Fish & Shellfish Immunology 40 (2014) 354–361

Contents lists available at ScienceDirect

Fish & Shellfish Immunology

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

Full length article

Molecular cloning and characterization of secretory and membrane-bound IgM of turbot

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ARTICLE INFO

Article history:
Received 20 March 2014
Received in revised form
8 July 2014
Accepted 8 July 2014
Available online 25 July 2014

Keywords:
IgM
Turbot
Cloning
Vibrio anguillarum
Phagocytisis of B cell

ABSTRACT

In recent years, increasing diseases especially bacterial diseases have brought a host of losses with the expansive cultivation of turbot (*Scophthalmus maximus*). In order to do more research about the immune system of turbot for better understanding the mechanism of resisting diseases, the immunoglobulin genes related to secretory and membrane-bound IgM (s-IgM and m-IgM) of turbot were cloned using homology sequences cloning and SMART RACE PCR method. The heavy chain of s-IgM cDNA is 1900 bp in length including a leader region, a variable region, four constant regions (CH1, CH2, CH3 and CH4) and a C-terminal while the cDNA of m-IgM is 1795 bp with the same leader region, variable region, three constant regions (CH1, CH2 and CH3) and two transmembrane regions (TM1 and TM2). The sequence of IgM gene was also obtained and the structure consisted of V–CH1–CH2–CH3–CH4-TM1-TM2 is similar to other fishes. The highest level of s-IgM expression was observed in spleen, followed by kidney, gills, eyes, skin of the healthy turbot whereas the same profile of m-IgM expression is found with low level. And s-IgM takes up dominant proportion of total IgM expression. Also the relative expressions of s-IgM and m-IgM were analyzed in turbot vaccinated with the live attenuated vaccine *Vibrio anguillarum*. Not only the transcriptions of both s-IgM and m-IgM in liver, spleen and kidney of turbot injected with *V. anguillarum* MVAV6203 were up-regulated but also the expressions of s-IgM and m-IgM in spleen, kidney, gut, skin and gills of bath-vaccinated turbot were increased. Comparing the ratio changes of relative expression of m-IgM and s-IgM in vaccinated turbot, we found that the proportion of m-IgM were increasing in both administration routes, which probably indicated that the increasing expression of m-IgM strengthen the phagocytic ability of B cells.

1. Introduction

In fish, humoral immune response is mediated by specific immunoglobulins secreted by B lymphocytes directed to neutralize specific antigens and activating the complement cascade [1]. In teleost, the primary structure and gene organizations of immunoglobulin heavy (H) and light (L) chains had been revealed [2]. The immunoglobulin heavy chain gene complex in teleost typically encodes three antibody classes: IgM, IgD and IgT (or IgZ) while transcripts of both secreted and membrane-bound forms are generated from the IgM and IgT genes. Most teleost appear to express primarily (or only) membrane bound IgD [3,4], except in channel catfish which possesses both secreted and membrane-forms of IgD and lacks IgT [5]. Very recently secreted IgD was found in rainbow trout [6]. Whereas IgD, and to some degree IgT, have been subjected to major structural divergence [7,8], the IgM monomer has been evolutionarily stable, with four constant Ig heavy chain domains, μ1-μ4. However, IgM makes up tetramers in teleost, in contrast to mammals and elasmobranch fish, where IgM typically forms pentamers [9,10].

Before 2005, there were supposed to be only two types of fish immunoglobulins, IgM and IgD, but recent studies have found that there are four types of fish Igs: IgM, IgD, IgZ/T and chimera IgM-IgZ. The genetic information of IgM and IgD in *Paralichthys olivaceus*

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http://dx.doi.org/10.1016/j.fsi.2014.07.011
1050-4648/

conformed that secretory IgM consists of CH1~CH4 while the membrane-bound type IgM consists of CH1~CH3 and two transmembrane regions TM1 and TM2. IgD gene was located in 900 bp downstream IgM gene, which contains seven exons and two membrane-bound regions [11]. In carp, chimera IgM-IgZ consists of the leader peptide, the variable region, CH1, the fourth constant region of IgZ and the end zone of secretory type. This chimera was transcripted only by the stimulation of toxin [12]. In mandarin fish, three types of Igs namely IgM, IgD and IgZ had been cloned. They had different immune responses to pathogens [13]. Recently, IgZ2 discovered in zebrafish was found to be different from IgT in rainbow trout [14]. Different molecular weight light chains were found according to denatured protein electrophoresis analysis of teleost Ig. Also, more and more research showed that there were many types of light chains indeed. Through comparative analysis of the sequence, there were at least three types of IgL in Atlantic salmon. Of which, IgL1 and IgL2 sequences were homologous to rainbow trout and CL region of the IgL3 is homologous to spotted wolffish and yellowtail, while the VL region is more similar to channel catfish. Also, the expression of light chain had distinct specificity of different tissues [15].

Turbot (*Scophthalmus maximus*), a kind of teleost, is one of species in Chinese aquaculture which had brought great benefits. Fish intensive culture favors the proliferation of pathogens and the consequent economic losses associates with disease outbreaks. As known, teleost possesses both innate and adaptive immunity which consist of humoral and cell-mediated immunity [16]. Hence, a comprehensive knowledge of the immune system of this commercially important fish species is required [17]. The immune-prophylactic control of fish diseases through vaccination, probiotics and immunostimulation has been undertaken since long ago [18–20], whereas genetic programs on disease resistance, specifically in turbot, clearly require further investigation. In recent years, though the information of genome and expressed sequence tags (ESTs) in turbot has been increasingly accumulated [21–22], gene sequence and structure of the important immune-related gene IgM were still unknown. In this study, IgM of turbot was cloned by homology-based candidate gene method and rapid-amplification of cDNA ends (RACE) method. After full-length cDNA sequences of IgM were obtained, gene structures, distribution and expression characteristics in different tissues of turbot were analyzed. Expression of IgM in turbot vaccinated with *Vibrio anguillarum* MVAV6203 [23] was further investigated to understand the protective mechanism of the vaccine and to provide immunological theoretical basis for prevention and treatment of the turbot disease.

2. Materials and methods

2.1. Fish rearing, immunization and sampling

Turbot (*S. maximus*) used for IgM cDNA cloning and vaccination with *V. anguillarum* MVAV6203 were cultured in TianYuan Aquatic Products Co., Ltd (Yantai, Shandong, China), weighting 30.0 ± 5.0 g and maintained at a temperature of 14–16 °C in aerated tanks supplied with a continuous flow of sand-filtered sea water. The salinity of the sea water was 3.0–3.1% and the dissolved oxygen was not less than 6.0 mg/L. Fish were fed twice daily with commercial turbot feed (Hayashikane, Sangyo, Japan).

V. anguillarum MVAV6203 was grown in high-salt Luria–Bertani (LB) medium at 30 °C for 16 h. Strains were counted as colony forming units (CFU) by spreading plate method. The cells were harvested by centrifugation at 8000 r/m for 8 min and rinsed twice in sterile physiological sea water (20 g of NaCl, 4.8 g of $MgCl_2 \cdot 6H_2O$, 3.5 g of $MgSO_4 \cdot 7H_2O$, 0.7 g of KCl, 0.11 g of $NaHCO_3$, 1.21 g of $CaCl_2 \cdot 2H_2O$ in a liter deionized water). Finally, cells were resuspended and diluted to 1×10^7 CFU/ml with physiological sea water. 50 fish were injected with *V. anguillarum* MVAV6203 (1×10^6 CFU/fish, 100 μl/fish) as a vaccination group while the same amount of fish were injected 100 μl physiological sea water as a control group. To bath-vaccination, 50 fish were bathed in 30 L sea water with 1×10^6 CFU/ml *V. anguillarum* MVAV6203 as vaccinated group and 30 L sea water only as control group for 10 min in aerated tanks respectively. All fish for sampling were euthanized in 300 ng/ml MS-222 for at least 10 min and were killed by scalpel. Kidney, thymus, spleen, gills, skin, hind gut, brain, muscle, and liver were immediately collected and put into RNAstore (Tiangen, Beijing, China) and stored at −80 °C until use. All animal experiments were conducted in accordance of the guidelines and approval of the respective Animal Research and Ethics Committees of East China University of Science and Technology or Yantai Science and Technology Bureau.

2.2. RNA extraction

Total RNA was extracted from each tissue of the turbot using the TRIzol reagent (Invirtrogen, Carlsbad, USA) according to the protocol. In order to remove residual genomic DNA, the RNA samples were incubated with DNA-free DNase I (Promega, Madison, USA) for 30 min at 37 °C according to the protocol. The qualities and quantities of the purified RNA were determined by measuring the absorbance at 260 nm/280 nm using a Nanodrop ND-1000 spectrophotometer (LabTech, Wilmington, USA). RNA integrity was further verified by electrophoresis through a 2.0% (w/v) agarose gel.

Table 1
Primers used in this study.

Designation	Sequence (5′ → 3′)	Function
MF	CTGTCAGGTCTCTTATTC	Partial cDNA
MR	ACCWBRCACACCAGAG	Partial cDNA
M13F	TGTAAAACGACGGCCAGT	Sequencing
M13R	CAGGAAACAGCTATGACC	Sequencing
MGSPR	GGGCTCATTGAGGCACCAACCATTTCG	5′RACE
MGSPF	ACAGCCACCCCAAAGGGACCAACTGTG	3′RACE
NGSPF	CTATTAATTGTCATGTCACGGTCAAC	Nested PCR
NGSPR	CAGAACCACATGGCATCAGAGG AAACACAGTTGGTCCCTTTGG	Nested PCR
UPM	CTAATACGACTCACTATAGGGCAAG CAGTGGTATCAACGCAGAGT)	5′, 3′RACE
NUP	AAGCAGTGGTATCAACGCAGAGT	Nested PCR
FH-F	GGAGTTCACAGCACGTCAGATTCAG	Full cDNA
FH-R	TTGATTTGAATCTGCATTAAAAAGACA	Full cDNA
mMR	GAGGAAGAGRAWGATRAAGG	Partial cDNA
mMF	ATTCGCTTGAACCATATAGGGTGCTG TATGAAAGGACTGCTGAAATC	3′RACE
mMF-R2	TGGTAATAACACTGTATTTTGTATC	Full cDNA
CH1-F	CTTCTACACGGGAGTCAG	Sequencing
CH2-F	ATACTGGTGACGAAAACGAGGCTTC	Sequencing
CH3-F	TTGTCATGTCACGGTCAACAAG	Sequencing
CH4-F	TTATGCTGCCACCAGTAGAACACAC	Sequencing
Tm1-F	CCTGAATGAATTGGAAAGCTCC	Sequencing
Tm2-F	CCTGAATGAATTGGAAAGCTCC	Sequencing
CH2-R	GAGGATTGTAGATCACAGATGTATT	Sequencing
CH3-R	AGGCACCAACCATTTCGT	Sequencing
CH4-R	GTACCCAGTGGATCGGACAATGGC	Sequencing
Tm1-R	CAATAGTGAACAGAAGAGTG	Sequencing
Tm2-R	CTTGCCTTGGCTCATTTAAC	Sequencing
s-IgM-F	TCCAGCGTCCCTCAGTGTTT	RT-qPCR
s-IgM-R	AACAAGCCAAGACACGAAAACC	RT-qPCR
m-IgM-F	GCTCCATGCAAAAAGAAGAAGATAAT	RT-qPCR
m-IgM-R	AGTGATGAGGAAGAGAAGGATGAAG	RT-qPCR
β-actin-F	GATGGTGGGTATGGGCCAGAAG	Control, RT-qPCR
β-actin-R	ATGTCACGCACGATTTCCCTCTC	Control, RT-qPCR