Fish & Shellfish Immunology 52 (2016) 23–30

Contents lists available at ScienceDirect

Fish & Shellfish Immunology

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

Short communication

Identification and functional characterization of fish-egg lectin in zebrafish

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ARTICLE INFO

Article history:
Received 3 November 2015
Received in revised form
3 March 2016
Accepted 10 March 2016
Available online 11 March 2016

Keywords:
Zebrafish
Fish-egg lectin
Maternal immunity
Phagocytosis
Opsonization

ABSTRACT

Fish-egg lectins (FELs) are identified in several species of fishes, but their activity and mode of action remain largely unknown in early life stages. Here we showed that zebrafish FEL (zFEL) was a maternal factor, which was capable of interacting with Gram-negative and Gram-positive bacteria and enhancing the phagocytosis of the bacteria by macrophages. Interestingly, microinjection of purified native zFEL into the embryos (resulting in the increase of zFEL in the embryos) markedly promoted the resistance of the embryos to the pathogenic *Aeromonas hydrophila*. Taken together, zFEL appears a maternal immune-relevant molecule capable of defending the developing embryos/larvae from pathogenic attacks.

1. Introduction

Lectins, carbohydrate-binding proteins, are part of the innate immune response throughout the animal kingdom. They are well-known pattern recognition receptors capable of specifically binding to the carbohydrate molecules on the surface of pathogens, and helping in their rapid clearance by enhancing opsonization and phagocytosis [1–6]. Animal lectins are classified into distinct families characterized by unique sequence motifs and structural folds: galectins, C-type lectins (including selectins, collectins and hyalectins), F-type lectins, I-type lectins, P-type lectins (Man-6-P-lectins), pentraxins, ficolins and cytokine lectins [7] (see also www.imperial.ac.uk/research/animallectins). Although lectins from the various families differ vastly in their domain architecture, they all possess at least one carbohydrate-recognition domain (CRD), conferring the proteins a carbohydrate-binding capacity [8].

Embryos and hatchings of most fishes are exposed to aquatic environment full of thousands of various microorganisms, including potential pathogens, before their immune system is fully developed. Currently, our knowledge is rather limited regarding the defense mechanisms how fish survive the pathogenic attacks in such a hostile environment during the earliest stages of life. It has been proposed that fish embryos/hatchings depend upon the maternal provision of immune-relevant molecules for protection against invading pathogens [9,10]. Previous studies on several fish species have shown that lectins are able to be transferred from mother to offspring [11–16]. Moreover, a novel group of lectins, termed fish-egg lectins (FELs), has been identified in the eggs of several fishes, including common carp (*Cyprinus carpio*) [17], goldfish (*Carassius auratus*) [18], rock bream (*Oplegnathus fasciatus*) [19], chum salmon (*Oncorhynchus keta*) [20], shishamo smelt (*Osmerus lanceolatus*) [21], catfish (*Silurus asotus*) [22] and ayu (*Plecoglossus altivelis*) [23]. FELs have the interesting property of binding rather specifically to rhamnose [12,22], a property that is shared by very few species outside the fish family [24]. In carp, FEL has shown a rather broad binding specificity to Gram-positive and Gram-negative bacteria [25]. However, the activity and mode of action of these lectins in early stages of fish life remain largely unknown. Recently, while using the mannan-agarose column to isolate mannan-binding lectin (MBL) from zebrafish (*Danio rerio*) eggs, we unexpectedly purified its egg lectin (zFEL). Therefore, the purpose of this study is to explore the function of zFEL in the developing embryos of *D. rerio*. Our results indicated that zFEL is a maternal factor involved in the immune defense of early embryos/

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http://dx.doi.org/10.1016/j.fsi.2016.03.016
1050-4648/

hatchlings.

2. Materials and methods

2.1. Fish and embryos

Wild-type zebrafish *Danio rerio* were purchased from a local fish dealer and maintained in the containers with well-aerated tap water at 27 ± 1 °C. The fishes were fed with live bloodworms and fish flakes (Tetramin, Germany) twice a day. Sexually mature *D. rerio* were placed in the late evening at a female to male ratio of 2:1, and the naturally fertilized eggs were collected in the next early morning and cultured at 27 ± 1 °C until use.

2.2. Purification and identification of zebrafish fish-egg lectin (zFEL)

Healthy fertilized eggs were rinsed three times with sterile 10 mM Tris–HCl buffer (pH 7.4) with 150 mM NaCl (TBS). After the excess TBS was withdrawn, the eggs were immediately homogenized on ice for 30 s, and centrifuged at 15,000 g at 4 °C for 30 min. The supernatant, egg cytosol, was pooled and added with protease inhibitor cocktail (Roche, Indianapolis, IN) and stored at −70 °C until used.

zFEL was purified according to the method of Takao *et al.* [26]. In brief, the embryo homogenate was adjusted to 10 mM Ca^{2+} by addition of $CaCl_2$ solution, which was mixed with mannan-agarose (Sigma) equilibrated with TBS containing 10 mM $CaCl_2$ (TBS/C), and incubated at 4 °C overnight. The agarose gel was packed in column and washed thoroughly with TBS/C. The column was then eluted with TBS containing 20 mM EDTA (TBS/E). The eluent was adjusted to 10 mM Ca^{2+}, mixed with mannan-agarose again and incubated at 4 °C overnight. The gel was packed in column, washed as above, and eluted with TBS/C containing 100 mM D-mannose. zFEL was recovered from the eluent, and analyzed on a 12% SDS-PAGE gel. The purified zFEL was also subjected to MALDI-TOF MS analysis (Beijing Protein Innovation Co., China).

2.3. Cloning and sequencing of zfel cDNA

Total RNAs were isolated from *D. rerio* using RNAiso Plus, and digested with recombinant DNaseI (RNA free; TaKaRa) to eliminate the genomic contamination. The first-strand cDNA was synthesized with reverse transcription system (TaKaRa) using oligo d(T) primer and stored at −20 °C till used. To amplify *zfel* cDNA fragment, a polymerase chain reaction (PCR) was conducted using the first-strand cDNA as template, in a total volume of 20 μl PCR reaction mixture containing 1 × PCR buffer, 0.5 unit of Ex Taq DNA polymerase and 0.4 μM of the specific primers S1 and AS1 (Table 1). The reaction was carried out at 94 °C for 5 min, followed by 35 cycles at 94 °C for 30 s, 54 °C for 30 s, 72 °C for 45 s, and a final extension at 72 °C for 7 min. After purification with DNA gel extraction kit (OMEGA), the PCR product was cloned into T/A cloning vector pGEM-T and then transformed into Trans5α *Escherichia coli*. The positive clones were selected and sequenced with ABI PRISM 3730 DNA sequencer.

2.4. Sequence and phylogeny analysis

The putative *zfel* cDNA was analyzed for coding probability with the DNASTAR soft ware package (version 5.0) and the protein domain analyzed using SMART program (http://smart.embl-heidelberg.de/). The molecular mass (MW) and isoelectric point (pI) of the deduced protein were determined using ProtParam (http://web.expasy.org/protparam/). Homology search in GenBank database was carried out by BLAST network server (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and multiple alignment of FELs including zFEL generated using the ClustalW program in the MegAlign of the DNASTAR software package (version 5.0). Phylogenetic tree was constructed by the neighbor-joining method within the package MEGA 6.0 software package using 1000 bootstrap replicates.

2.5. Quantitative real-time PCR (qRT-PCR)

Total RNAs were extracted with RNAiso Plus from 1- and 512-cell stage embryos, and the embryos at 6, 10, 18, 25, 35 and 48 h post fertilization (hpf) as well as the different tissues of adult zebrafish, including intestine, eye, muscle, liver, ovary, testis, gill, heart and skin. After digestion with RNase-free DNaseI to eliminate the genomic contamination, cDNAs were synthesized and used as template. One pair of the primers specific of *zfel*, S2 and AS2 (Table 1), was designed using Primer Premier 5.0 program (version 5.0). The reaction mixture (final volume 20 μl) consisted of 10 μl of SYBR@ Premix Ex Taq™, 0.4 μl ROX Reference DyeII, 0.5 μl of templates and 200 nM of each sense and antisense primers. The housekeeping gene *β-actin* was selected as the reference for internal standardization. The amplification was carried out on ABI 7500 real-time PCR system (Applied Biosystems) and the expression level of *zfel* relative to that of *β-actin* was calculated by the comparative C_T method ($2^{-\triangle\triangle CT}$) [27]. All qRT-PCR experiments were performed in triplicate to ensure the accuracy.

2.6. Expression, purification and refolding of recombinant fish-egg lectin (rzFEL)

The complete coding region of *zfel* was amplified with specific primers, S3 and AS3 (Table 1), according to the following steps: an initial denaturation at 94 °C for 5 min, followed by 35 cycles each of denaturation at 94 °C for 30 s, annealing at 55 °C for 45 s, extension at 72 °C for 45 s, and a final extension at 72 °C for 7 min. The PCR product was digested with EcoRI and BamHI, and sub-cloned into the plasmid expression vector pET-32a previously cut with the same restriction enzymes. The DNA insert was verified for authenticity and the plasmid was named *pET32a/zfel*.

Table 1
The sequences of primers used in this study.

Primer	Sequence (5′–3′)	Sequence information
S1 (sense)	GTTATCACTTTGACTGTTGAATCCT	*zfel* clonging primer
AS1 (antisense)	TGTCTGTTAATGATTTCTTTAGGGT	
S2 (sense)	TGATGAAGGGTGTGTCCAGTAATG	*zfel* Real-time PCR primer
AS2 (antisense)	AGCAGGGTTAGACCGACTCACA	
S3 (sense)	CGGGATCCTTAGACTGTACCATAATGAATGGTA	*zfel* Recombinant primer
AS3 (antisense)	CGGAATTCTTATAGAGTACATTTACGGATGGAG	
S4 (sense)	GATGCGGAAACTGGAAAGGG	*β*-actin Real-time PCR primer
AS4 (antisense)	AGGAGGGCAAAGTGGTAAACG	