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Cloning and Expression of Partial Japanese Flounder (*Paralichthys olivaceus*) IgD

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The cDNA sequence of the Japanese flounder (*Paralychthys olivaceus*) IgD has been previously reported (GenBank accession no. *AB052658*) and this was followed by the detection of IgD mRNA expression in some flounder organ tissues. However, it has not been determined whether the flounder IgD gene is virtually expressed into IgD protein. To characterize the flounder immunoglobulins utilized in elucidating the mechanism, evolution and diversity of the flounder immune system, antibodies specific to IgD and IgM were necessary. In the present study, partial flounder recombinant IgD (rIgD), IgM (rIgM) and the conserved regions of IgD and IgM (rCIg) were produced by cloning the cDNA sequence using isotype specific primers which were designed to produce unique fragments of IgD and IgM specific amino acid sequences. The production of recombinant Igs was ascertained by SDS-gel electrophoresis and immunoblot analysis using anti-T7·Taq antibody. The produced recombinant Igs were purified using affinity columns, and used as immunogens. Antibodies specific to the isotype of flounder Igs were generated by immunizing rabbits with rfIgs and the antibodies produced were identified by enzyme-linked immunosorbent assay (ELISA) and immunoblotting. Specificities of the generated antibodies were evaluated by testing cross-reactivity between recombinant IgM and IgD. By ELISA, rabbit antibodies against the rfIgD fragment (anti-rfIgD) failed to recognize any kind of flounder serum Igs, whereas respective antibodies against rfCIg (anti-rfCIg) and rfIgM fragments (anti-rfIgM) reacted with serum Igs. Likewise, in immunoblot assays, though anti-rfIgD did not, both anti-rfCIg and anti-rfIgM bound with the ~85 kd flounder IgM heavy chain. By flow cytometry analysis, anti-rfCIg, anti-rfIgD and anti-rfIgM reacted with 6%, 3% and 6.5% of cells, respectively, suggesting that flounder IgD is not secreted in serum but expressed on flounder B-like cell surfaces as in mammals. Antibodies produced against recombinant flounder Igs could be used to develop sandwich assay systems for detecting flounder Igs and for further investigating the flounder immune system.

Keywords: cDNA, ELISA, Flounder, IgD, Recombinant protein

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Introduction

Teleost fish possess both humoral and cell-mediated immunity (Graham and Secombes, 1990). Humoral immune response in fish involves the secretion of specific immunoglobulins (Igs) directed to neutralize antigens and to activate the complement cascade (Rijkers *et al*., 1980). In teleosts, accumulated evidence has revealed the primary structure and gene organizations of Ig heavy (H) and light (L) chains (Warr, 1995; Lundgvist *et al*., 1996; Nakao *et al*., 1998). The secretory form of the teleost IgH chain was found to be homologous to mammalian m chain. Furthermore, IgM heavy chain genes were cloned by using the cDNA libraries of some fishes (Aoki *et al*., 1999; Lee *et al*., 2001). Moreover, recently, full IgD heavy chain gene sequences have been determined in channel catfish (Wilson *et al*., 1997), Atlantic salmon (Hordvik *et al*., 1999), Atlantic cod (Stenvik and Jorgensen, 2000), and Japanese flounder (Hirono *et al*., 2003), which raises the possibility that IgD, in teleosts, could be an ancestral type of Igs in addition to IgM. Little is known about the expression of teleost IgD. In salmon and cod, only transcripts of surface IgD have been found, whereas a secretory variant has been identified in catfish (Wilson *et al*., 1997; Miller *et al*., 1998). Lee *et al*. (2001) reported that two different H chains were found in flounder serum with distinct size and antigenicity. Similarly,

Jang *et al*. (2004) suggested (on a molecular weight basis) that two different H chains are present in flounder serum.

The humoral immune systems of fish at the molecular and protein levels are interesting topics for study, but at present information available on the proteomics and molecular mechanisms in fish are inadequate to allow an understanding of the fish immune system, although molecular evolutionary studies of fish MHC, T cell receptor α and β, and Ig genes have already been performed. Specific antibodies, polyclonal and/or monoclonal, to Igs and their subunits have been found to be valuable tools in immunological research and immunological assays. During the last decade, these have also been extensively used in various studies on fish immune systems (DeLuca *et al*., 1983; Thuvander *et al*., 1990; Magnadottir and Gudmundsdottir, 1992; Estevez *et al*., 1994). In the present study, partial recombinant flounder IgD and IgM heavy chains, and conserved regions in both Igs were manufactured and polyclonal antibodies were produced against the respective recombinant proteins.

These antibodies against recombinant flounder Igs may be useful in investigations on the differentiation of flounder B-like cells, for evaluating and developing effective vaccines for flounder, and for monitoring the course of therapy in the pathogenically infected flounders.

Materials and Methods

Tissue preparation and RNA extraction. Kidneys were collected from 1-year-old Japanese flounders (Paralichthys olivaceus). To harvest tissues, fish were sedated in an ice bath and sacrificed by decapitation. Tissues were immediately removed by dissection, frozen in liquid nitrogen and stored at −80ºC until analyzed. Total RNA was isolated from flounder kidney by the acid guanidinium thiocyanate-phenol chloroform extraction method according to the manufacturer's instructions. RNA samples were kept at −80ºC until required.

Cloning of flounder Ig cDNA and expression vector construction. First-strand cDNA was synthesized from total flounder RNA extracted under RNase-free conditions. The reaction was performed with 10 µg of total RNA using a ProSTAR™ First-Strand RT-PCR Kit (Stratagene, La Jolla, CA). Briefly, a mixture of 10 µg of total RNA templates and 3 µl of oligo (dT) primer (100 ng/µl) in DEPC-treated water to a total volume of 38 ml was incubated at 65ºC for 5 min, and then slowly cooled at room temperature (RT). 1× first-strand buffer, 1.25 U RNase block ribonuclease inhibitor, 4 mM dNTPs and 1 U StrataScript™ reverse transcriptase were then added to produce a final volume of 50 µl which was incubated at 42ºC for 1 h.

To amplify the coding region of flounder IgD, IgM and the conserved sequence of both Igs (CIg) by PCR, specific primers were designed to introduce *Bam*HI and *Eco*RI restriction sites at both ends of the PCR product since the expression vector, pET28a contains the same restriction enzyme sites. Specific primers were prepared (as detailed in Table 1) based on flounder IgD and IgM cDNA sequences (GenBank Accession nos. ***AB052658*** and ***AB052744***, respectively). The three kinds of flounder Ig PCR products produced were adjusted to about 0.7 kb by each specific primer set, and cloned into an expression vector which carried an integrated copy of the T7 · Taq RNA polymerase gene under the control of inducible lac UV5 promoter (Fig. 1). The sequences of the insert and frame were confirmed by DNA sequencing analysis (Korea Basic Science Research Institute, Daejon, Korea).

Table 1. Primers used in RT-PCR

	Primer	Position
CIg	5'-CGGGATCCTTGGCAGCTGGATCATATGTG-3' **BamHI**	66 → 86
	5'-CGGAATTC**TTA**TTTTGGCTTTTGGAGGG-3' **EcoRI** end code	746 → 729
IgD	5'-CGGGATCCAGTCGGGTTGTCTCTCCAAAC-3' **BamHI**	747 → 767
	5'-CGGAATTC**TTA**GAGCACAGCACTTTGTCCCTT-3' **EcoRI** end code	1427 → 1407
IgM	5'-CGGGATCCGTATTATTTAGTTCACCCG-3' **BamHI**	725 → 742
	5'-CCGAATTC**TTA**TGGCATCATAAACACTGA-3' **EcoRI** end code	1396 → 1413

Expression and purification of recombinant flounder Ig fragments (rfIg). Expression vectors with/without insert were used to transform *E. coli* strain BL21 (DE3)-competent cells. Transformed cells were grown at 37ºC in Luria-Bertani medium supplemented with kanamycin (50 µg/ml) to a cell density of A660 nm = 0.4-0.6 and induced with 1mM isopropyl-β-D-thiogalactoside (IPTG) for 2 h at 37ºC. Cells were then pelleted and resuspended in sample buffer and the presence of rflgs fragments in eluted fractions were confirmed by denatured SDS-PAGE analysis and Western blotting using anti-T7 · Taq monoclonal Ab. To purify the rfIg fragments, about 1 liter of cultured transformed cells were centrifuged and then pellets were resuspended in 5 ml of PBS (pH 7.4) containing 1 mM PMSF, sonified, and were centrifuged (8000 rpm) at 4ºC for 10 min. To determine the solubilities of rfIg fragments, pellets and supernatants were analyzed by SDS-PAGE. The resulting lysates containing rfIg fragments (CIg, IgD or IgM) were purified by T7 · Taq polymerase antibody-labeled affinity chromatography (Novagene, Germany) according to the manufacturer's instructions. Purified rfIg fragments were dialyzed against PBS (pH 7.4) and protein concentrations were determined by the Bradford method (Bradford, 1976).

Production of polyclonal antibodies against rfIg fragments. To produce polyclonal antibodies against the purified rfIg fragments, CIg, IgD, and IgM, New Zealand rabbits were subcutaneously immunized with 100 µg of the rfIg fragments emulsified 1 : 1 in Freund's complete adjuvant (FCA), and made up to a total volume of 1 ml per rabbit. Boosters injections were administered on days 7, 14 and 21 at the same rfIg fragment dose in Freund's incomplete adjuvant (FIA). Final booster injects were administered on day 28 at the same rfIg fragment doses in 1 ml of sterile Hank's balanced

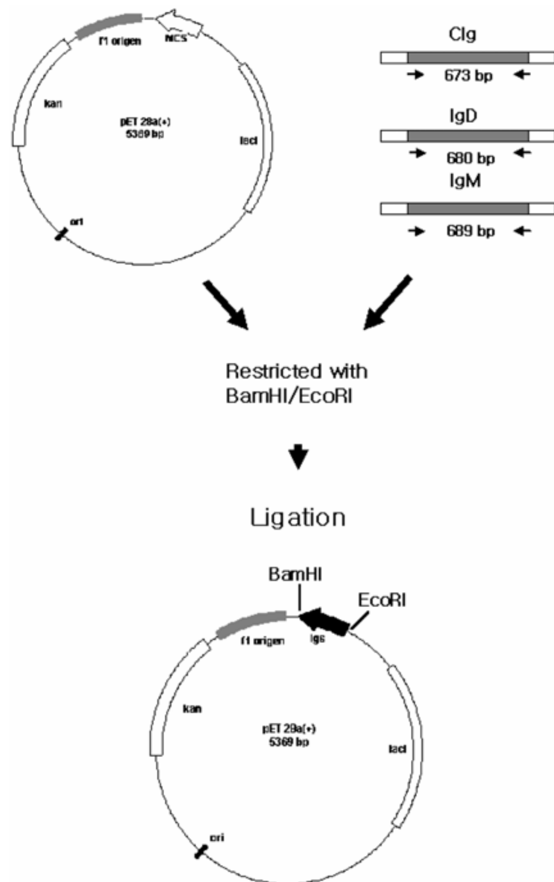

Fig. 1. A schematic representation of the construction of the expression vector (pET28a) used to produce rfCIg, rfIgD, or rfIgM in *E. coli* under the control of T7 promoter. MCS, molecular cloning site; Kan, kanamycine resistant gene.

salt solution (HBSS). Following each boosting injection, serum samples were harvested for serological study. Antibody titers were determined by enzyme-linked immunosorbent assay (ELISA) using a slight modification of the method described by Choi *et al*. (2002). Briefly, normal flounder serum (NFS) was diluted 1 : 50 in coating buffer (15 mM sodium carbonate, 34 mM sodium bicarbonate, 0.02% sodium azid). 100 µl aliquots of the diluted NFS were dispensed into each well of a immuno module (Nunc). lates were incubated overnight at 4°C, and wells were then washed three times with PBS (PH 7.4), supplemented with 0.1% Tween-20 (Sigma) (T-PBS) and blocked for 1 h at 37°C with 100 µl of 2% (weight/volume, w/v) skimmed milk in T-PBS to prevent nonspecific protein binding. Plates were then washed and reincubated for 30 min at RT with 100 µl of serially diluted antibodies against recombinant CIg, IgD or IgM, respectively. Diluted normal rabbit serum (1 : 100) was applied as a negative control and rabbit antibody against purified flounder serum IgM (Jang, 2004) as a positive control. After removing unresponsed supernatants, alkaline phosphate-conjugated goat anti-mouse IgG (Promega, USA) was administered as a probe. All plates were washed three times after each step, except for the substrate incubation. After incubation for 30 min at RT, plates were washed five times. Color reactions were initiated by adding 100 µl of substrate solution (BCIP/NBT in substrate buffer) to each well, and absorbances were read at 405 nm (A405) using a DigiScan ELISA Reader.

Immunoblot analysis. To determine whether rabbit antibodies against rfIg fragments recognize the natural flounder Igs present in normal flounder serum, immunoblot analysis was performed. Total flounder serum proteins were diluted 1 : 50 and reduced and denatured by boiling for 5-6 min in loading buffer (62 mM Tris-HCl (pH 6.8), containing 2% [w/v] SDS, 10% [v/v] glycerol, 0.05% [w/v] bromophenol blue and 0.1 M dithiothreitol), electrophoresed in 10% SDS polyacrylamide gel, and electrotransferred to nitrocellulose membranes (BioRad) for 1 h at 250 mA. Membranes were then blocked for 30 min in Tris-buffered saline (TBS) containing 2% skimmed milk, and washed in TBS. The three different rabbit antibodies diluted 1 : 50 were then applied to each membrane for 1 h, respectively. After washing, goat anti-rabbit IgG-alkaline phosphatase (AP) conjugate diluted 1/3000 in TBS (pH 7.5), containing 0.05% (v/v) Tween-20 and 2% skimmed milk (S-TTBS) was incubated with the membranes for 1h at RT, which were then washed three times. The presence of immune complexes was detected by incubating membranes in substrate (NBT & BCIP) (Promega, Madison, WI) for 15 min.

Flow cytometry. Flounder kidney cell suspensions were prepared by rubbing tissue samples between two slide glasses in serum-free DMEM. Cell suspensions were layered over Histopaque-1077 (Sigma) and centrifuged at 2,500 rpm for 20 min at 4°C to remove erythrocytes. White cells were collected from Histopaque-1077 layers and washed twice with serum-free DMEM. Prepared cells were suspended in ice-cold PBS. Approximately, 2×10^6 cells were then labeled for 30 min on ice containing 100 µl of anti-rfCIg, anti-rfIgD, and anti-rfIgM diluted 1 : 50 in PBS. Normal rabbit serum diluted 1 : 50 was applied to cells as a negative control. Nonspecific binding and background fluorescence were quantified by analyzing negative control samples. After washing cells twice with ice-cold PBS, 100 µl of FITC-conjugated goat anti-rabbit Ig (H + L) antibody (Immunoresearch, USA) was added to the cells, which were then incubated for 30 min on ice. Cells were then washed twice with ice-cold PBS and fixed in 2% (wt/vol) paraformaldehyde containing 200 µl of PBS. Fluorescence-positive cells were analyzed using a Coulter FACScan (Beckman Coulter, Fullerton, CA). Each of the 2×10^3 cells per sample was collected for fluorescence-activated cell sorter (FACS) analysis from lymphocyte gates. Percentages of cells stained with antibodies were determined from forward (FS) and sideways (SS) scatter patterns.

Results

Characterization of flounder IgD and IgM cDNA sequences. The cDNA sequences of Japanese flounder (*Paralychthys olivaceus*) IgD and IgM were deposited in the DNA database using NCBI program (GenBank Accession nos. ***AB052658*** and ***AB052744***). IgD cDNA of 3244 bp and IgM cDNA of 1877 bp coded 999 and 579 amino acid residues, respectively. The nucleotide sequences of flounder IgD and IgM used in this study showed 95% and 97% homologies, respectively,

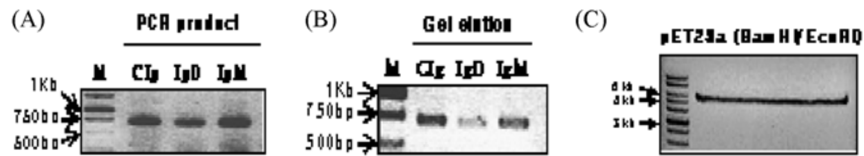

Fig. 2. PCR amplifications of CIg, IgD or IgM and eluted pET28a vector. A; PCR products of CIg, IgD, and IgM. B; Elution of amplified CIg, IgD and IgM genes from agarose gels. C; Elution of pET28a vector restricted with *Bam*HI and *Eco*RI.

with previously registered sequences (Accession nos. ***AB052658 and AB052744***) (data not shown). The homology of the conserved sequences of Japanese flounder IgD and IgM (CIg) was 97% (data not shown). The amino acid sequences deduced from the open reading frame (ORF) of flounder CIg, IgD and IgM cDNAs were compared with known amino acid sequences of the immunoglobulins of 5~7 other species. The amino acid sequences of CIg, IgD and IgM fragments showed less than 40% identity with other fish species by BLAST (data not shown).

Cloning of target genes and the selection of recombinants. Flounder Ig cDNAs were amplified by PCR and purified for ligation with pET28a expression vector, which can direct the expression of Ig genes with T7 Tag fusion protein. Amplified PCR products were detected by 1% agarose electrophoresis, and DNA segments of about 680 bp were observed, as was expected (Fig. 2A). PCR products of flounder Ig cDNAs were successfully purified from gel (Fig. 2B). The plasmids (pET28a) of transformed cells were recovered and confirmed using restriction enzymes *Bam*HI and *Eco*RI (Fig. 2C). Pure PCR products and pET28a vector were digested with *Bam*HI and *Eco*RI, and then purified and ligated with T4 DNA ligase (Fig. 3A). To confirm if the successful ligation of PCR products and pET28a, pET28a vectors with inserts were digested with *Bam*HI and *Eco*RI. Fig. 3B shows that the inserts were correctly ligated into the pET28a vectors.

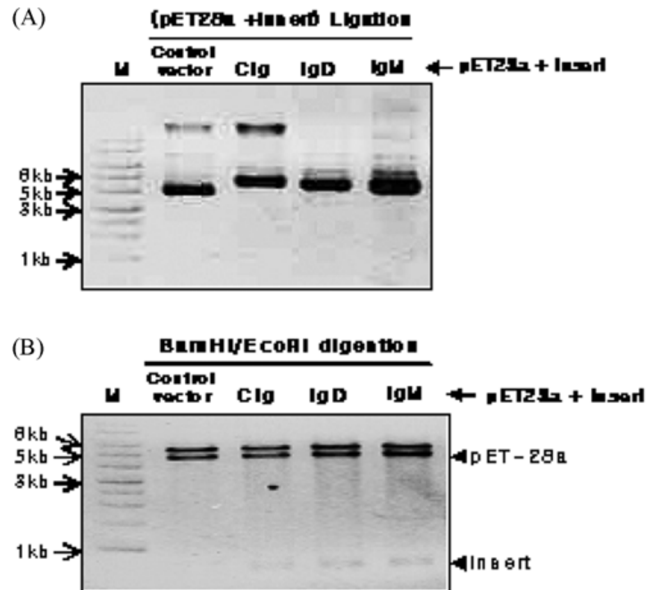

Fig. 3. Construction of pET28a vectors ligated with fCIg, fIgD or fIgM inserts. A, pET28a vectors ligated with fCIg, fIgD, or fIgM inserts; B, *Eco*RI and *Bam*HI-digested pET28a vectors with fCIg, fIgD, or fIgM inserts.

Expression and purification of recombinant flounder Igs. The recombinant proteins produced by transformed *E. coli* BL21 (DE3) host cells were analyzed by SDS-PAGE. As shown in Fig. 4A, rfIg fragments, CIg, IgD and IgM of ~30 kDa were successfully induced. On the other hand, the *E. coli* BL21 control containing pET28a did not have any related protein bands (Fig. 4A). Expressed recombinant proteins were recovered as inclusion bodies in pellets. Culture of transformed BL21 cells without IPTG induction failed to increase the solubility of recombinant proteins (data not shown). Expressions of recombinant proteins was determined by immunoblotting using anti-T7 · Taq monoclonal antibody (Fig. 4B), and the predicted ~30 kDa recombinant protein bands were visualized as shown in Fig. 4B.

Production of polyclonal antibodies and identification of their specificities. To investigate whether rabbit antibodies against rfIg fragments recognize natural flounder Igs in normal flounder serum, ELISA and immunoblot assays were conducted. By ELISA, rabbit antibodies against rfIgD fragment (anti-rfIgD) failed to recognize any kind of flounder serum Igs, whereas the respective antibodies against rfCIg (anti-rfCIg) and rfIgM fragments (anti-rfIgM) did react with serum Igs (Fig. 5). To further confirm this result, immunoblot assays were performed. As shown in Fig. 6, normal rabbit serum (NRS) appeared non-specifically bound with ~75 kd of some flounder serum proteins, which reacted with rabbit antibodies against anti-rfIgs. Anti-rfCIg and anti-rfIgM (but not anti-rfIgD) bound to ~85 kd of flounder IgM heavy chain (lanes 4 and 6).

Flow cytometry analysis. To determine whether antibodies against rfCIg, rfIgD or rfIgM can recognize immunoglobulin receptors on flounder B-like cell surfaces, flow cytometry was performed. As shown in Fig. 7, anti-rfCIg, anti-rfIgD, or anti-rfIgM reacted with 6%, 3% and 6.5% of cells, respectively. Although anti-rfIgD showed only a weak response in 3% of cells, these positive results indicate that flounder IgD is not expressed in serum but only on the surfaces of B-like cells.

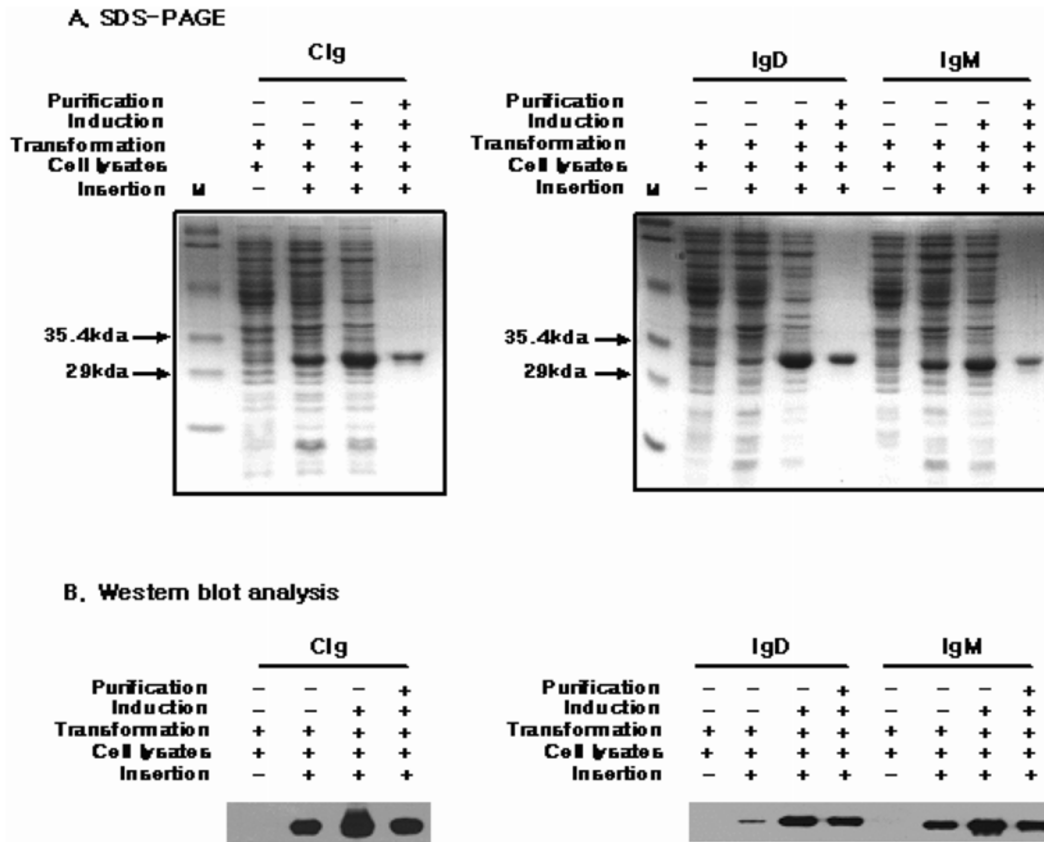

Fig. 4. Expression of recombinant proteins and Western blot analysis. A, The expressions of rfCIg, rfIgD, or rfIgM fusion proteins on SDS-PAGE; B, Western blot analysis using anti-T7 · Tag monoclonal antibody. Purification was performed using T7 · Taq polymerase antibody-labeled affinity chromatography; Induction, induced using 1mM IPTG for 2 h at 37°C; Cell lysates were prepared by sonicating transformed cells treated with PMSF. rfCIg, rfIgD and rfIgM PCR products were inserted into; +, positive; -, negative.

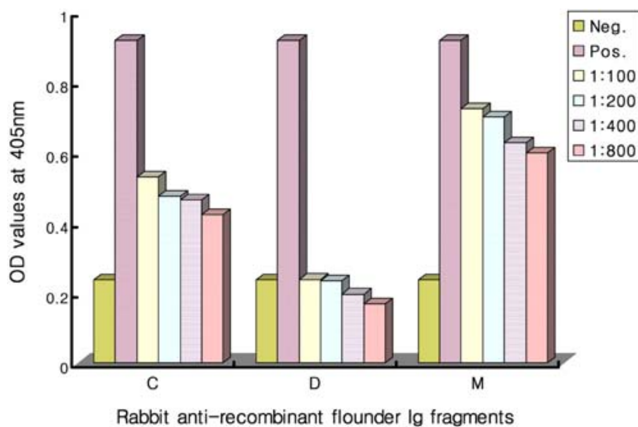

Fig. 5. ELISA of rabbit antibodies against flounder immunoglobulins present in pre-immune serum. Fifty-fold diluted flounder pre-immune serum was coated on plates overnight, blocked with 2% skimmed milk, and incubation with serially diluted rabbit polyclonal antibodies against recombinant flounder Ig fractions. Alkaline phosphatase color reactions were developed using diethanolamine substrate solution and OD values were read at 405 nm using an ELISA reader. Result are representative of more than three different experiments. The OD values shown are the mean values of triplicate wells.

Discussion

IgM is the only antibody isotype found universally in gnathostomes, and until 1997, teleosts (bony fish) were thought to only possess IgM. However, studies on catfish (Wilson *et al*., 1997) and Atlantic salmon (Hordvik *et al*., 1999) provided evidence for the existence of IgD in teleosts, thus suggesting that IgD had an important function during the early phase of evolution (Miller *et al*., 1998). In channel catfish, the IgH locus encodes two distinct delta genes that represent both the membrane-bound and secreted forms of IgD (Bengten *et al*., 2002). Nevertheless, the cDNAs representing the secreted versions of Japanese flounder IgD have not been identified. The characterizations of fish immunoglobulins can help to elucidate the mechanism underlying the evolution and diversity of the fish immune system. In addition, knowledge of the DNA sequences of immunoglobulin genes can be useful during the development of vaccines, and makes it possible to detect variations in the immunoglobulin variable region after vaccination (Hirono *et al*., 2003). Recently, several cDNA clones encoding partial cDNA fragments of IgD and IgM from Japanese flounder were cloned (Aoki *et al*., 2000). According to Hirono *et al*.

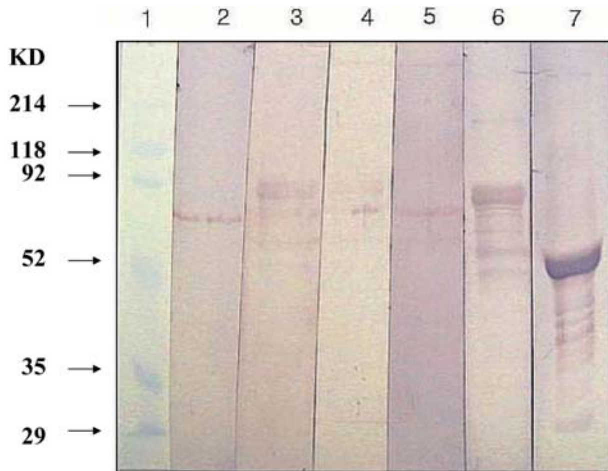

Fig. 6. Immunoblot analysis of rabbit antibodies against flounder immunoglobulins present in pre-immune serum. SDS-PAGE and electrophoretic transfer were performed as described in Materials and Methods. In lanes 2~6, normal flounder sera diluted 1 : 50 were applied to SDS-PAGE and transferred to nitrocellulose membrane filters. Only normal rabbit serum was applied to lane 7. Lane 1, molecular marker; lane 2, normal rabbit serum diluted 1 : 50; lane 3, rabbit anti-flounder IgM as a positive control; lanes 4~6, rabbit anti-rfCIg, rfIgD or rfIgM, respectively; lane 7, anti-rabbit IgG (another positive control).

(2003), the amino acid sequence of the conserved region of Japanese flounder IgD shares 40-53% identity to the sequences of previously reported teleost full-length IgDs. These amino acid sequence identities of fish Ig heavy chain constant regions are lower than those found in mammals, suggesting a higher divergence of fish Ig heavy chains. One possible reason for this is that fish need to adapt to different environmental conditions and are exposed to a wider variety of pathogens in their environments.

Previously, we developed monoclonal antibodies (MAbs) against flounder IgM molecules that were purified from antigen-sensitized flounder serum (Jang *et al*., 2004). These MAbs were found to react with surface immunoglobulin (sIg) receptors which were regarded as IgM molecules. FACS analysis demonstrated that 25-35% of the lymphoid gate is composed of sIgM + B cells (Jang *et al*., 2004). However, if an sIg molecule (e.g., sIgD) other than sIgM is present on the cell surface, it is uncertain whether the produced MAbs against serum IgM bind specifically with a sequence of sIgM or simultaneously a conserved one between IgD and IgM. It was, Therefore, it necessary to obtain sIgM and sIgD specific antibody molecules. To make those antibodies, both IgD and IgM specific antigen peptides are required, and because it is too difficult to purify sIgD molecules, we attempted to make recombinant proteins based on the previously reported cDNA

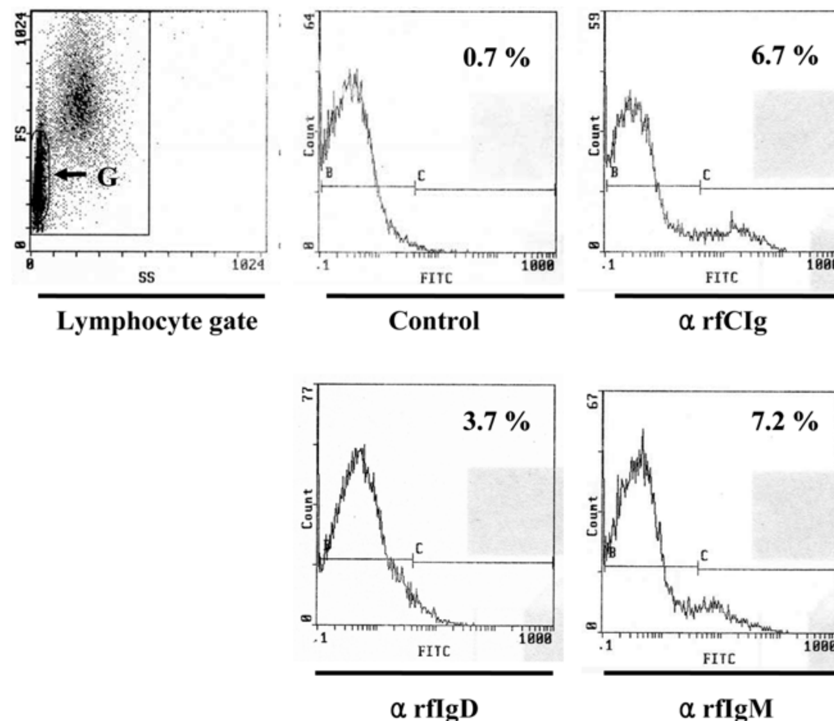

Fig. 7. Flow cytometry analysis of isolated flounder kidney cells. Cells were incubated with normal rabbit serum, rabbit anti-rfCIg, anti-rfIgD, or anti-rfIgM and stained with FITC-conjugated anti-mouse Ig. The presence of kidney leukocytes were demonstrated by FSC/SSC dot plots of gated lymphoid cells (G). The fluorescence intensities of different antibody reactive G-gated cells are shown in the histograms.

gene sequences of flounder IgD and IgM (GenBank Accession nos. ***AB052658*** and ***AB052744***).

Basis on a sequence of about 0.7 kb with an open reading frame (ORF), three different primers, specific for flounder δ and μ chains and the conserved region of both chains (CIg) were manufactured, and PCR products based on these primers were successfully amplified. The three DNA inserts eluted from agarose gel were ligated to pET28a vector restricted with *Bam*HI and *Eco*RI. To confirm that the pET28a constructs were correctly made, the constructs were treated with restriction endonucleases and this was followed by agarose gel electrophoresis. As shown in Fig. 4B, pET28a constructs were not fully digested due to the short enzyme treatment times used. The upper bands just above pET28a in the figure, indicate the pET28a constructs which were not digested by the enzymes. The lower weak bands are insert DNAs taken from enzyme-treated constructs, which suggests that the recombinant expression vectors were successfully manufactured.

E. coli BL21 (DE3) cells were transformed using recombinant plasmid (pET28a) expression vectors. Transformed cells containing the constructed plasmids were cultured with IPTG to induce recombinant Igs using T7 · Taq fusion protein partner, which was in favor of the purification of the recombinant proteins. When induced for 2 h at 37°C, expression reached a maximum (data not shown). Protein expression, as analyzed by Western blotting, proved that had the antigenic activity of T7 · Taq fusion protein. To examine the relative distribution of the expressed recombinant protein in soluble and insoluble fractions, both supernatants and pellets of cell lysate were investigated after sonication, and it was found that the recombinant proteins were predominantly expressed in the insoluble fraction.

To determine whether rabbit antibodies against rfIg fragments recognize natural flounder Igs in normal flounder serum, both ELISA and immunoblot assays were performed. According to our ELISA findings anti-rfIgD failed to detect flounder IgD-like molecules, while both anti-rfCIg and anti-rfIgM recognized serum proteins regarded as flounder IgM molecules. However, unexpectedly, the degree of binding activity by anti-rfCIg was found to be less than that of anti-rfIgM, suggesting that the antigenicity of rfCIg is weaker than that of rfIgM. Likewise by Western blotting only rfIgM and rfCIg detected the ~85 kd of flounder IgM heavy chain in normal flounder serum. Bands migrating at ~75 kd that reacted with antibody were found to cross-react with pre-immune serum. Considering these results, it appears, that as has been reported in mammals, flounder IgD molecules are unlikely to be secreted in serum and that they are preferably expressed on B-like cell surfaces. To ascertain whether anti-rfCIg, anti-rfIgD, or anti-rfIgM can recognize immunoglobulin receptors on flounder B-like cell surfaces, FACS analysis was performed. The selected gate for lymphocytes was expected to segregate mainly dead cells, but actually most were identified as lymphoid cells by Wright staining (data not shown). Although anti-rfIgD reacted with only 3% of cells weakly, this positive result indicates that flounder IgD is not expressed in serum but rather on B-like cell surfaces.

The aim of the present study was to overexpress flounder IgD fragment protein and to raise antibodies against that could be as a tool for studies on the development of the fish immune system. Detailed knowledge of immune system function is an essential part of devising disease prevention strategies, such as, the development of vaccines and for monitoring therapeutic processes in microbially infected fishes.

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