Fish & Shellfish Immunology 55 (2016) 717–728

Contents lists available at ScienceDirect

Fish & Shellfish Immunology

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

Full length article

Apolipoprotein A-I in *Labeo rohita*: Cloning and functional characterisation reveal its broad spectrum antimicrobial property, and indicate significant role during ectoparasitic infection

Amruta Mohapatra [a], Sweta Karan [b], Banya Kar [a], L.C. Garg [c], A. Dixit [b], P.K. Sahoo [a, *]

[a] Fish Health Management Division, ICAR-Central Institute of Freshwater Aquaculture, Kausalyaganga, Bhubaneswar, 751002, India
[b] Gene Regulation Laboratory, School of Biotechnology, Jawaharlal Nehru University, New Delhi, 110 067, India
[c] Gene Regulation Laboratory, National Institute of Immunology, Aruna Asaf Ali Marg, New Delhi, 110 067, India

ARTICLE INFO

Article history:
Received 12 April 2016
Received in revised form
24 June 2016
Accepted 27 June 2016
Available online 28 June 2016

Keywords:
Aeromonas hydrophila
Antimicrobial property
Apolipoprotein A-I
Argulus siamensis
Labeo rohita
Poly I:C

ABSTRACT

Apolipoprotein A-I (ApoA-I) is the most abundant and multifunctional high-density lipoprotein (HDL) having a major role in lipid transport and potent antimicrobial activity against a wide range of microbes. In this study, a complete CDS of 771 bp of *Labeo rohita* (rohu) ApoA-I (*LrApoA-I*) encoding a protein of 256 amino acids was amplified, cloned and sequenced. Tissue specific transcription analysis of *LrApoA-I* revealed its expression in a wide range of tissues, with a very high level of expression in liver and spleen. Ontogenic study of *LrApoA-I* expression showed presence of transcripts in milt and 3 h post-fertilization onwards in the larvae. The expression kinetics of *LrApoA-I* was studied upon infection with three different types of pathogens to elucidate its functional significance. Its expression was found to be up-regulated in the anterior kidney of *L. rohita* post-infection with *Aeromonas hydrophila*. Similarly following poly I:C (poly inosinic:cytidylic) stimulation, the transcript levels increased in both the anterior kidney and liver tissues. Significant up-regulation of *LrApoA-I* expression was observed in skin, mucous, liver and anterior kidney of the fish challenged with the ectoparasite *Argulus siamensis*. Immunomodulatory effect of recombinant *LrApoA-I* (rApoA-I) produced in *Escherichia coli* was demonstrated against *A. hydrophila* challenge *in vivo*. *L. rohita* administered with rApoA-I at a dose of 100 μg exhibited significantly higher protection (~55%) upon challenge with *A. hydrophila* 12 h post-administration of the protein, in comparison to that observed in control group, along with higher level of expression of immune-related genes. The heightened expression of ApoA-I observed post-infection reflected its involvement in immune responses against a wide range of infections including bacterial, viral as well as parasitic pathogens. Our results also suggest the possibility of using rApoA-I as an immunostimulant, particularly rendering protection against *A. hydrophila*.

1. Introduction

Antimicrobial peptides are significant molecules of the innate immune system which play an essential role in disease prevention in vertebrates as well as invertebrates. Many protein molecules including histone derived proteins, haemoglobin derived peptides, hemocyanin derived peptides, high density lipoproteins (HDL) and others have been shown to possess antimicrobial property [1]. The characteristic functions of HDL involve reverse cholesterol transport and lipid metabolism [2]. However, only one-third of HDL proteins take part in lipid metabolism, while the rest have been reported to be involved in playing major roles in innate immune responses such as acute phase response, complement regulation and many other primary defence mechanisms along with their LPS binding activity [3].

The apolipoprotein AI (ApoA-I), a major and abundant plasma HDL protein of mammals and fish, is known to have diverse protective roles. ApoA-I neutralizes lipopolysachharides (LPS), has anti-viral activity and inhibits inflammatory cytokines [4,5]. This protein has a highly flexible structure which helps it to exist in lipid free, lipid rich and lipid poor states [6]. Structural analysis of ApoA-I reveals the presence of two independently folded domains, the N-terminal domain and the C-terminal domain [7]. The C-terminal

* Corresponding author.
E-mail address: pksahoo1@hotmail.com (P.K. Sahoo).

http://dx.doi.org/10.1016/j.fsi.2016.06.045
1050-4648/

lysine residues significantly enhance the lipid binding affinity, facilitating interaction with the bacterial membrane components and contributing towards its bactericidal activity [6]. ApoA-I protein can form cationic amphipathic α helices (a common feature reported for many fish antimicrobial peptides) which intercalate into lipid bilayers, bringing about a broad range of antimicrobial effects. The amphipathic helix motif also inhibits virus penetration, cell to cell spread and virus-induced cell fusion [8]. Antiviral activity of ApoA-I has been successfully demonstrated in mammals against herpes simplex virus (HSV), xenotropic murine virus and human immunodeficiency virus (HIV) [8–10]. The presence of ApoA-I has been observed in seminal plasma and spermatozoa of mammals describing its role in capacitation, acrosome reaction and sperm motility [11]. The presence of ApoA (ApoA-I and ApoA-II) in seminal plasma of rainbow trout indicates that these not only help in maintenance of sperm membrane integrity, but also give protection to sperm and reproductive tissue along with antimicrobial activity against *Escherichia coli* and *Aeromonas hydrophila* [12]. Besides that up-regulation in ApoA-I transcript levels was observed in most of the tissues after *A. hydrophila* infection in channel catfish [13].

ApoA-I has already been isolated from many fish species viz., brown bullhead (*Ameiurus nebulosus*), sea turtle (*Chrysemys picta*), sea bream (*Sparus aurata*), skate (*Raja erinacea*), cod (*Gadus morhua* L) and eel (*Anguilla japonica*) [14–18]. Its antimicrobial role has also been reported in many fishes like rainbow trout (*Oncorhynchus mykiss*), carp (*Cyprinus carpio*), and striped bass (*Morone saxatilis*) [2,19,20]. ApoA-I expression has been detected in all immunologically relevant tissues of many fishes including carp and trout seminal plasma [12,21]. Purified ApoA-I from striped bass plasma contributes to potent antibacterial activity against *Streptococcus* sp., *E. coli*, and *Mycobacterium marinum* at an inhibitory concentration (IC_{50}) of 250 μg mL^{-1}, 125 μg mL^{-1} and 250 μg mL^{-1}, respectively [20]. Anti-bacterial and anti-viral role of synthetic peptides derived from ApoA-I and ApoA-II have been demonstrated in *C. carpio* [19]. However, the role of this molecule during parasitic infection, if any, has not been investigated.

The present study was undertaken to characterize ApoA-I in one of the economically important Indian major carp species, *Labeo rohita* (rohu). The kinetics of its expression was evaluated in three different pathogen models (bacterial: *A. hydrophila*, viral: poly I:C and parasitic: *Argulus siamensis*) in few targeted tissues of *L. rohita*. In addition, we also evaluated immunomodulatory properties of this protein against *A. hydrophila in vivo* using recombinant ApoA-I produced using heterologous *E. coli* expression system.

2. Materials and methods

2.1. Fish

L. rohita juveniles (50–60 g) showing no signs of disease (after meticulous microscopic examination of skin, gill, intestine and kidney tissues of representative samples) were collected from farm facilities of the ICAR-Central Institute of Freshwater Aquaculture, Kausalyaganga, Bhubaneswar, India. Fishes were kept in 700 L capacity fiber reinforced plastic tanks under optimal aeration. Commercial pellet feed was given to the fish twice daily at 3% of their body weight. About one-third of the water in the tanks was exchanged with freshwater regularly to remove the waste materials and to maintain good water quality. The basic physico-chemical water parameters were measured systematically at seven-day intervals to maintain optimal levels (temperature: 28–31 °C, dissolved oxygen: 5.65 ± 0.73 mg L^{-1}, pH: 7.9 ± 0.81, nitrites: 0.016 ± 0.009 mg L^{-1}, ammonia: 0.109 ± 0.024 mg L^{-1}) throughout the experiment.

2.2. Cloning and characterisation of ApoA-I mRNA sequence

A primer set to amplify the full CDS of ApoA-I of *L. rohita* was designed from the consensus ApoA-I sequences of few teleosts available in NCBI database [*Hemibarbus mylodon* (ACI15890.1), *O. mykiss* (NP_001117720.1) and *C. carpio* (CAC34942.1)] (Table 1), using Primer Premier 5 (version 5.0, Premier Biosoft International, Palo Alto, CA) (Integrated DNA Technologies, Coralville, IA, USA). Naïve *L. rohita* liver samples were collected in RNAlater. RNA was isolated using TRI reagent (Sigma-Aldrich, St. Louis, MO, USA), following manufacturer's instructions and used for cDNA synthesis as detailed later. The *ApoA-I* gene was amplified using gene specific (ApoA-I CDS) primers (Table 1) using the following PCR conditions: initial denaturation at 95 °C for 2 min, followed by denaturation at 95 °C for 45 s, annealing at 55 °C for 45 s and extension at 72 °C for 1 min 30 s for 40 cycles, followed by a final extension at 72 °C for 10 min. PCR products were analyzed in 1% agarose gels and the amplicon was purified using gel purification kit (Bangalore Genei, Pvt. Ltd., India). The purified PCR product was ligated into T-vector and transformed into competent *E. coli* DH5α cells, using InsTAclone PCR Cloning Kit (Thermo Scientific, USA) according to the manufacturer's instruction. Putative clones were further confirmed by DNA sequencing (Xcelris, India). Sequence alignment was done using ClustalW multiple alignment tool implemented in BioEdit version 7.0.0 [22].

Complete coding sequence was deduced using ORF Finder (http://www.ncbi.nlm.nih.gov/gorf/gorf.html) to get the amino acid sequence. The signal peptide sequence was identified using Signal P bioinformatics tool (http://www.cbs.dtu.dk/services/SignalP/). Isoelectric point and molecular weight of the mature protein were calculated using Compute PI/Mw tool implemented in ExPASy Bioinformatics Resource Portal (http://web.expasy.org/compute_pi/). The functional domains were identified using SMART domain architecture analysis (http://smart.embl-heidelberg.de/).

Available amino acid sequences of ApoA-I protein of various fish species (indicated in the legend to Fig. 1) were retrieved from the NCBI database and aligned with the amino acid sequence of the encoded ApoA-I of *L. rohita*. Phylogenetic analysis was performed using MEGA 6.0 and the phylogenetic tree was constructed using Neighbor joining method. The evolutionary distances refer to unit equivalent to number of amino acid substitutions per site. All positions containing gaps and missing data were removed during analysis [23].

2.3. Sample collection

2.3.1. Tissue specificity and ontogeny study

To check tissue specific expression of ApoA-I, naive *L. rohita* juveniles (n = 3) were anaesthetized with an over-dose of MS222 (tricaine methanesulfonate, Sigma-Aldrich, St. Louis, MO, USA) and different tissues (skin, muscle, liver, anterior and posterior kidneys, spleen, fore gut, hind gut, heart, gill, eye and brain) were aseptically collected. Milt, eggs (both before and after fertilization) and larvae were collected at different time periods (0, 1, 3, 6, 9, 12, 18, 24 and 48 h, 3, 4, 7 and 15 days post-fertilization) from three pairs of broods for developmental expression analysis [24]. All the samples (eggs, hatchlings and tissues) were collected in RNA*later* (Sigma-Aldrich, St. Louis, MO, USA) aseptically and preserved at −20 °C until further use.

2.3.2. Bacterial infection

The LD_{50} dose of *A. hydrophila* in *L. rohita* was determined following the method of Reed and Muench [25,26]. Further, *L. rohita* juveniles (60 numbers) were challenged (in triplicate, 20 per group) with live *A. hydrophila* in 0.1 ml PBS (phosphate buffered