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Generation of polyclonal antiserum to olive flounder (*Paralichthys olivaceus*) immunoglobulin by immunization of rabbit with plasmids containing heavy chain gene of olive flounder immunoglobulin

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In fish vaccinology, the secondary antibodies against fish immunoglobulins (Igs) are necessary to measure specific humoral immune responses in immunized fish. In the present study, polyclonal antiserum against olive flounder (*Paralichthys olivaceus*) IgM heavy chain was generated by intramuscular immunization of rabbit with *Escherichia coli*/eukaryotic shuttle vector containing open reading frame (ORF) of olive flounder IgM heavy chain. Western blot analysis demonstrated the specific activity of the rabbit antiserum with reduced olive flounder serum H chain at dilutions up to 1:1000. Titer of immunized rabbit serum against olive flounder serum was significantly higher than that of pre-immunized rabbit serum when determined by ELISA.

Key Words: Genetic immunization, Polyclonal antiserum, Olive flounder, Immunoglobulin

Introduction

In fish vaccinology, the secondary antibodies against fish immunoglobulins (Igs) are necessary to measure specific humoral immune responses in immunized fish. In order to develop antibodies against fish Igs, highly purified fish Igs extracted from sera are needed. Although purification and quantification of Ig from the serum of several fish species have been performed by specific antigen/antibody reactions (Sanchez *et al*., 1991; Buchmann *et al*., 1992; Kofod *et al*., 1994; Palenzuela *et al*., 1996; Bryant *et al*., 1999; Swain *et al*., 2004), lectin-binding (mannan binding protein) (Nevens *et al*., 1992; Cobb *et al*., 1998) and bacterial cell wall proteins (staphylococcal protein A) (Estevez *et al*., 1993a,b; Kanlis *et al*., 1995; Scapigliati *et al*., 1996, 1997; Morrison and Nowak, 2001), the extraction efficiency and purity were highly variable according to fish species.

The *in vivo* synthesis of antigen by DNA-based immunization is a potential alternative to immunization with purified antigen. Immunization by plasmid DNA has many advantages including the purity, ease of production, stability of episomal DNA, and the possibility to use only a given part of the gene. Recently, Timmusk *et al*. (2003) had reported the generation of monoclonal antibodies against trout two isoforms of the Ig light chain proteins (L1 and L2) of rainbow trout (*Oncorhynchus mykiss*) by genetic immunization.

In the present study, we have generated rabbit

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antibody against immunoglobulin of olive flounder (*Paralichthys olivaceus*) by immunization with *Escherichia coli*/eukaryotic shuttle vector containing open reading frame (ORF) of olive flounder IgM heavy chain. The applicability of antibody produced by genetic immunization was assessed using Western blot and ELISA.

Materials and Methods

Generation of DNA constructs

Total RNA was extracted from healthy Japanese flounder (*Paralichthys olivaceus*) spleen using TRIZOL (Invitrogen, CA, USA). The purified total RNA was reverse transcribed into cDNA using the AMV reverse transcriptase first-stand cDNA synthesis kit (Promega, WI, USA). The open reading frame (ORF) of *P. olivaceus* immunoglobulin heavy chain (Ig-hc) was amplified by polymerase chain reaction (PCR) from the cDNA using primers complimentary to the N-terminal (**GAATTC**GGCACGAGACCATAAACCAT; bolds represent *EcoR I* site) and C-terminal (GTCGACAATAGCATCTACTGGGCCTT) ends of the coding sequence (GeneBank accession number, AF226284). PCR was performed with an initial denaturation step of 3 min at 95℃, and then 30 cycles were run as follows: 30 s of denaturation at 95℃, 30 s of annealing at 55℃, and 7 min of extension at 72℃. The reacted products were electrophoresed on a 0.7% agarose gel. The PCR product was subcloned into pGEM-T easy vector (Promega) and the amplified product was ligated into the shuttle vector, pcDNA3.1 (Invitrogen). The DNA construct was subjected to DNA sequencing to verify that the olive flounder Ig-hc DNA sequence was in frame with the coding sequence of the vector. Transformed *Escherischia coli* colonies were amplified in LB containing 50 ㎍/㎖ ampicillin and the plasmid DNA constructs were purified by alkaline lysis method.

Immunization

A male 4-month-old New Zealand white rabbit was injected with 500 ㎍ of the constructed plasmids in a total volume of 0.3 ml phosphate buffered saline (PBS) into the cervical muscle and boosted 4 times with 2 weeks intervals with the same procedure of the primary immunization. Blood was sampled via the ear vein prior to the first inoculation and each boosting. After 2 weeks of the last boosting, the rabbit was euthanized and bled out.

Immunoblotting

Serum of olive flounder was solublized in SDS-PAGE loading buffer (2% SDS, 14.4 mM β-mercaptoethanol, 25% glycerol, 0.1% bromophenol blue, 60 mM Tris-HCl, pH 6.8), boiled for 5 min and fractionated on a 10% SDS-PAGE gel. Proteins were transferred to nitrocellulose membrane. The membrane was blocked with blocking solution (3% Bovine serum albumin in TBS; 150 mM NaCl, 10 mM Tris-HCl, pH 7.5) for 2 h at room temperature (RT), washed with TTBS (0.05% Tween 20 in TBS, pH 7.5) and incubated with a diluted rabbit serum (1:500 or 1:1000) for 2 h at RT. The membranes were washed three times with TTBS and incubated with alkaline phophatase conjugated mouse anti-rabbit IgG (1:2000, Santa Cruz Biotechnology Inc., CA, USA) for 2 h at RT. After washing off unbound secondary antibody, the specific antigen-bound antibody was visualized with NBT-BCIP substrate buffer (Sigma Co., St Louis, MO, USA).

ELISA

96-well ELISA plate (Corning, NY, USA) was coated with olive flounder serum (150 ㎍ protein/well) in PBS (pH 7.2) for 2 h at 60℃. The

plate was washed with PBST (0.1% Tween 20 in PBS) and blocked with blocking buffer (2% BSA in PBS) for 1 h at 37℃. After three times washing with PBST, 2-fold serially diluted immunized and pre-immunized rabbit sera (initial dilution titer, 1:50) were applied into the ELISA plate and incubated for 30 min at 37℃. The plate was then washed with PBST and incubated with alkaline phophatase conjugated mouse anti-rabbit IgG (1:1000, Santa Cruz Biotechnology Inc.) for 30 min at 37℃. The plate was washed with PBST and developed with p-nitrophenyl phosphate (p-NPP, Sigma) substrate solution for 30 min in the dark chamber. The developed colour was measured at 415 nm in a microplate reader (Bio-Rad laboratories, Hercules, CA, USA). An unpaired Student's *t*-test was used to compare the means of immunised and pre-immunised rabbit sera.

Results

Olive flounder IgM heavy chain gene ORF was amplified from total RNA of spleens by RT-PCR (Fig. 1), and was confirmed by comparison of the sequences with previously reported sequences (the sequence data are not shown).

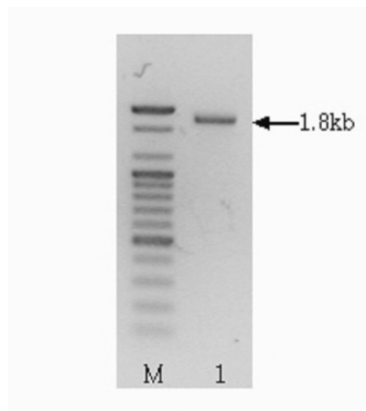

Fig. 1. Amplication of the mRNA of olive flounder (*Paralichthys olivaceus*) IgM heavy chain by RT-PCR (Lane 1). Lane M, 100 bp ladder (Bioneer Corp., Daejeon, Korea).

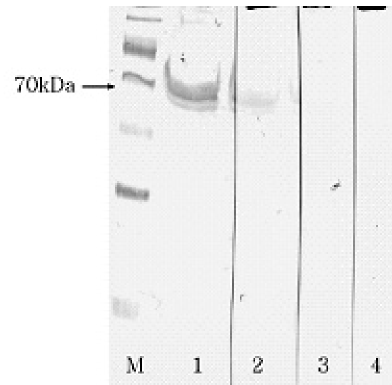

Fig. 2. Western blot analysis of rabbit anti-olive flounder IgM heavy chain serum against olive flounder serum. Lane M, TriChromRanger prestained protein marker (Pierce, Rockford. IL, USA); Lane 1 and 2, rabbit serum produced by intramuscular injection of plasmids containing olive flounder IgM heavy chain ORF at dilution 1:500 and 1:1000, respectively; Lane 3 and 4, pre-immune rabbit serum at dilution 1:500 and 1:1000, respectively.

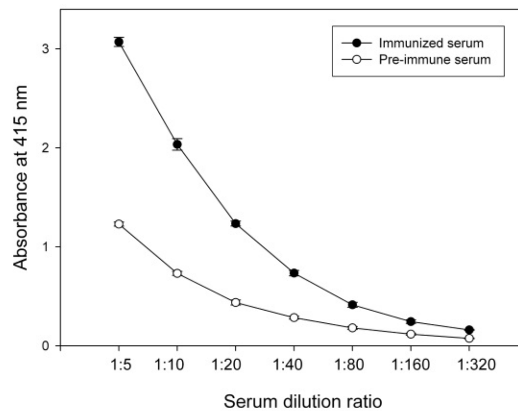

Fig. 3. Titration of rabbit anti-olive flounder IgM heavy chain serum in an ELISA using olive flounder serum as the coating antigen (mean ± standard error, n=4).

Western blot analyses demonstrated the specific activity of the rabbit antiserum with reduced olive flounder serum H chain at dilutions up to 1:1000 (Fig. 2). Pre-immunized rabbit serum was negative to reduced olive flounder serum.

Titer of immunized rabbit serum against olive flounder serum was significantly higher than that of

pre-immunized rabbit serum when determined by ELISA (Fig. 3).

Discussion

In the present study, polyclonal antibody against olive flounder IgM heavy chain was generated by intramuscular immunization of rabbit with plasmid containing olive flounder IgM heavy chain gene. The role of skeletal muscle in antigen production and antigen presentation to the immune system after intramuscular genetic immunization has been debated (Torres *et al*., 1997; Klinman *et al*., 1998; Corr *et al*., 1999; Stan *et al*., 2001). Generally, to produce antibodies against T-dependent antigens, helper T cell must recognize epitopes presented on the MHC II of antigen presenting cells (APCs) such as dendritic cell, macrophage or B cell. Although, in this study, the mechanism of Ab production in rabbit by genetic immunization was not investigated, rabbit antiserum produced by genetic immunization with olive flounder Ig heavy chain gene was applicable to Western blotting, suggesting presentation of olive flounder Ig epitopes on the MHC II of rabbit APCs by intramuscular genetic immunization.

One problem generally encountered in genetic immunization was the relatively low immunogenicity of plasmid DNA vaccines compared to their protein counterparts (Zhu *et al*., 2001). Recently, several approaches have been used to improve the immunogenicity of DNA vaccines (Barouch *et al*., 2000; Widera *et al*., 2000; Scheerlinck, 2001; Bagley *et al*., 2003). In the present study, although the ELISA titers of the rabbit serum produced by genetic immunization of olive flounder IgM heavy chain were significantly higher than those of pre-immunized rabbit serum, the titers were not high as in protein immunization, suggesting requirement of further improvements in vector construction to enhance specific humoral immune responses.

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