characterisation of liver-expressed antimicrobial peptide genes in ancient chondrostean sturgeons

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ARTICLE INFO

Keywords:
Antimicrobial peptide
Liver-expressed antimicrobial peptide 2
Innate immunity
Sturgeon

ABSTRACT

Liver-expressed antimicrobial peptide 2 (leap-2) is an evolutionarily ancient molecule that acts as the key component in vertebrate innate immunity against invading pathogens. Leap-2 has been identified and characterised in several teleosts, but not yet in chondrosteans. Herein, the complete coding sequences of leap-2b and leap-2c were identified from expressed sequence tags (ESTs) isolated from Dabry's sturgeon (*Acipenser dabryanus*) and Chinese sturgeon (*A. sinensis*), designated as adleap-2b, adleap-2c, asleap-2b, and asleap-2c, respectively. Adleap-2b and adleap-2c sequences share 98% and 100% sequence identity with asleap-2b, and asleap-2c, respectively. Sequence alignment revealed that all four genes contain four cysteine residues, conserved in all fish leap-2 homologs, that form two disulfide bonds. Comparative analysis of the exon-intron structure revealed a three exon/two intron structure for that leap-2 genes in animals, but intron 1 is much longer in sturgeons than in other species. The adleap-2c gene was expressed mainly in the liver of Dabry's sturgeon, and transcription of adleap-2c was significantly up-regulated ($p < 0.05$) in the liver and midkidney in response to *Aeromonas hydrophila* challenge. These results suggest adleap-2c may contribute to the defence against pathogenic bacterial invasion. The findings further our understanding of the function of adleap-2c and the molecular mechanism of innate immunity in chondrosteans.

1. Introduction

Antimicrobial peptides (AMPs), present in all living organisms from bacteria to humans, are an essential component of the innate immune system [1,2]. AMPs exhibit a broad spectrum of activity against bacteria, fungi, yeast, protozoa, and viruses [3]. Recently, besides their antimicrobial activity, novel physiological effects of AMPs have been documented, such as endotoxin neutralisation, chemotactic and immunomodulatory activities, induction of angiogenesis, and wound repair [4–9]. Thus, these ancestral molecules are crucial components of the innate immune system, and attractive candidates for novel therapeutic approaches [10]. AMPs are typically less than 100 amino acids in length, display hydrophobic and cationic properties, and adopt an amphipathic structure [2,6]. Based on their three-dimensional structure, AMPs are broadly classified into five major groups: α-helical structures, peptides rich in cysteine residues (cysteine-rich peptides), β-sheet structures, peptides rich in regular amino acids (including histidine, arginine, and proline), and peptides composed of rare and modified amino acids [11]. Since the discovery of cecropins in insects, more than 2800 AMPs have been found and deposited in the Antimicrobial Peptide Database (http://aps.unmc.edu/AP/about.php) [12]. However, to date, only 113 sequences have been reported from fish species, the largest vertebrate group containing over 23,000 species [13]. Major AMPs in fish include defensins, cathelicidins, hepcidins (also known as liver-expressed antimicrobial peptide 1, leap-1), piscidins, and leap-2 [14].

Two liver-expressed antimicrobial peptides (leaps) with four or two pairs of disulfide bonds (leap-1 and leap-2, respectively) have been isolated from human blood [15,16]. Leap-1 is one of the most widely-studied AMPs, but research on leap-2 is minimal [17]. Human leap-2 was first isolated in 2003 as the second blood-derived peptide with antimicrobial activity [16,18]. Human leap-2 is synthesised in the liver and secreted into the blood as a number of splice variants [16]. Both leap gene types consist of three exons and two introns, and encode prepropeptides which are subsequently cleaved to mature peptides [17]. Leap-1 is present in actinopterygian and non-actinopterygian fish [14],

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https://doi.org/10.1016/j.fsi.2018.05.023
Received 10 October 2017; Received in revised form 7 May 2018; Accepted 11 May 2018
Available online 15 May 2018
1050-4648/ © 2018 Published by Elsevier Ltd.

while leap-2 has only been found in actinopterygian fish, including grass carp (*Ctenopharyngodon idella*) [19], channel catfish (*Ictalurus punctatus*) [20], blue catfish (*Ictalurus furcatus*) [20], rainbow trout (*Oncorhynchus mykiss*) [21], Japanese flounder (*Paralichthys olivaceus*) [22], common carp (*Cyprinus carpio*) [23], blunt snout bream (*Megalobrama amblycephala*) [17], and large yellow croaker (*Larimichthys crocea*) [24]. AMPs from ancient chondrosteans are yet to be studied.

Sturgeons (infraclass Chondrostei, order Acipenseriformes) are a very ancient fish group distributed in the Palearctic hemisphere and represented by about 25 species [25]. Dabry's sturgeon (*Acipenser dabryanus*) and its relative Chinese sturgeon (*Acipenser sinensis*) are primarily distributed in the main stream of the upper Yangtze River and its tributaries [26,27]. The natural population has declined drastically during the last two decades due to anthropogenic activities such as overfishing, damming, pollution, and habitat alteration and destruction [27]. For commercial interest and conservation, both species have been intensively reared via aquaculture over the past several decades. To better understand the roles of leap-2 in ancient sturgeons, particularly in host immune responses to pathogenic bacterial invasion, molecular cloning and expression analysis is needed. Therefore, in the present study, we aimed to (1) obtain and analyse the deduced amino acid sequences of leap-2, and determine the evolutionary relationships between sturgeon peptides and those of other teleosts, (2) examine leap-2 mRNA levels in various tissues, and (3) investigate changes in leap-2 mRNA expression in four tissues following bacterial challenge. The results will help to better understand the evolution and function of leap-2 in innate immunity in sturgeons, and provide fundamental information for further study of the antimicrobial activities of AMPs in ancient chondrosteans.

2. Materials and methods

2.1. Experimental fish and cDNA sequences

All fish handling and experimental procedures were approved by the Animal Care and Use Committee of the Yangtze River Fisheries Research Institute, Chinese Academy of Fishery Sciences (YFI). A total of 65 F2 Dabry's sturgeon (35 ± 2 g) were cultured for tissue distribution and bacterial challenge in Taihu hatchary in YFI. Fish were maintained in aerated water tanks at 25 °C for 1 week to ensure health prior to sampling in the School of Animal Science, Yangtze University. A strain of *Aeromonas hydrophila* H3 isolated and identified from diseased Chinese sturgeon (*A. sinensis*) was found to be pathogenic to Dabry's sturgeon, and was stored in YFI. The complete coding sequences of leap-2b and leap-2c from Chinese sturgeon and Dabry's sturgeon were identified from transcriptome sequencing using BLAST searches of the web servers of the National Center for Biotechnology Information (NCBI), and designated as adleap-2b, asleap-2b, adleap-2c, and asleap-2c. In addition, we further amplified the complete genes in Chinese sturgeon and Dabry's sturgeon by 3′- and 5′- RACE.

2.2. Isolation of genomic sequences

Genomic DNA was isolated from sturgeon fin using a genomic DNA isolation kit (Qiagen, UK). The resulting DNA was dissolved in sterile H_2O to a concentration of 350 ng/mL PCR was performed under the conditions described above, whereas 2 μL of genomic DNA was used as template with primers designed to bind the 3′-UTR. Obtained products were cloned and sequenced as described below. All products obtained by PCR were directly cloned into the pGEM-T Easy vector (Promega, Madison, USA). Plasmid DNA was isolated from bacterial colonies carrying an appropriately sized insert using the Qiaprep spin miniprep kit (Qiagen, London, UK). Three randomly selected clones representing each product were sequenced by MWG-Biotech (Germany).

2.3. Sequence and phylogenetic analyses

The cDNA sequences were translated into the amino acid sequence using the Translate tool (http://www.expasy.org/tools/dna.html). Analysis of ORFs of cDNA sequences was performed using ORF Finder (http://www.ncbi.nlm.nih.gov/projects/gorf/). Putative signal peptides were analysed using the SignalP 3.0 server (http://www.cbs.dtu.dk/services/SignalP/). Multiple sequence alignment was conducted using ClustalW software (http://www.ebi.ac.uk/Tools/msa/clustalw2/). The deduced amino acid sequences of asleap-2b, adleap-2b, asleap-2c, and adleap-2c were aligned with the corresponding sequences from various animal species using ClustalW. Protein identity was established using the MegAlign 5.01 program with the Clustal W method to determine the amino acid distance. Different 3D structures were predicted using the Swiss-model protein structure prediction program (https://swissmodel.expasy.org/interactive/Cvmtvg/models/), and structures were validated by Rasmol 2.7.5 software (http://www.openrasmol.org/). Based on the alignment, a phylogenetic tree was constructed with MEGA7 software using the neighbour-joining (NJ) method [28]. Bootstrap analysis [29] was used with 1000 replicates to test the relative support for branches produced in the NJ analysis. All analysed sequences were retrieved from GenBank or SWISS-PROT databases (Table 2).

2.4. Tissue distribution and immune challenge

To examine the tissue specificity of adleap-2b and adleap-2c gene expression, four healthy Dabry's sturgeons were sacrificed and 12 tissues (gill, skin, muscle, liver, spleen, head kidney, kidney, intestine, eye, brain, heart, and blood) were removed for RNA extraction. To evaluate the immune responses of adleap-2b and adleap-2c, fish maintained at 25 °C were randomly divided into two groups: one group was injected intraperitoneally with 200 μL of *A. hydrophila* suspension in sterile 0.1 M PBS buffer (1.9×10^7 cfu/mL), and the other (control) group was injected intraperitoneally with 200 μL of sterile 0.1 M PBS buffer (pH 7.2). Four tissues (blood, liver, midkidney, and spleen) from four animals were sampled at 3, 12, 24, and 36 h post injection (hpi), and immediately soaked in RNA save Tissue storage solution (Biological Industries, USA) and stored at −80 °C.

Tissue and organ samples described above were used for total RNA extraction, and cDNA samples were prepared with a first strand cDNA synthesis kit (Fermentas, Canada). Specific primers (Table 1) were designed to amplify the corresponding gene, and pre-tested to ensure that each primer pair was unable to amplify genomic DNA using quantitative real-time PCR. KAPA SYBR FAST qPCR Master Mix (Kapa Biosystems) and a Step-one Plus real-time PCR system (ABI) were used to determine the expression of adleap-2b and adleap-2c as described previously [30,31]. The Dabry's sturgeon β-actin gene (GenBank no. MF536662) was amplified using the same RT-qPCR temperature profile as an internal reference gene. RT-qPCR was performed essentially as described previously [30,31]. In brief, for comparison, a standard was constructed using a mixture of equal mole amounts of purified PCR products of each gene amplified from cDNA. A serial dilution of the standard was run along with the cDNA samples in the same 96-well PCR plate and served as a reference for quantification. The expression level of each gene was calculated as arbitrary units normalized to the expression of β-actin. Statistical analysis was performed using SPSS 21.0 software. One way-analysis of variance (ANOVA) followed by LSD post hoc tests were used to evaluate transcript expression levels, and t-tests were performed to determine significant differences ($p < 0.05$) between sets of samples.

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