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Blunt Snout Bream *Megalobrama amblycephala* Yih 1955 Interleukin-10: Characterisation, Comparative Homology Modelling and Expression

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Abstract

Interleukin 10 (IL-10) is important in the inflammatory cytokine mechanisms of many organisms. This study describes the identification and characterisation of the IL-10 homolog obtained from transcriptome profile of blunt snout bream *Megalobrama amblycephala* Yih 1955 (Cyprinidae): *MaIL-10*. It contained an ORF of 534 bp encoding a putative protein of 177 amino acids. *Ma*IL-10 protein contained a conserved IL10 family domain. Physicochemical characterisation analysis revealed that it was soluble, basic, hydrophilic, unstable based on the instability index, and thermostable based on its aliphatic index. Alpha helix (56.50%) was predominant among the secondary structure elements, followed by random coil (25.42%), extended strand (12.43%) and beta turn (5.65%), while the remaining elements were absent. *Ma*IL-10 3-D model was generated by comparative modelling using a human IL-10-like (PDB ID: 1lqs.1.D) as the template. The acceptable model was validated using Procheck's Ramachandran plot, ProQ and ProSA-web tools. Immune challenge of blunt snout bream with *Aeromonas hydrophila* bacterium (1.8×10^5 cfu $fish^{-1}$) demonstrated a significant differences in the expression of *maIL-10* in liver, spleen and kidney within 120 h post-infection ($P<0.01$). The results of this study indicate that *ma*IL-10 plays an important role in the innate immune system of blunt snout bream.

Introduction

The immune system plays an important role in animal organisms, protecting animals from the invasion of pathogens, and participating in the inflammatory reactions, healing process in case of tissue damage, as well as in the processes of development and growth (Magnadottir 2010).

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Similar to mammals, fish possess both innate and adaptive immunity (Tort et al. 2003; Whyte 2007; Alvarez-Pellitero 2008). Innate immunity is the first line of defence against pathogen invasion, as well as an internal stimulator for the development of antigen-specific acquired immune responses and homeostasis (Tort et al. 2003; Takeda and Akira 2004; Basu et al. 2012; Rauta et al. 2012). Cytokines are low-molecular weight proteins that are produced in a variety of cells; they monitor cell interactions important in regulating the progress of the immune response, including the stimulation of the innate response, promotion of cytotoxic T cells and induction of antibodies (Savan and Sakai 2006; Reyes-Cerpa et al. 2012). In response to stimuli, the activation of cytokines promotes the expression of immunity-related genes involved in various signalling pathways important for the initiation of the immune responses in an autocrine or paracrine manner by linking to their corresponding receptors (Reyes-Cerpa et al. 2012). Interleukin 10 (IL-10), one of the cytokines, is a helical glycoprotein of 30-40 kDa in size that typically acts as a cytokine inhibitory synthesis factor (Savan and Sakai 2006). Interleukin 10 is induced by multiple types of cells and performs anti-inflammatory function by downregulating the expression of other cytokines (Aste-Amezaga et al. 1998). It affects three functions of monocytes/macrophages: induction of immune mediators, antigen presentation, and phagocytosis (Harun et al. 2011). Teleost IL-10s are known to be involved in the functioning of inflammatory cytokines in response against the pathogen invasions (Savan et al. 2003; Inoue et al. 2005; Zhang et al. 2005; Pinto et al. 2007; Seppola et al. 2008). The gene has been cloned and characterised in a number of fish species, such as the common carp (*Cyprinus carpio*) (Linnaeus 1758), Cyprinidae (Savan et al. 2003; Feng et al. 2012), rainbow trout (*Oncorhynchus mykiss*) (Walbaum 1792), Salmonidae (Inoue et al. 2005; Harun et al. 2011), zebrafish (*Danio rerio*) (Hamilton 1822), Cyprinidae (Zhang et al. 2005), sea bass (*Dicentrarchus labrax*) (Linnaeus 1758), Morinidae (Pinto et al. 2007), Atlantic cod (*Gadus morhua* Linnaeus 1758), Gadidae (Seppola et al. 2008), and Indian major carp (*Labeo rohita*) (Hamilton 1822), Cyprinidae (Swain et al. 2011). In this study, the IL-10 of the blunt snout bream (*Megalobrama amblycephala* Yih 1955, Cyprinidae), an important freshwater cultured fish in China (Zhou et al. 2008), was identified from the previously obtained transcriptome profile (Tran et al. 2015) and *in silico* analysis applied to characterise its physicochemical and functional properties. Three-dimensional (3-D) structure of the blunt snout bream IL-10 (*ma*IL-10) protein was predicted by the comparative homology modelling method. mRNA transcript expression profile of *maIL-10* gene was investigated in liver, spleen and kidney of blunt snout bream after a challenge with *Aeromonas hydrophila*. This study provides basic information about the immunity-related functions of *ma*IL-10.

Materials and Methods

Experimental fish, bacterial preparation and challenge test

The bacterium, *A. hydrophila* D4, previously isolated from diseased haemorrhagic blunt snout bream in Dongxihu (Hubei, China), was cultured on the agar medium (including 3 g of beef extract, 10 g peptone, 5 g NaCl and 15 g agar in 1,000 mL distilled water) and incubated at 28 ^{o}C for 24 h.

Sterile physiological saline (0.85% NaCl) was added into the incubated bacterial test tubes, and surface colonies were crushed using a sterile loop to dilute the bacterial suspension. The desired densities of bacteria (1.8×10^6 cfu·mL^{-1}) were determined using a Neubauer haemocytometer and confirmed using a method for plate count by serial dilution. A total of 238 healthy blunt snout bream (average body weight: 27.3±6.8 g) used in this study were obtained from a farm in Huanggang District, Hubei Province, China. Fish were acclimatised in a 1 m^3 tank with aeration at about 28 °C for 2 weeks in the laboratory at the College of Fisheries, Huazhong Agricultural University. Fish were fed commercial pelleted feed twice a day. For the analysis of mRNA expression, fish were challenged with *A. hydrophila*. Fish were divided into two groups: 87 fish in the control group (intraperitoneally injected with 0.1 mL of physiological saline fish^{-1}) and 151 fish in the experimental group (injected with 0.1 mL of bacterial suspension fish^{-1}). Six fish from each group were sampled at 4, 12, 24, 72 and 120 h post infection (hpi), anesthetised in MS-222 (Sigma-Aldrich, Saint Louis, USA) (at 100 mg·L^{-1} concentration) and immediately killed. Liver, spleen and kidney were sampled separately from the specimens, immediately flash-frozen in liquid nitrogen and stored at -80 °C until further use. All fish were handled and experiments conducted in accordance with the "Experimental Animal Regulation in Hubei Province", Standing Committee of People's Congress of Hubei Province (code No. 50; issue date: July 29, 2005).

Total RNA preparation and cDNA synthesis

Total RNA was extracted from each sample with RNAisoPlus Reagent (Takara Bio Inc., Dalian, China), according to the manufacturer's instructions. Quality and quantity of the extracted RNA were evaluated using electrophoresis in 1% agarose gel and Nanodrop 2000 spectrophotometry (Thermo Scientific, Delaware, USA). Equal amounts of the total RNA of six specimens sampled at each time-point were pooled. cDNA libraries were synthesised using PrimeScript® RT reagent Kit with gDNA Eraser (Takara Bio Inc., Dalian, China) following the manufacturer's instructions, serially diluted 10-fold and used as the template for qRT–PCR.

Identification of maIL-10

The sequence of the blunt snout bream IL-10 (*MaIL-10*) gene was obtained from the blunt snout bream transcriptome profile constructed in a previous study using Solexa/Illumina technology (Tran et al. 2015). The *maIL-10* gene was identified through BLAST homology search against the GenBank database (http://blast.ncbi.nlm.nih.gov/Blast). The open reading frame (ORF) and amino acid sequence of *ma*IL-10 were inferred using NCBI's Open Reading Frame Finder (http://www.ncbi.nlm.nih.gov/gorf/orfig.cgi), signal peptide using the SignalP server (http://www.cbs.dtu.dk/services/SignalP-3.0) and domain structures using simple modular architecture research tool (SMART) server (http://smart.embl-heidelberg.de/). Thirty-seven teleost amino acid sequences (with over 50% similarity to *ma*IL-10) were retrieved from GenBank (http://www.ncbi.nlm.nih.gov) for phylogenetic analysis. The sequences were aligned with ClustalW2 using default parameters (http://www.ebi.ac.uk/Tools/msa/clustalw2/). Neighbour-joining (N-J) phylogenetic tree was constructed using MEGA 6.0 (Tamura et al. 2013).

The reliability of the phylogenetic tree was evaluated by the bootstrap method with 1,000 replications.

Protein physicochemical and functional characterisation

Expasy's ProtParam server (http://web.expasy.org/protparam/) was used to determine the physicochemical properties of the *ma*IL-10 (177 amino acids) polypeptide chain. These included molecular weight, amino acid composition, theoretical isoelectric point (pI), total number of negative (Aspartic acid+Glutamic acid) and positive (Arginine+Lysine) residues, extinction coefficient (EC), instability index (II), aliphatic index (AI) and grand average of hydropathicity (GRAVY). SOSUI server (http://harrier.nagahama-i-bio.ac.jp/sosui/) was used to identify the type of the protein: soluble or membrane. CYS_REC server (http://linux1.softberry.com/) was applied to predict the presence of disulphide bonds and their bonding patterns.

Protein structure prediction

Secondary structure of *ma*IL-10 was predicted using the Self-optimized prediction method by alignment (SOPMA) server (Geourjon and Deleage 1995), with default parameters: window width - 17, similarity threshold - 8, number of states - 4. 3-D structure was predicted on the basis of sequence identity and similarity with templates deposited in the Protein Data Bank (PDB) (Fiser 2010). Model was built by SWISS-MODEL (http://swissmodel.expasy.org/) tool using the best PDB match (in terms of fold recognition) as the template. Stereochemical quality and accuracy of the predicted model were analysed using Procheck's Ramachandran plot analysis, ProQ protein quality prediction and ProSA-web protein structure analysis. (Structural match between the predicted model and its template was evaluated through TM-score values in TM-Align server (http://zhanglab.ccmb.med.umich.edu/TM-align/). TM-score values (0~1) were interpreted as follows: TM-score <0.3 indicates random structural similarity, whereas TM-score >0.5 implies generally the same fold between two structures (Zhang and Skolnick 2005).

Quantitative real-time PCR (qRT-PCR) and data analysis

qRT–PCR was performed using SYBR® Premix Ex Taq™ (Takara Bio Inc., Dalian, China) in a Rotor-Gene Q real-time PCR cycler (Qiagen, Dusseldorf, Germany). The total reaction volume of 10 mL contained 5 µL of SYBR® Premix Ex Taq II (2×), 0.4 µL of each forward and reverse primer (10 µM), 0.8 µL of cDNA template and 3.4 µL of dH_2O. Primers (forward: 5'-atttgaccatatcccgcttg-3' and reverse: 5'-atcctgcgcttctacttgga-3') were designed using Primer Premier 5 software (Premier Biosoft, Palo Alto, USA) and synthesised by Sangon Biotech (Shanghai) Co., Ltd. *18S rRNA* was used as the reference gene (Luo et al. 2014). Thermal conditions were as follows: denaturation at 95 °C for 30 s, followed by 40 cycles of 95 °C for 5 s, annealing temperature at 54 °C (*maIL-10*)/60 °C (*18S rRNA*) for 20 s and elongation at 72 °C for 15 s. All reactions were performed in triplicate. Data were analysed using Rotor-Gene Q series software 1.7 (build 94) (Qiagen, Dusseldorf, Germany).

To maintain consistency, a threshold value of 0.05 for the Ct values for the two genes was manually set. Comparative Ct method, expressed as $2^{-\Delta\Delta Ct}$ (Livak and Schmittgen 2001) was used to analyse the expression levels of *maIL-10*. The expression of target genes was normalised to an endogenous reference gene (*18S rRNA*), where ΔCt was calculated as $Ct_{Test} - Ct_{18S\ rRNA}$, and ΔΔCt was calculated as $\Delta Ct_{Test} - \Delta Ct_{Control}$.

The control group expression level was designated as 1, so values >1 indicate upregulation and values <1 indicate downregulation. Statistical analyses were performed using one-way analysis of variance (ANOVA) and mean comparisons were performed by Duncan's multiple range tests using SPSS 16.0. Differences were considered statistically significant at $P< 0.05$ and $P< 0.01$.

Results

Sequence analysis

The *maIL-10* sequence with an ORF of 534 bp encoding a putative protein of 177 amino acids (molecular weight: 20,461 Daltons) was obtained from the transcriptome profile. The start (ATG) and stop (TAA) codons were determined from the 5' end of the sequence (Fig. 1). *N*-terminal signal peptide (Methionine 1 to Glycine 16) and the cleavage position of the signal sequence (between Glycine16 and Arginine17) were predicted (Fig. 1).

None potential *N*-linked glycosylation sites were found in the protein sequence. SMART analysis predicted the presence of a conserved IL10 family domain (comprising 111 residues at positions Aspartic acid 61 - Phenylalanine 171) in its sequence (Fig. 1). BLASTP analysis showed that the *ma*IL-10 amino acid sequence shared the highest sequence similarity with the IL-10 orthologs of *Ctenopharyngodon idella* (Valenciennes 1844), Cyprinidae (97%), *Hypophthalmichthys molitrix* (Valenciennes 1844), Cyprinidae (97%), *L. rohita* (89-90%), *C. catla* (89%) and *C. carpio* (89%). Orthologs from other organisms exhibited at least 32% sequence similarity.

Multiple alignment of *ma*IL-10 with a number of other fish IL-10 orthologs (Fig. 2), constructed using ClustalW2, showed a high level of amino acid sequence conservation, particularly at the conserved family domain IL-10 (aspartic acid 61 - phenylalanine 171). The first 16 amino acid residues, comprising the *N*-terminal signal peptide of the *ma*IL-10, were also almost identical in all studied teleosts, with the exception of the *L. rohita* IL-10 ortholog (*Lr*IL10 - ADD63992) (Fig. 2). An N-J phylogenetic tree based on the amino acid sequences of *ma*IL-10 and its homologs in different teleosts was constructed (Fig. 3). The result showed that *ma*IL-10 formed a clade with *H. molitrix* and *C. idella* orthologs, and that it exhibited an overall high similarity with other teleost IL-10 orthologs.

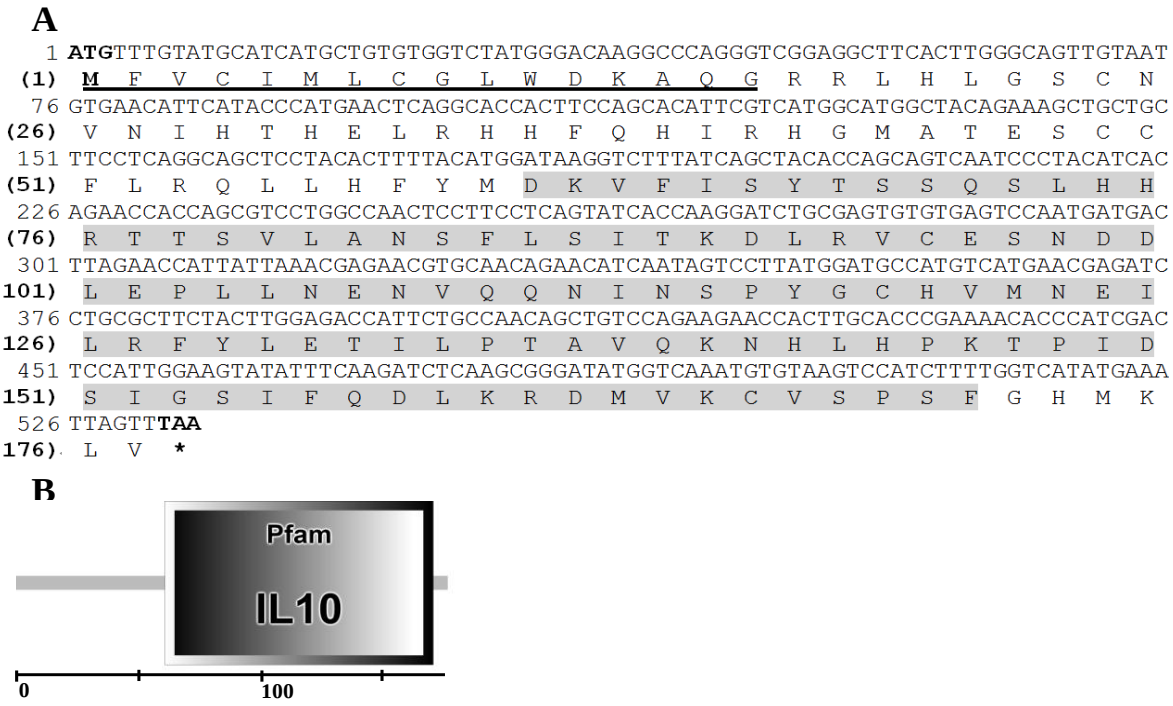

Fig. 1. Sequence and domain topology of the *Ma*IL-10 identified from the blunt snout bream transcriptome profile. (A) Nucleotides (upper row) and deduced amino acids (lower row) sequence are numbered on the left; the latter numbers are bolded and bracketed. Start codon (ATG) and stop codon (TAA) are bolded and boxed. The *N*-terminal signal peptide (methionine 1 - glycine 16) is underlined. Asterisk mark (*) indicated the stop codon. (B) The architecture of the domain topology of *ma*IL-10 protein, rendered by Simple Modular Architecture Research Tool (SMART) server, showing the conserved IL10 domain.

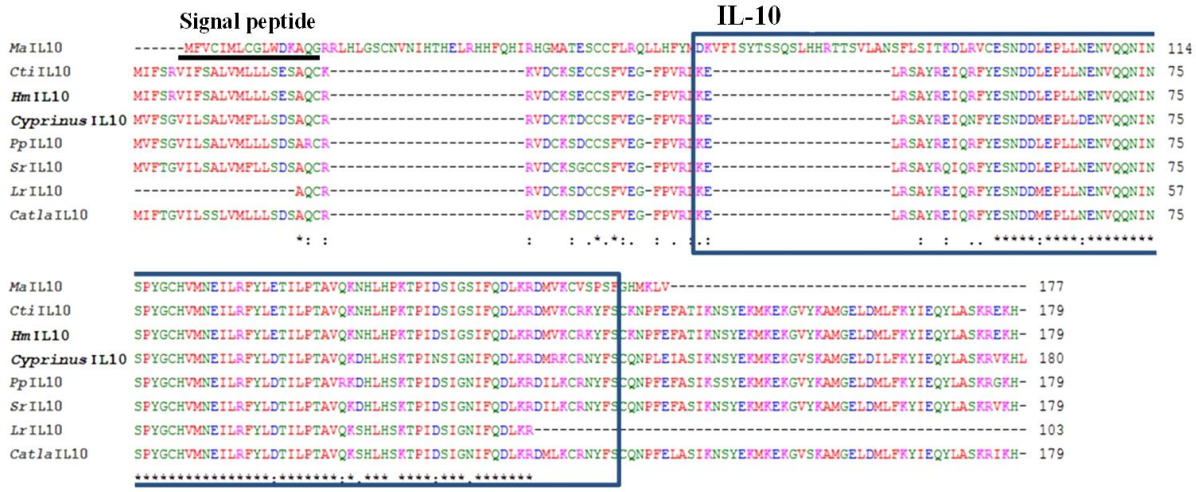

Fig. 2. Multiple amino acid sequence alignment of *ma*IL-10 and seven fish orthologs: *Ctenopharyngodon della* (*Ci*IL10- Acc. no. AEA50953.1), *Hypophthalmichthys molitrix* (*Hm*IL10- AAY99196.1), *Cyprinus carpio* (*Cc*IL10- BAC76885.1), *Percocypris pingi* (*Pp*IL10- AIN25991.1), *Sinocyclocheilus rhinocerous* IL-10 (*Sr*IL10- XP_016394327.1), *Labeo rohita* (*Lr*IL10- ADD63992.1) and *Catla catla* (*Ccat*IL-10- ADQ74794.1). Sequences are numbered on the right. *N*-terminal signal peptide (Methionine 1-Glycine 16) is underlined, and IL10 domain is boxed. Asterisk marks (*) indicate identical amino acids, colons (:) indicate conserved substitutions, baseline dots (.) indicate semi-conserved substitutions, and dashes (-) indicate deletions.

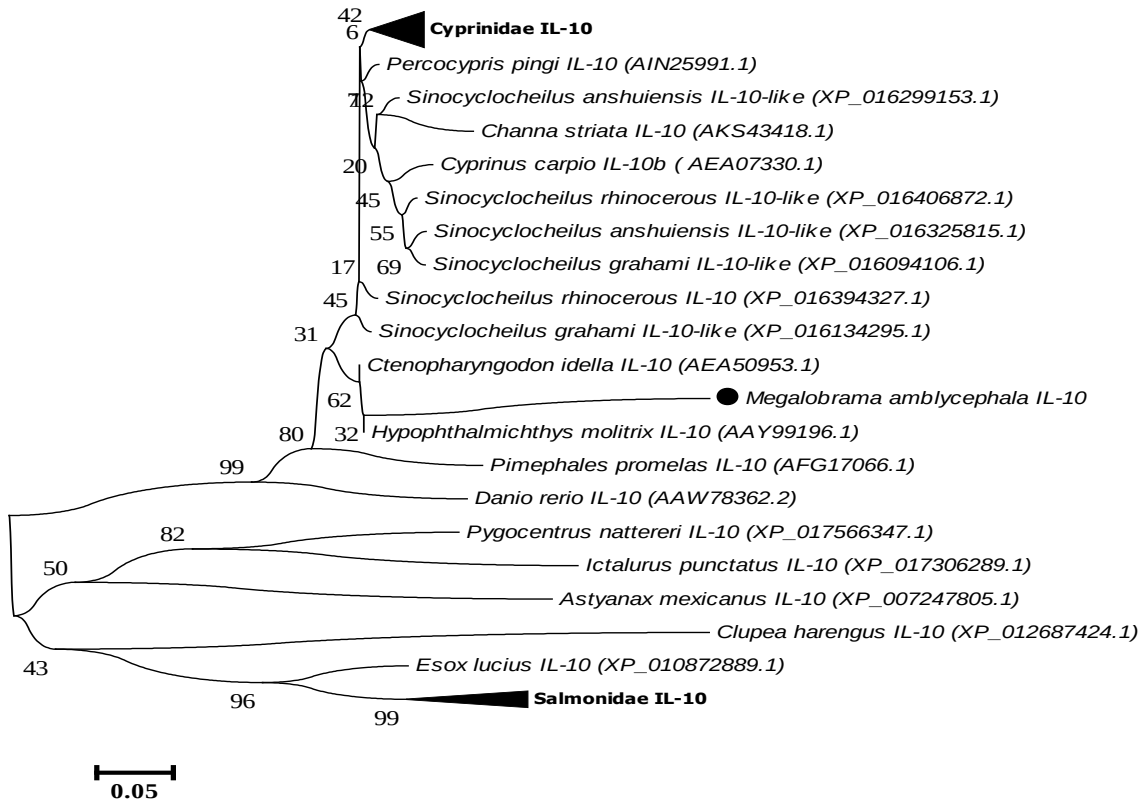

Fig. 3. Neighbour-joining phylogenetic tree showed the relationships between *ma*IL-10 and its homologs in selected animals (accession numbers in the parentheses). The numbers at the branches represent bootstrap support values (1000 replications). The bar (0.05) indicates genetic distance. *Ma*IL-10 is marked by a black dot.

Physicochemical and functional characterisation

Physicochemical properties of *ma*IL-10 protein were analysed. The protein was rich in leucine (11.9%), serine (8.5%) and histidine (7.9%). The theoretical isoelectric point (pI) was 8.15. The number of negative (aspartic acid + glutamic acid) and positive (arginine + lysine) residues was 15 and 17, respectively. The extinction coefficient (EC) measured at 280 nm was 11,960 $M^{-1}cm^{-1}$ (assuming all pairs of cysteine residues form cysteines) and 11,460 $M^{-1}cm^{-1}$ (assuming all cysteine residues are reduced). The N-terminal of the sequence was methionine. The estimated half-life of the protein was 30 h in mammalian reticulocytes *in vitro*, more than 20 h in yeast *in vivo*, and above 10 h in *Escherichia coli in vivo*. The instability index (II), aliphatic index (AI) and grand average hydropathicity (GRAVY) values were 60.9, 90.8, and -0.156, respectively. *Ma*IL-10 was classified as a soluble protein and there were eight cysteine residues found in its sequence; however, no cysteine pairing was predicted.

Protein structure prediction and model validation

Alpha helix (56.50%) was predominant among the secondary structure elements, followed by the random coil (25.42%), extended strand (12.43%) and beta turn (5.65%) (Fig. 4). The rest of the secondary structure elements (3_{10} helix, Pi helix, Beta Bridge, bend region and ambiguous states) were not predicted.

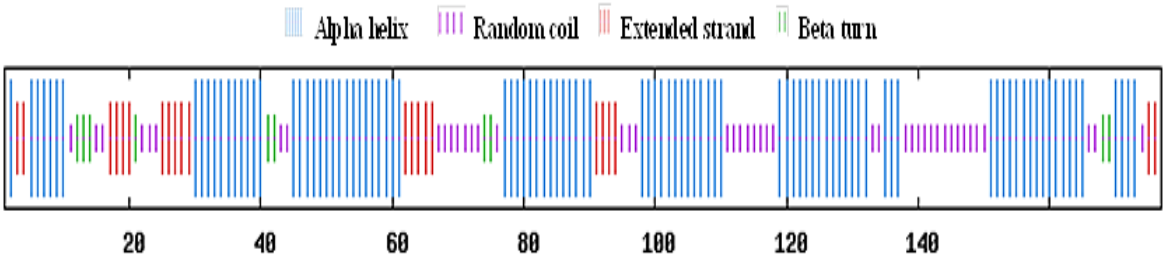

Fig. 4. Secondary structure model of *ma*IL-10, rendered using self-optimized prediction method by alignment (SOPMA) server.

The search using SWISS-MODEL server suggested that the human IL-10-like (PDB ID: 1lqs.1.D) at 2.7Å resolution was the best template to generate the 3-D structure of *ma*IL-10, with 27.47% sequence identity and 34% similarity. The query coverage (51%) ranged from glycine 22 to serine 168 residues, which includes a section of the conserved IL-10 domain. The predicted model for *ma*IL-10 is shown in Fig. 5A. TM-scores were 0.61 and 0.63 (if normalised for the length of *ma*IL-10 and human interleukin-10-like), respectively, which indicates that *ma*IL-10 and its template have the same fold and are structurally almost identical (Fig. 5B).

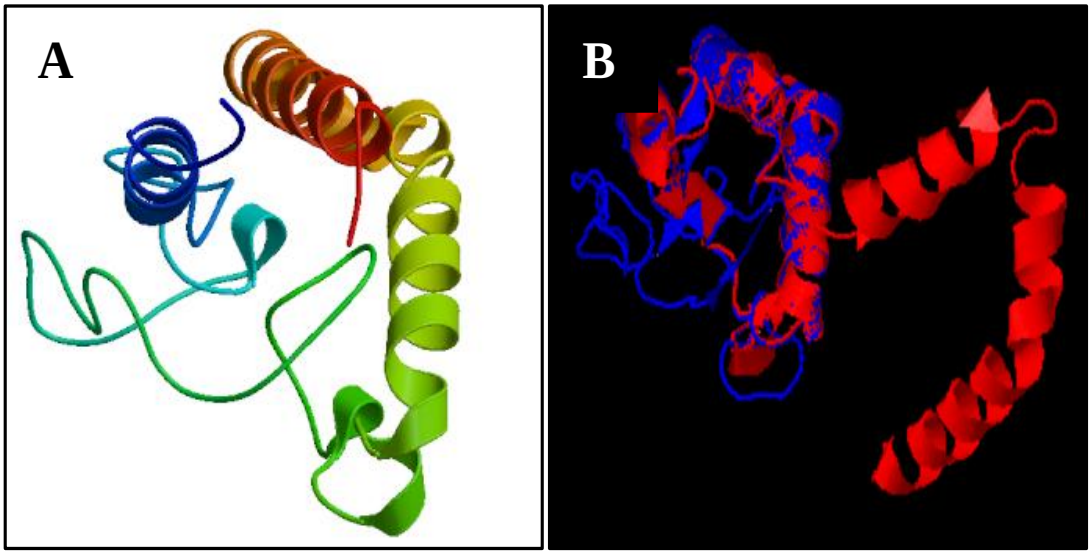

Fig. 5. Three-dimensional (3-D) structure of the *ma*IL-10 protein: A) 3-D homology model with the residue coverage of the model: glycine 22 to serine 168, rendered by the SWISS-MODEL program. B) Human interleukin-10-like (PDB ID: 1lqs.1.D) structure (red colour) superimposed upon the *ma*IL-10 protein (blue colour), rendered by TM-Align server.

The predicted 3-D model was validated using several web tools. Ramachandran plot analysis showed that 72.3%, 24.1%, 2.9% and 0.7% of residues were found in the most favoured, additional allowed, generously allowed and disallowed regions, respectively. An overall average G-factor of dihedral angles and main-chain covalent forces for the models was -0.33 (Fig. 6A). The Lgscore and MaxSub values were 1.19 and 0.12, respectively. Z-Score value (ProSA analysis) was -2.66 and the plot of residue energies contained negative values (Fig. 6B&C).

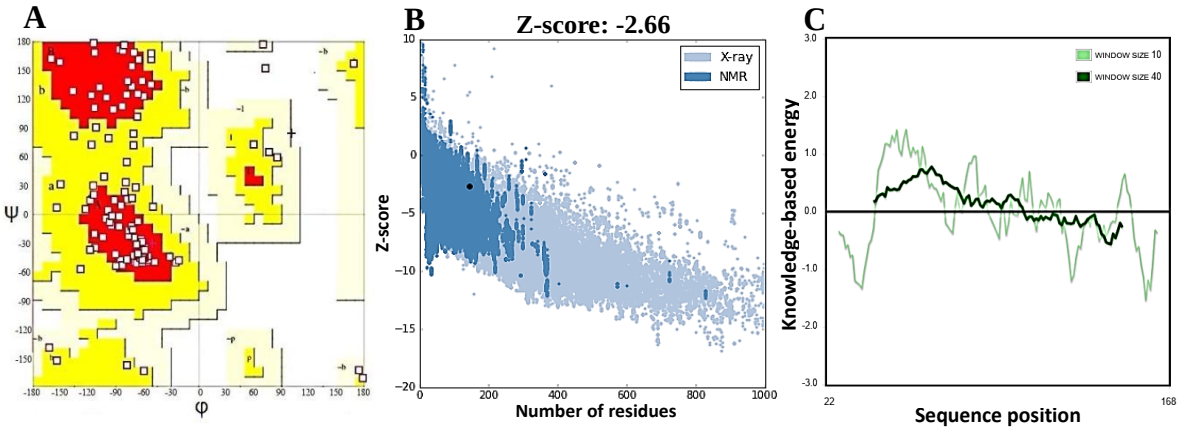

Fig. 6. Validation results for the three-dimensional (3-D) structure of the *ma*IL-10 protein: A) Ramachandran plot analysis, indicating residues in the favoured regions (red), allowed regions (yellow), generously allowed regions (light yellow) and disallowed regions (white). B) Z-score (highlighted as a black dot) displayed in a plot that contains the Z-scores of all experimentally determined protein chains currently available in the Protein Data Bank. Groups of structures from different sources (X-ray and NMR) are distinguished by different colours (light- and dark-blue, respectively). C) Plot of single residue energies, where window sizes of 40 and 10 residues are distinguished by dark- and light-green lines, respectively. Positive values indicate problematic or erroneous parts of the structure.

Expression of maIL-10 after A. hydrophila infection

After *A. hydrophila* infection, *maIL-10* transcripts were significantly (*P<0.01*) upregulated in liver (at 12 hpi), spleen (from 4 to 24 hpi, max. at 12 hpi), and kidney (4 and 12 hpi) (Fig. 7). In liver, *maIL-10* was upregulated from 4 to 72 hpi, reaching a peak at 12 hpi (15.4-fold, *P<0.01*), and then rapidly declined towards the eventual downregulation at 120 hpi (0.65-fold). In spleen, upregulation was observed during the first 24 h after the challenge, reaching a peak at 12 hpi (89.8-fold, *P<0.01*). The expression in kidney peaked at 4 hpi (9.62-fold, *P<0.01*) and then slowly (12 hpi = 7.06-fold, *P<0.01*) declined.

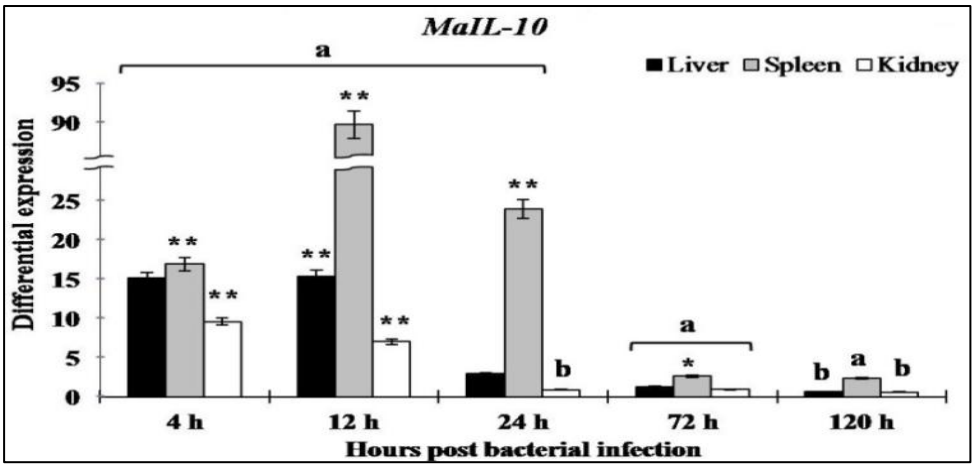

Fig. 7. Expression profiles of *maIL-10*, inferred *via* qRT-PCR, in liver, spleen and kidney of blunt snout bream at 4, 12, 24, 72 and 120 hpi after the *A. hydrophila* infection. Expression in both control and experimental groups was normalised to *18S rRNA* as a reference gene. The control group expression level is designated as 1, so values >1 indicate upregulation (a), whereas values <1 indicate downregulation (b). Each histogram represents the mean ± SE of three replicates. Statistical significance was assessed by one-way analysis of variance (ANOVA). Statistically significant differences in comparison to the control group are marked with * (*P*<0.05) and ** (*P*<0.01).

Discussion

Interleukin 10 was originally reported as an inhibitory factor for the cytokine synthesis in mouse, attending to reactive oxygen and nitrogen intermediates in many cells and skewing the immune response from Th1 to Th2 (Fiorentino et al. 1989; Inoue et al. 2005). To date, IL-10s have been reported in both invertebrates and vertebrates, but its presence in blunt snout bream remained unexplored so far. In this study, the cDNA encoding *ma*IL-10 was identified from the previously obtained transcriptomic profile of the blunt snout bream (Tran et al. 2015). *ma*IL-10 consisted of an ORF of 534 bp encoding a putative protein of 177 amino acids (molecular weight: 20,461 Dalton). The comparison with orthologs in other cyprinids (grass carp, common carp and silver carp), all of which have 179 amino acids, suggesting that the sequence is complete. An *N*-terminal signal peptide (methionine 1 - glycine 16) and a cleavage position of the signal sequence for the proenzyme (between glycine 16 and arginine 17) were predicted. A signal peptide and a cleavage site were also found in a number of other fish species, such as zebrafish (*D. rerio*) (Zhang et al. 2005), sea bass (*D. labrax*) (Pinto et al. 2007), and Indian major carp (*L. rohita*) (Swain et al. 2011). High similarity (97%) between the IL-10 orthologs in blunt snout bream, grass carp and silver carp was further corroborated by the clustering of these sequences in the phylogenetic tree. The presence of a conserved family domain (IL10) in *ma*IL-10 indicates that it probably has the same function as its homologs in other fish species, such as the Atlantic cod (*G. morhua*) IL-10 (Seppola et al. 2008). Taken together, these results suggest that *ma*IL-10 is indeed a member of the teleost IL-10 family.

Protein structure plays an important role in full understanding of the biological functions of proteins at the molecular level. Alpha helix was dominant in the secondary structure of *ma*IL-10, followed by random coil, extended strand and beta turn. This is comparable to the grass carp (AEA50953.1), silver carp (AAY99196.1), and common carp (BAC76885.1) homologs (computed in this current study), where alpha helices were also predominant, ranging between 59.22 and 64.25%. Human IL-10-like (PDB ID: 1lqs.1.D) was identified as the best template for generating the 3-D model of *ma*IL-10. Ramachandran plot analysis indicates a good quality (96.4% residues in the most favoured and additional allowed regions combined) of the model. The overall average G-factor (-0.33) was within the acceptable values range (>-0.50), suggesting that the model is reliable (Ramachandran et al. 1963). LGscore (1.19) and MaxSub (0.12) indices also indicated the acceptable quality of the model (Cristobal et al. 2001). The Z-Score (-2.66) was within the range of scores typically found for native proteins of similar sizes, and the plot of its residue energies showed that the calculated values were mostly negative, which both indicate a good quality model (Wiederstein and Sippl 2007).

Overall, the validation results confirmed the acceptable quality of the predicted *ma*IL-10 model. Physicochemical parameters computed using Expasy's Protparam tool indicated that the theoretical isoelectric point (pI) was higher than 7 (pI=8.15), indicating that *ma*IL-10 was basic in character. This value is useful for the protein purification carried out using an isoelectric method on a polyacrylamide gel.

The extinction coefficient (EC) value indicates the particular wavelength at which a protein absorbs light, and is directly associated with the concentration of cysteine, tryptophan and tyrosine in a protein. In this study, the ECs of *ma*IL-10 were 11,960 and 11,460 M^{-1} cm^{-1} (when it was assumed that all pairs of cysteine residues form cysteines and all cysteine residues reduced, respectively), which is lower than the EC (13,785 and 13,410 $M^{-1}cm^{-1}$, respectively) of the grass carp IL-10 (AEA50953.1) (computed in this current study), implying a lower concentration of these amino acids in its sequence. The instability index (II) value (60.9) indicates that *ma*IL-10 is an unstable protein in a test tube (II>40) (Guruprasad et al. 1990). Aliphatic index (AI), defined as the relative volume occupied by aliphatic side chains (alanine, valine, isoleucine, and leucine), is believed to be in a positive correlation with the thermostability of globular proteins (Ikai 1980). Herein, high AI value (90.8) suggests a high thermostability of this protein. A low GRAVY value (-0.156) implies that the protein is hydrophilic in natural conditions.

The upregulation of *maIL-10* in the blunt snout bream after *A. hydrophila* infection was quicker than that of its human homolog in monocytes after LPS stimulation, which could be detected at 7 hpi and peaked only between 24 and 48 hpi (de Waal Malefyt et al. 1991). However, expression patterns of IL-10 orthologs in other fish species were rather similar to the one observed in the blunt snout bream: sea bass (*D. labrax*) IL-10 peaked at 6 hpi in head kidney after a challenge with UV-inactivated *Photobacterium damselae* ssp. *piscicida* (Pinto et al. 2007), whereas rainbow trout (*O. mykiss*) IL-10 peaked at 1-6 hpi in head kidney and 3-12 hpi in spleen after an LPS injection (Inoue et al. 2005). Additionally, high expression of IL-10 was also observed in gills and liver of the Indian major carp (*L. rohita*) 24 h after the infection with *A. hydrophila* (Swain et al. 2011). High expression of IL-10 in spleen and kidney of blunt snout bream upon the bacterial stimulation may be explained by the presence of phagocytic cells in these tissues (Inoue et al. 2005). Taken together, these results suggest that *maIL-10* may be important in the immune system of blunt snout bream for responding to bacterial infections.

Conclusion

In this study, IL-10 was identified and characterised in blunt snout bream for the first time. Alpha helix was predominant among the secondary structure elements, followed by random coil, beta turn and extended strand. The remaining secondary structure elements were not predicted. The 3-D structure of *ma*IL-10 was predicted and validated as reliable and of acceptable quality. The results showed that the regulation of IL-10 increased within 72 hpi in liver, spleen and kidney, suggesting its association with the immune system of the blunt snout bream.

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