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Cloning and expression of the constant region of rainbow trout (*Onchorhynchus mykiss*) µ immunoglobulin chain in *Escherichia coli*

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Abstract

The importance of rainbow trout (*Onchorhynchus mykiss*) in Iran aquaculture industry on one hand, and increasing the mortality of this fish due to outbreaks of infectious diseases, on the other hand, indicate the requirement for more profound understanding the rainbow trout immune system and access to laboratory tools for definitive diagnosis of its diseases. One of the most important defense mechanisms of vertebrates including fish is the production of immunoglobulin against microbial pathogens. In rainbow trout, dominant immunoglobulin in serum is immunoglobulin M (IgM). The purpose of this study was the cloning and expression of the constant region of rainbow trout IgM heavy chain (µ chain) gene in *Escherichia coli*. Therefore, RNA of the targeted gene was extracted from spleen and head kidney of rainbow trout and the constant region of µ chain was amplified by RT-PCR. The amplified fragment was ligated to pMALc2x vector and transferred to DH5α strain of *E. coli*. Recombinant vector transformed and expressed into *E. coli* Rosetta strain. SDS-PAGE analysis indicated the production of a recombinant protein with an expected molecular weight of 75 KDa. Thereafter, the recombinant protein was purified by amylose resin and its antigenicity was accessed by immunoblotting. Positive reaction of the expressed protein with anti-trout serum indicated that the expressed constant region of trout µ chain possess antigenic epitopes and could be applied in future immunological studies.

Keywords: Immunoglobulin M, Rainbow trout, Immunoblotting, pMALc2x

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Introduction

The immune system of vertebrates consists of innate and acquired immune responses. Acquired immune responses also named as specific or adoptive responses include two types: the humoral and cell-mediated immunity (Graham and Secombes, 1991; Shin *et al*., 2006; Uribe *et al*., 2011). Humoral immune responses consist on production of specific immunoglobulins (Igs) by host against foreign antigens (Tizard, 2000).

Immunoglobulins protect the host by removing the pathogens or preventing their dissemination in blood and host organs. Specific responses are also able to distinguish and differentiate between various antigens (Choi *et al*., 2007; Abbas *et al*., 2012). All classes of immunoglobulins are heterodimers, composed of four polypeptide chains including two similar heavy chains (H) with molecular weight of 55-70 KDa and two light chains (L) with molecular weight of 24 KDa, connected by disulfide bindings to each other (Acton *et al*., 1971; Andersson and Matsunaga, 1993; Pilstrom and Bengtén, 1996; Tizard, 2000; Cheng *et al*., 2006).

Mammalian immunoglobulins have been classified according to their heavy chains (μ, δ, γ, ε, and α) in IgM, IgD, IgG, IgE, and IgA isotypes (Savan *et al*., 2005; Shin *et al*., 2006). Fish has four classes of immunoglobulins which includes IgM (Warr, 1995), IgD, IgZ and IgT (Shin *et al*., 2006; Vesly *et al*., 2006). However, IgM is the dominant fish immunoglobulin and plays a major role in immune responses of fish (Sanchez *et al*., 1991; Magnadottir *et al*., 1996). In cartilaginous and teleost fishes IgM is pentameric (Kunihiko *et al*., 1984) and tetrameric, respectively (Magnadottir *et al*., 1998).

A better understanding of fish IgM structure and its performance is crucial to respond the aquaculture needs to prevent and control various diseases of fish. Some recent studies in this context include purification of IgM of Catfish (*Ictalurus punctatus*) (Ourth and Phillips, 1986), Goldfish (*Carassius auratus*) (Wilson *et al*., 1985), Common carp (*Cyprinus carpio*) (Vesely *et al*., 2006), Rainbow trout (*Onchorhynchus mykiss*) (Sanchez *et al*., 1991) and Atlantic salmon (*Salmo salar*) (Magnadóttir., 1998) and cloning and sequencing of IgM gene of some teleosts such as Atlantic cod (*Gadus morha*) (Bengten *et al*., 1991), Atlantic salmon (Hordvik *et al*., 1997, 1999) and Rainbow trout (Lee *et al*., 1993; Hansen *et al*., 1994).

Among cultured fish, trout is one of the most important freshwater fish in the world. Intensive culture of this fish in farms leads to outbreak of various diseases and sometimes heavy economic losses (Kibenge *et al*., 2012; Haghighi *et al*., 2017). Despite the high volume

of rainbow trout production in Iran, there have been relatively few studies on the immune system of this species. Analysis of the total IgM level and specific IgM production in serum are indices that can be used to recognize infectious diseases in fish and evaluate the success of vaccinations programs (Van der Heijden *et al*., 1995; AL-Harbi *et al*., 2000).

For this purpose, access to monoclonal and polyclonal antibodies against fish IgM is indispensable (Choi *et al*., 2007). To meet this need of the Iranian aquaculture industry, present study was undertaken to clone and express the constant region of trout µ chain gene in *E. coli* for production of monoclonal or polyclonal antibodies against rainbow trout IgM in future studies.

Materials and methods

Bacterial strain

Escherichia coli strains DH5α and Rosetta were used for cloning and expression of µ chain gene, respectively (Razi Institute, Iran). Both strains were cultured in Luria Bertani (LB) broth (Merck, Germany) at 37ºC (Choi *et al*., 2007).

RNA extraction

Rainbow trout, weighting 150±20 gr, were used to extract mRNA of µ chain gene. Fish were anesthetized and killed by a blow to the head. Head kidney and spleen samples (20-25 mg) were collected and stored in RNA later solution (Qiagen, Germany) until RNA extraction (Anderson *et al*., 1993). RNA was extracted using a commercial solution, RNAx (Ceinnagene, Iran), according to manufacturer's protocol and dissolved in 40 µl diethyl pyro carbonate (DEPC) treated water and stored at -70 °C (Choi *et al*., 2007; Dadar *et al*.,2014).

RT-PCR amplification of the constant region of µ chain gene

Trout's gene sequence of µ chain was extracted from world Genebank with accession number Y08598 and its constant region was selected to design primers. Primers were initially designed based on the previous studies and also using primer3 software. Then suitable restriction sites (*BamH*I and *Sal*I) were added to 5' ends of forward and reverse primers and the final sequences of primers were determined. Complementary DNA (cDNA) synthesis was conducted using the Accupower lyophilized master mix (production of Bioniz Company), as per the manufacturer instruction and the Reverse primer (5´- GCT GTC GAC TCA GTC AAC AAG CCA AGC CAC -3´). After synthesis of cDNA, it was amplified by PCR using both reverse and forward (5´- CCT GGA TCC GCC TCC CTC ACC TTC AAA TG-3´) primers and a commercial PCR master mix (Amplicon, Denmark). PCR reactions consisted on 5 µL of cDNA, 1 µL (20 pmols) of both primers, 25 µL of master mix and 18 µL DEPC water in a final volume of 50 µL. Thermal program of PCR was as follows: initial denaturation for 2 min at 94ºC, 30 cycles (94ºC for 30 sec, 55ºC for 45 sec

and 72ºC, 90 sec) of amplification and a final 7 min extension at 72ºC. The PCR product (972 bp) was analyzed by electrophoresis on 1.5% agarose gel in TAE buffer (Sanchez *et al*., 1991; Magnadottir *et al*., 1996).

Cloning of PCR product

PCR product was purified using GF1 Ambiclean (Gel & PCR) kit (Vivantis, Malaysia), according to manufacturer's protocol (Vivantis, Malaysia) and stored at -20ºC. For cloning, PCR product was digested by enzymes *BamH*I (Fermentas, Lithuania) and *Sal*I (Fermentas, Lithuania) to create sticky sequences at both ends. Digested PCR product was again purified using GF1 Ambiclean (Gel & PCR) kit and verified by electrophoresis on a 1.5% agarose gel. Then it was ligated to pMAL-c2x expression vector, and digested by similar enzymes. After transforming the DH5α strain of *E. coli* by the ligation product, the bacterium was cultured on LB agar medium supplemented with Ampicillin (50 µg mL^{-1}). The grown colonies were randomly selected and screened by PCR using gene specific primers. Then, recombinant plasmids were extracted from three PCR positive colonies, using a plasmid extraction kit (Vivantis, Malaysia) and sent to Korea Bioniz Company for sequencing the inserts, using malE and M13 universal primers.

Expression and purification of MBP-µ chain recombinant protein

For protein expression, one of the sequenced plasmids was transferred to competent Rosetta strain of *E.coli*. A transformed colony of the bacteria was then cultured in LB broth medium supplemented with 50 µg mL^{-1} Ampicillin (Jaber-Ebne Hayyan Company, Iran) and incubated overnight at 37ºC in a shaker incubator (Haas *et al*., 1991). One hundred ml of fresh LB broth was inoculated by 1 mL of the overnight culture and incubated at 37ºC in a shaker incubator, until the OD_{600nm} reached to 0.6. Then, Isopropyl β-D-thiogalactopyranoside (IPTG) was added to a final concentration of 1 mM and incubation was continued for 3h after adding IPTG. Bacterial cells were harvested by centrifugation at 3,000 × g for 10 min. A sample of the culture before adding IPTG and a sample taken of 4h incubation in presence of IPTG were evaluated for expression of MBP-µ chain protein, by SDS-PAGE analysis (Laemmli , 1970). Maltose binding protein (MBP) is a fusion partner of about 42.5 kDa encoded by pMAL-c2x plasmid vector at the N-terminus part of the recombinant protein. Finally, the recombinant protein was purified by chromatography in a column of amylose resin (New England Bio Labs, USA), recommended for purification of MBP tagged fusion proteins (Dagert and Ehrlich, 1979; Wei *et al*., 2009).

Evaluation of immunogenicity of the MBP-µ chain protein

Immunogenicity of the recombinant MBP-µ chain protein was assessed

by immunoblotting using a mouse polyclonal serum prepared against trout serum. The purified MBP-μ chain and purified MBP were initially electrophoresed on a 10% polyacrylamide gel. Then, the electrophoresed proteins were transferred to a nitrocellulose membrane in a wet transfer system (Paya Pajuhesh, Mashhad), adjusted on 60V during 3 hours. In order to prevent nonspecific binds, blocking of the membrane was carried out overnight at 4ºC with phosphate buffered saline (PBS) containing 0.05% Tween 20 and 5% skim milk. All subsequent incubations were at room temperature and the membrane was washed three times with PBST (PBS containing 0.05% Tween 20) after each step. After washing, the membrane was incubated in a 1×100^{-1} dilution of the mouse anti trout serum in blocking buffer for 2 h. The membrane was washed again and then incubated for 1 h in 1×4000^{-1} dilution of a commercial peroxidase conjugated anti-mouse IgG (Komabiotech, Korea) in blocking buffer. Finally the membrane was washed and then 4-chloronaphthol-H_2O_2 (chromogen substrate solution) was added (Sakai and Savan, 2004).

Results

Amplification, cloning and sequence determination of the constant region of rainbow trout's μ chain gene

After cDNA synthesis and performing PCR using the designed primers, amplification of a 972bp DNA segment was expected. Electrophoresis of PCR product on 1.5% agarose gel beside a 1kb size marker (Fermentas, Lithuania) showed that PCR has successfully led to amplification of a DNA band of about 972bp (Fig. 1). Following amplification of this segment and performing the process of cloning, appearance of grown colonies on LB agar medium containing Ampicillin indicated the success of bacterial transformation by the plasmid. To ensure that the transformed bacteria contain a recombinant plasmid, bacterial colonies were screened by PCR. The results of screening for some colonies are illustrated in Fig. 2.

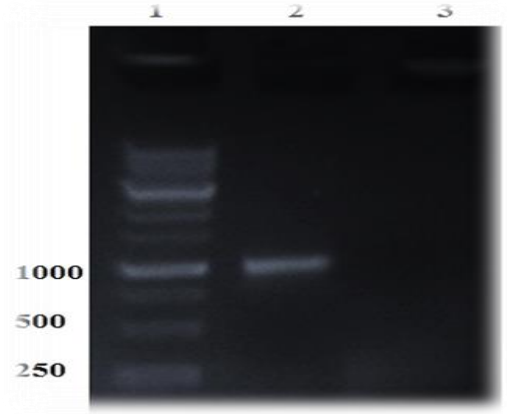

Figure 1: Electrophoresis of PCR product. Lane1, 1kb DNA ladder (sizes of some ladder bands have been shown in left), lane 2, RT-PCR product and lane 3, negative control.

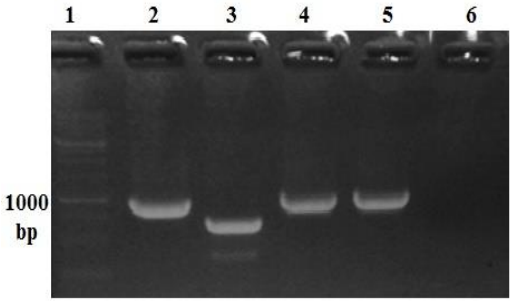

Figure 2: colony PCR of randomly selected colonies.Lane1, 1kb DNA ladder, lanes 2, 4 and 5: colonies possessing recombinant plasmid, lanes 3 and 6, negative colonies.

Sequencing of the insert in 3 recombinant plasmids followed by evaluation of sequences by BLAST searching in Gene Bank (NCBI), revealed that the cloned DNA segment had 100% similarity to sequence of the constant region of trout μ chain with the accession number Y08598. Sequencing also indicated that the insert was successfully cloned in pMAL-c2x plasmid, in frame with translation of MBP.

Expression and purification of recombinant MBP-μ chain protein

Expression of recombinant MBP-μ chain protein was evaluated by transformation of Rosetta strain of *E. coli* by recombinant plasmid followed by electrophoresis in polyacrylamide gel (Fig. 3). As it is observed, bacterium transformed with the recombinant plasmid, compared to a bacterium possessing the non-recombinant pMAL-c2x plasmid, has expressed a protein of about 75kDa, consistent with molecular weights of MBP (42 kDa) and the constant region of μ chain (33.4 kDa). Fig. 3 also indicates that the expressed protein is soluble and therefore could be purified from bacterial lysate by amylose resin, without any need for treatment with chemical substances like urea.

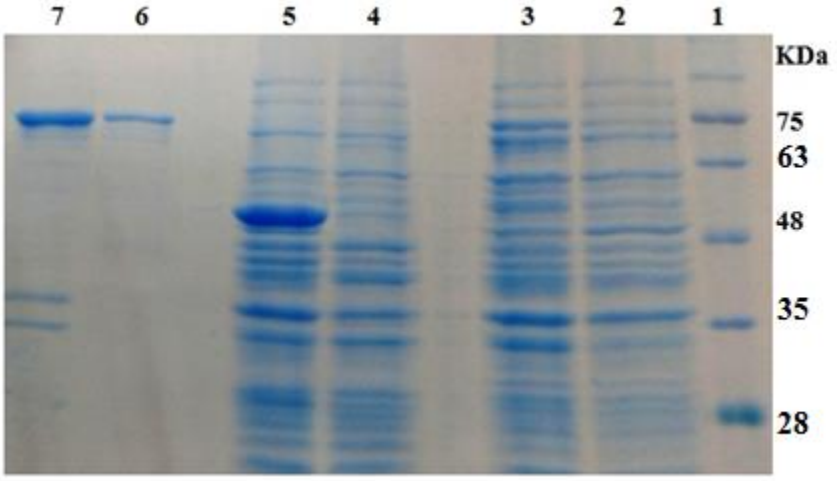

Figure 3: Analysis of protein expression by SDS-PAGE. Lane 1, Standard of protein molecular weight lane 2,bacteria containing a recombinant plasmid before induction with IPTG, lane 3, bacteria containing a recombinant plasmid after induction with IPTG, lane 4, bacteria containing pMALc2x plasmid, before induction with IPTG, lane 5, bacteria containing pMALc2x plasmid, after induction with IPTG, lanes 6 and 7, non-soluble and soluble proteins of bacteria expressing the recombinant MBP-μ chain protein respectively, prepared by centrifugation of sonicated bacteria.

Immunoblotting

Antigenic property of the expressed MBP-μ chain protein was analyzed by immunoblotting, using a mouse polyclonal serum prepared against trout serum. Results of this examination showed that the recombinant protein could be recognized by anti-trout serum

(Fig. 4). Thus, the expressed protein appears to maintain at least some of antigenic epitopes of the native µ chain protein.

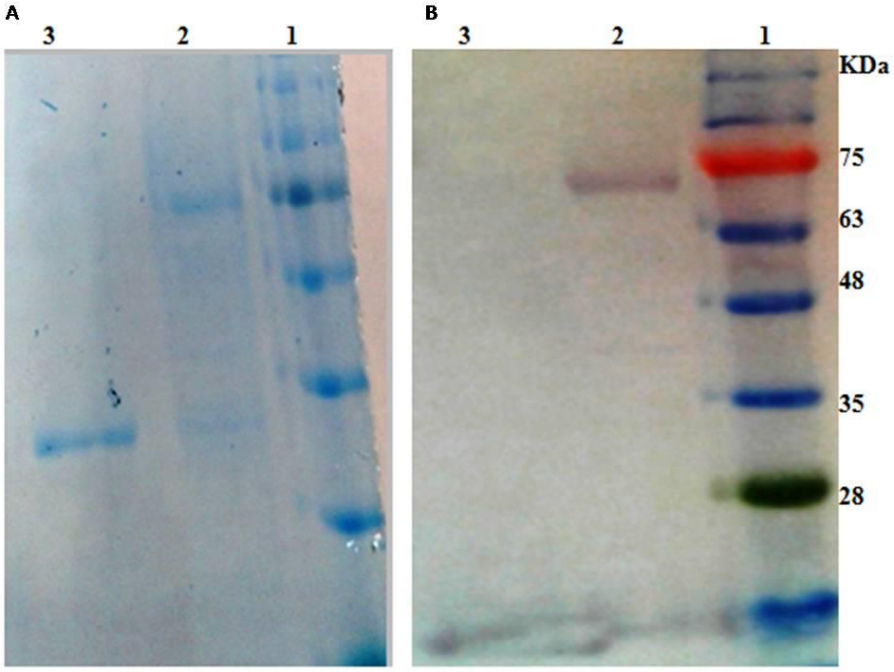

Figure 4: SDS-PAGE (A) and Immunoblot (B) analysis of MBP-µ chain expressed protein. Lane 1, standard of protein molecular weight, lane 2, purified MBP-µ chain recombinant protein, lane 3, purified MBP.

Discussion

Findings the characteristics of fish immune system including the immunoglobulins can be helpful to elucidate the evolution and diversity of fish immune system, diagnosis of fish infectious diseases and for vaccine development. So far, several studies have been performed on the structure and biochemical characteristics of fish immunoglobulins and in all studies, IgM has been mentioned as the major immunoglobulin in fish serum (Sanchez *et al*., 1991; Magnadottir *et al*., 1996; Tizard, 2000).

In fact, until 1997, IgM was the only known immunoglobulin class in fish but recent investigations revealed that fish possess 3 other classes of immunoglobulins including IgD, IgZ and IgT (Shin *et al*., 2006; Vesely *et al*., 2006). Some studies have indicated lower sequence identity of the constant regions of fish immunoglobulin compared to those of mammals and therefore suggested more divergence of fish immunoglobulin heavy chains (Choi *et al*., 2007). This diversification of immunoglobins may be due to fish need for compatibility with various environmental conditions and facing to an extensive spectrum of pathogens in their environment.

Detection of pathogen specific IgM in serum and quantification of total serum IgM can indicate the rate of fish exposure to pathogens and therefore, is important with regard to diagnosis of fish infectious diseases. For example, Olesen and Jorgensen (1986) showed

that total serum IgM of trout is higher in fish exposed to viral hemorrhagic septicemia and red mouth bacterial disease than the fish kept in aquarium conditions. Measurement of antigen specific or total serum IgM requires the access to an anti-fish IgM antibody which is usually prepared by immunization of laboratory mammals with purified fish serum IgM.

Unfortunately, purification of fish serum IgM is a relatively difficult process which requires several chromatography steps. However, with the advent of modern technology for production of recombinant proteins, now it is possible to produce the desired protein by molecular cloning of the gene which encodes the target protein. In the current study, we tried to produce the constant region of trout µ chain, as a recombinant protein in *E. coli*, in order to use the protein in future researches. The primers designed for PCR amplification of trout µ chain gene allowed to amplify the gene successfully. PCR product was then ligated to pMal-c2x expression plasmid and the protein was expressed in Rosetta strain of *E. coli*. The fact that, we were able to purify the protein by chromatography and the purified protein reacted with a polyclonal anti-trout serum indicates that the protein has been expressed in soluble form and it maintained at least some native antigenic epitopes.

Literature review showed that there have been only a few studies conducted on the cloning and expression of Fish immunoglobulins genes in *E. coli*. Choi *et al*. (2007) expressed segments of immunoglobulin D and M heavy chains in Flounder with PET28a vector in *E. coli*, BL21 strain and produced anti recombinant protein antibodies which reacted to respective native heavy chains in immunoblotting.

Jingzhuang *et al.* (2014) have also tried to produce the constant region of trout µ chain in *E. coli*. These researchers used PET-27b expression plasmid in their research and performed protein expression in Rosetta strain of *E.coli*. According to the results, a recombinant protein of 47.7 kDa was expressed in *E.coli* which was completely insoluble and its expression led to formation of inclusion bodies in bacterium. Nonetheless, after purification, it was used for immunization of rabbit and the rabbit serum was shown to react with trout serum in ELISA.

Generally, one disadvantage of heterologous proteins expression in *E. coli*is production of insoluble inclusion bodies, as reported by Jingzhuang *et al.* (2014). However, using pMAL-c2x plasmid for protein expression alleviates this problem efficiently, because MBP coded by this plasmid is one of the most effective solubilizing fusion partners (Fox *et al*., 2003). Apart from coding the MBP, pMAL-c2x plasmid contains a strong promoter that leads to more expression of recombinant proteins as compared to many prokaryotic expression plasmids. Another advantage of using pMAL-c2x plasmid is that MBP coded at N-terminal of recombinant proteins, enable the purification of fusion proteins by amylose resin. Amylose

resin is one of the cheapest resins which well absorb the MBP tailed proteins with high yield (Motamedi *et al*., 2011). In conclusion, it appears that the genetic construction we developed for expression of the constant region of trout μ chain is an efficient plasmid for production of the protein in *E. coli* and the expressed recombinant protein is an appropriate candidate for future applications, including production of monoclonal antibody against trout IgM.

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