Fish & Shellfish Immunology 54 (2016) 639–647

Contents lists available at ScienceDirect

Fish & Shellfish Immunology

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

Full length article

Cloning, characterization and mRNA expression of interleukin-6 in blunt snout bream (*Megalobrama amblycephala*)

Chun-Nuan Zhang [a, *], Ji-Liang Zhang [a], Wen-Bin Liu [b], Qiu-Jue Wu [a], Xiao-Chan Gao [a], Hong-Tao Ren [a]

[a] College of Animal Science and Technology, Henan University of Scientific and Technology, Luoyang 471003, PR China
[b] Key Laboratory of Aquatic Nutrition and Feed Science of Jiangsu Province, College of Animal Science and Technology, Nanjing Agricultural University, No.1 Weigang Road, Nanjing 210095, PR China

ARTICLE INFO

Article history:
Received 10 November 2015
Received in revised form
1 March 2016
Accepted 3 March 2016
Available online 8 March 2016

Keywords:
Megalobrama amblycephala
Interleukin-6
Expression
Ammonia stress
Aeromonas hydrophila

ABSTRACT

In the present study, the interleukin-6 gene (IL-6) cDNA in blunt snout bream (*Megalobrama amblycephala*) was identified and its expression profiles under ammonia stress and bacterial challenge were investigated. The IL-6 sequence consisted of 1045 bp, including a 696 bp ORF which translated into a 232 amino acid (AA) protein. The protein contained a putative signal peptide of 24 AA in length. IL-6 expression analysis showed that the it is differentially expressed in various tissues under normal conditions and the highest IL-6 level was observed in the intestine tissue, followed by the liver, and then in the gills. Under ammonia stress, the IL-6 mRNA level both in spleens and intestine increased significantly ($P < 0.05$), with the maximum levels attained at 6 h, 12 h (72, 10-fold, respectively). Thereafter, they all significantly decreased ($P < 0.01$) and returned to the basal value within 48 h. Whereas, in livers it slightly decreased at 3 h firstly (0.5-fold), and then significantly ($P < 0.05$) increased with the maximum level attained 12 h (3-fold). Further expression analysis showed that the mRNA level of IL-6 in spleens, intestine and livers of blunt snout bream all increased significantly ($P < 0.05$), with maximum values attained at 6 h, 3 h, 6 h (10, 6, 18-fold, respectively) after *Aeromonas hydrophila* (*A. hydrophila*) injection, and then decreased to the basal value within 24 h which suggested that IL-6 was involved in the immune response to *A. hydrophila*. The cloning and expression analysis of the IL-6 provide theoretical basis to further study the mechanism of anti-adverseness and expression characteristics under stress conditions in blunt snout bream.

1. Introduction

Cytokines are potent mediators of immune responses in vertebrates, and in recent years a number of teleost cytokine genes with homology to mammalian genes have been discovered [1–5]. IL-6, one of them, is not only a major mediator of host response to tissue injury and infection, but also a key factor for contributing the inflammatory, and autoimmune processes [6]. In mammals, it is produced by many different cell types and have been shown to conduct pleiotropic functions. In fish, The IL-6 gene has been discovered in the Japanese pufferfish (*Fugu rubripes*) [7], Olive flounder (*Paralichthys Olivaceus*) [8], gilthead seabream (*Sparus aurata*) [9], zebrafish (*Danio rerio*) [10] and rainbow trout (*Oncorhynchus mykiss*) [11]. However, to date, the IL-6 gene has not been identified in blunt snout bream (*Megalobrama amblycephala*). In the present investigation, we report the molecular cloning and sequencing of IL-6 gene in blunt snout bream.

IL-6 occupies a central role in fish immunity [12] because it acts as a proinflammatory agent in response to microbial infections [13], promoting acute-phase reactions, hematopoiesis, and immune cell differentiation [14]. It is important to investigate the functions and expression characteristics of IL-6. In fish, IL-6 can not only be induced by response to viral or bacterial pathogenic invasion and proinflammatory cytokines, such as IL-1, tumor necrosis factor (TNF)-a, or platelet-derived growth factor (PDGF); but also by stressors, such as ultraviolet light and hypoxia [15–17]. Given that IL-6 is an important cytokine, it has been postulated that this cytokine might determine how the organs cope with stress and enhance its immunity. So, it is interesting to characterize the IL-6 gene and to evaluate the relationship between the gene and the

* Corresponding author.
E-mail address: zhangchunnuan12@163.com (C.-N. Zhang).

http://dx.doi.org/10.1016/j.fsi.2016.03.005
1050-4648/

stress and pathogenic agents, especially for its role in response to *Aeromonas hydrophila* (*A. hydrophila*) which causes high levels of mortality in freshwater fish and crustaceans. However, no information could be accessible regarding its function in the regulation of immune responses through the transcriptional activation of IL-6 genes in herbivorous freshwater fish. In this work we studied the full-length cDNA of IL-6 gene from the liver tissue of blunt snout bream. Moreover, we analyzed its mRNA expression under ammonia stress and bacterial challenge. The data obtained here may be useful to increase the knowledge about fish IL-6 even if, a clear immune function may also be demonstrated.

2. Materials and methods

2.1. Animals

Blunt snout bream were purchased from a local fish hatchery (Nanjing, China) with an initial weight of 75 ± 0.5 g. Prior to the experiment, fish were acclimated to experimental conditions for 4 weeks. During the acclimation period, fish were fed a commerical diet thrice a day. Water temperature ranged from 26 °C to 28 °C, pH fluctuated between 6.5 and 7.6 and dissolved oxygen was maintained approximately at 5.0 mg L^{-1}. All experiments and handling of the animals were conducted according to the research protocols approved by the Institutional Animal Care and Use Committee, Henan University of Science and Technology.

2.2. Stress treatment

2.2.1. High ammonia stress

24 fish in every tank (three tanks as one group) were subjected to high ammonia stress. The desired ammonia levels (10 mg L^{-1} of fresh water) were elevated by gradually adding ammonium chloride (NH_4Cl) into the water. Levels of ammonia were measured thrice a day according to the spectrophotometric method described by Solorzano [18]. Water temperature was maintained at 26 °C, and adequate dissolved oxygen was provided. Samples of 3 fish in every tank were randomly collected at 0 h (the control), 3 h, 6 h, 12 h, 24 h and 48 h. Individual liver, spleen and intestine were dissected on an ice bed and washed thoroughly with chilled saline (0.89 g NaCl L^{-1}), dried quickly over a piece of filter paper and stored at −80 °C for total RNA extraction.

2.2.2. Bacterial challenge

Another 24 fish in every tank (three tanks as one group) were selected for challenge of bacteria *Aeromonas hydrophila* (*A. hydrophila*), BSK-10, provided by Freshwater Fisheries Research Center, Chinese Academy of Fishery Sciences (Wuxi, Jiangsu Province, China). According to the methods detailed by Zhang et al. [19]. *A. hydrophila* was diluted with sterile phosphate buffer solution (PBS) to a final concentration of 5×10^5 CFU mL^{-1}. Each fish was injected with 1.0 mL suspended bacteria intraperitoneally. Another six blunt snout breams each received the same amount of PBS were used as the control group. The injected fish were stocked in tanks, ensuring adequate dissolved oxygen. Samples sampled and stored as described above.

2.3. Total RNA extraction

Six healthy blunt snout bream were anaesthetised in diluted MS-222 (tricaine methanesulfonate, Sigma, USA) at the concentration of 100 mg L^{-1}, killed and 10 tissues including: kidneys, spleens, gills, intestine tissue, livers, adipose tissue, brains, hearts, muscle tissue and eyes were sampled.

Total RNA was extracted from tissues using Trizol reagent (Invitrogen, USA) according to the manufacturer's instructions. Then it was resuspended in DEPC-treated water. Agarose gel electrophoresis at 1% was tested its integrity and spectrophotometric analysis (A260: 280 nm ratio) were used to assess RNA quantity. The purified RNA generally had OD260/OD280 ratio of 1.8–2.0.

2.4. Cloning and sequence

A first-strand cDNA was synthesized using Reverse Transcriptase XL (AMV) (Takara, Japan) according to the standard protocol. A pair of generated primers were designed based on the available sequences of zebra fish (*Danio rerio*), common carp (*Cyprinus carpio*) and grass carp (*Ctenopharyngodon idella*) which were of high homology to the sequences of blunt snout bream analyzed by Clustal W program. A first PCR reaction was performed with the IL-6-F1 and IL-6-R1 primers (Table 1). One PCR product (about 700 bp) was obtained by 1.0% (w/v) agarose gel electrophoresis and was delivered to Shanghai Invitrogen Biotech Service Co. Ltd. (Shanghai, China) for sequencing. According to the sequence information of this fragment, gene-specific primers were designed for 3′ RACE and 5′ RACE.

5′ Rapid amplification of cDNA ends (RACE) was performed using a SMART RACE cDNA amplification kit (Clontech) to obtain the full-length IL-6 cDNA sequence. First strand cDNA is synthesized from total or poly(A)$^+$ RNA using a gene-specific primer (5′GSP1) that the user provides for SuperScript™ II. After the first strand cDNA synthesis, the original mRNA template is removed by treatment with the RNase Mix (mixture of RNase H, which is specific for RNA and DNA heteroduplex molecules, and RNase T1). Following purification, 5′-end sequences are amplified using Abridged Anchor Primer (AAP), Abridged Universal Amplification Primer (AUAP), and a user-defined, nested gene-specific primers (5′GSP-2, 5′GSP-3). This single-cycle installation, followed by a purification step to remove excess oligo(dT) primer and the potential for internal mispriming by the primer during each PCR cycle, resulted in a higher proportion of "full-length" amplification products. PCR product of 5′ RACE was cloned into pMD18T vector and sequenced (Invitrogen, China).

The 3′-RACE cDNA was synthesized using the SMART™RACE cDNA Amplification Kit (Clontech, Japan) following the manufacturer's instructions. The first round of RACE PCR was performed with primer 3′GSP and primer UPM, then the second RACE PCR used 2 μL of the first round products as template with primer 3′GSP and primer NUP. Both reaction conditions were 94 °C for 5 min, followed by 30 cycles of 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 2 min, and then a final elongation step at 72 °C for 10 min. PCR product of 3′ RACE was cloned into pMD18-T vector and sequenced (Invitrogen, China).

2.5. Sequence analysis

The protein sequence was edited and analyzed using the program EditSeq in DNAStar Package to search open reading frame (ORF), and then translated into amino acid (AA) sequence using standard genetic codes. The molecular weight (MW) and isolectric point (PI) of IL-6 protein was both predicted using the compute pI/Mw software at http://cn.expasy.org/tools/pi_tool.html. The signal peptide was predicted using the SignalP 3.0 software at http://www.cbs.dtu.dk/services/signalP/ [20]. Putative transmembrane regions were predicted with HMMTOP v2.0 at http://www.enzim.hu/hmmtop/ [21]. The phylogenetic tree was constructed based on the deduced full-length amino acid sequences using the neighbor-joining (NJ) algorithm in MEGA version 5.0, and analysis reliability was assessed by 1000 bootstrap replicates [22].