Purification of Two Different Immunoglobulins (Igs) from Olive Flounder *Paralichthys olivaceus* and Analysis of *Lactococcus garvieae* Antigens by the Igs

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ABSTRACT—Immunoglobulins (Igs) were purified from sera obtained from olive flounder *Paralichthys olivaceus* immunized with goat IgG using immunoaffinity and mannan-binding protein (MBP) affinity columns and designated IMMIg and MBPIg respectively. SDS-PAGE and two-dimensional gel electrophoresis (2-DE) analyses demonstrated that the olive flounder serum contained at least two different types of Ig in terms of molecular weight and pI of heavy chain. 2-DE separation of the Igs followed by peptide mass fingerprinting using matrix-assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF MS) revealed that these two Igs were IgM and IgM precursor of olive flounder. Monoclonal antibodies (MAbs) were raised against the IMMIg and MBPIg and proteome analysis of *Lactococcus garvieae* using these MAbs and olive flounder immune sera demonstrated that IMMIg and MBPIg recognized different antigens of *L. garvieae*. This suggested that different Igs are possibly involved in protecting olive flounder against *L. garvieae* infection.

Keywords: *Paralichthys olivaceus*, *Lactococcus garvieae*, immunoglobulin, affinity chromatography, immunoproteomics, monoclonal antibodies, olive flounder

The olive flounder *Paralichthys olivaceus* is an important mariculture fish species in Korea, China and Japan. In Korea, this species is mainly cultured in land-based tanks, and its production reached 32,141 metric tons in 2004 (2005 Statistics, Ministry of Maritime Affairs & Fisheries, Korea Government). However, the intensive culture of this species has led to the emergence of various parasitic, bacterial and viral diseases, which have triggered severe economic losses. A more thorough understanding of fish defense mechanisms against various infectious diseases is highly needed for the development of appropriate health management strategies and sustainable aquaculture production.

The defense mechanisms of vertebrates are gener-

ally divided into the innate and acquired immune responses. Acquired immune responses involve the production of specific immunoglobulins (Igs) by the host following antigen stimulation. The produced Igs protect the host by removing the pathogen or preventing it from spreading into the blood or organs. Mammals possess five classes of IgM, IgG, IgA, IgE and IgD (Tizard, 2000). On the other hand, fishes have been reported to have 3 isotypes of the Ig in genomic study, namely IgM (Lee *et al.*, 2001; Hordvik *et al.*, 2002), IgD (Hirono *et al.*, 2003), IgZ/IgT (Danilova *et al.*, 2005; Hansen *et al.*, 2005). Of these Igs, tetramer IgM has been reported to play an important role in humoral immune response of fish (Kaattari and Piganelli, 1996; Tizard, 2000). On the other hand, two forms of IgMs, high molecular protein (HMW) and low molecular protein (LMW) IgM, have been reported from some fish species, such as olive

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flounder (Bang *et al.*, 1996), giant grouper *Epinephelus itaira* (Clem, 1971), rainbow trout (Elcombe *et al.*, 1985), channel catfish *Ictalurus punctatus* (Lobb and Olson, 1988) and carp *Cyprinus carpio* (Rombout *et al.*, 1993). In olive flounder, LMW IgM purified from hyper-immune sera against *Edwardsiella tarda* showed different agglutination titer from that of the HMW IgM (Bang *et al.*, 1996).

A large variety of techniques have been used to purify Igs from fish serum, including precipitation in saturated ammonium sulfate, gel filtration, ion exchange chromatography and affinity chromatography. Affinity columns bound with specific ligands, such as protein A, mannan binding protein (MBP) and specific protein, have been widely used for purifying IgM from fish sera due to their methodological simplicity and specificity to IgM (Suzuki *et al.*, 1990; Al-Harbi *et al.*, 2000; Watts *et al.*, 2001; Crosbie and Nowak, 2002; Bromage *et al.*, 2004).

Immunoproteomic tools to study immune response, such as two-dimensional gel electrophoresis (2-DE), immunoblotting and peptide mass fingerprinting (PMF) using matrix-assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF MS), have been widely used for separation of crude proteins/antigens and their identification from cells, tissues, body fluids, or organism lysates (Görg *et al.*, 2000; Mini *et al.*, 2006; Twine *et al.*, 2006). In these ways, proteomic tools have been proven highly useful for studying pathogenesis, exploring markers for diagnosis and vaccines development.

Lactococcus garvieae has been identified as a causative agent of fish streptococcosis (Roberts, 2001) in many species worldwide, including olive flounder (Shin *et al.*, 2006a), rainbow trout *Oncorhynchus mykiss* (Chang *et al.*, 2002), and yellowtail *Seriola quinqueradiata* (Kusuda *et al.*, 1976). In spite of importance as a fish pathogen, there were few studies about antigens of this bacterium recognized by host fish (Kang *et al.*, 2006; Ooyama *et al.*, 2002). Recently, we constructed partial 2-DE reference map for *L. garvieae* (Shin *et al.*, 2006b) which might be highly useful in identifying antigenic proteins on 2-DE immunoblotting profile. In the present study, we produced monoclonal antibodies (MAbs) for two different IgMs purified from olive flounder. Using these MAbs and olive flounder immune serum, immunoproteomic analysis of *L. garvieae* was carried out to understand humoral immune response of olive flounder against *L. garvieae* infection.

Materials and Methods

Fish and preparation of immune sera

Olive flounders (body weight 300–400 g) were purchased from a commercial fish farm and maintained in fiber reinforced plastics (FRP) tanks provided with recir-

culated seawater maintained at 18–20°C. Fifteen fish were immunized by intraperitoneal injection with goat IgG (250 µg/100 g body weight) mixed 1:1 (v/v) with Freund's complete adjuvant. Identical second and third booster doses were administered at two-week intervals. One week after the third injection, the fish were bled from their caudal veins, and the collected sera were kept at –70°C until use. Ten fish injected with phosphate buffered saline (PBS; 140 mM NaCl, 2.7 mM KCl, 1.5 mM KH₂PO₄, and 8.1 mM Na₂HPO₄, pH 7.2) were used as controls and harvested for non-immunized sera.

Purification of fish antibodies

Protein A, mannan binding protein (MBP) and immunoaffinity columns were used to purify Igs from immunized sera.

(a) Protein A affinity columns

Protein A beads (Sigma, USA) were used to purify Igs from immunized olive flounders, according to Bollag *et al.* (1996) with slight modification. Briefly, 1 mg of protein A beads were packed in disposable polystyrene columns (0.8 × 4 cm, Bio-Rad, USA) and washed with binding buffer (10 mM Tris, 1.25 M NaCl, 20 mM CaCl₂, 0.02% NaN₃, pH 7.4). Serum samples were mixed with binding buffer (1:1) and applied to the columns. Unbound materials were removed with 150 mL of binding buffer, and the Igs were eluted using elution buffer (10 mM Tris, 1.25 M NaCl, 0.02% NaN₃, pH 7.4) with 1 mL fractions collected in microtubes containing 0.1 mL of 0.1 M Tris buffer, pH 7.4.

(b) Mannan binding protein (MBP) affinity columns

Igs were purified from immunized olive flounder using the ImmunoPure[®] IgM Purification Kit (Pierce, USA). Briefly, the 5 mL columns prepacked were pre-washed with 5 mL of preparation buffer and then equilibrated with 20 mL of binding buffer. Serum samples were mixed with binding buffer (1:1) and applied to the columns, which were then incubated for 30 min at 4°C. After unbound materials were removed with 42 mL of binding buffer, columns were loaded with 3 mL elution buffer and incubated for 1 h at room temperature (R.T.), and the elutes were fractionated as above.

(c) Immunoaffinity columns

Igs were immunoaffinity purified from immunized olive flounder sera according to the method of Smith (1992). Briefly, columns (0.8 × 4 cm, Bio-Rad, USA) containing 2 mg of goat IgG agarose beads (Sigma, USA) were washed with 150 mL PBS and loaded with serum samples mixed 1:1 with PBS. After the columns were washed with 150 mL PBS, the Igs were eluted with elution buffer (0.1 M glycine-NaOH, pH 11), and 1 mL fractions were collected in microtubes containing 0.1 mL of 0.1 M Tris buffer, pH 7.4. The elutes containing Igs were desalted and concentrated by centrifugation with PBS in Centricon (Falcon, USA). The concentrations of

purified proteins were measured using the Bradford reagent (Bio-Rad, USA) with spectrophotometer at 595 nm.

SDS-PAGE and two-dimensional gel electrophoresis (2-DE) of purified Igs

SDS-PAGE was performed under reducing to elucidate the molecular weights of the purified Igs. For reducing conditions, purified Igs (0.3 μ g) were mixed with SDS-PAGE sample buffer (60 mM Tris-HCl, 25% glycerol, 2% SDS, 14.4 mM 2-mercaptoethanol, 0.1% bromophenol blue) and boiled for 10 min at 100°C. The denatured proteins were resolved on 12.5% gels at 50 V for 15 min and then at 100 V until the dye reached the bottom of the gel. 2-DE was performed as described by Görg *et al.* (2000), with slight modification. Affinity column purified Igs were diluted to 1 mg/mL with rehydration buffer (9 M urea, 4% CHAPS, 20 mM DTT, 0.5% IPG buffer, pH 4–7, and trace amounts of bromophenol blue), then microcentrifuged at $16,000 \times g$ for 10 min (R.T.). The supernatants were subjected to isoelectric-focusing with immobilized pH gradient (IPG) strips (Immobiline DryStrip, pH 4–7, $0.5 \times 3 \times 70$ mm; Amersham Biotech, Sweden) using the IPGphor system (Amersham). The automated run consisted of 6 h at 30 V, 6 h at 60 V, and a gradual voltage increase to 4,500 V for a total 56 kVh. The focused IPG strips were placed on 12.5% SDS-PAGE gels, sealed with 0.5% (w/v) agarose and electrophoresed until the dye reached the bottom of the gel. The bands on SDS-PAGE and spots on 2-DE gels were visualized with CBB G-250, digitized with an Agfa Arcus 1200™ image scanner (Agfa-Gevaert, Mortsel, Belgium) and analyzed using the Phoretix™ 2D software (Ver. 5.01, NonLinear Dynamics Ltd., UK). Identification of Igs was conducted by peptide mass fingerprinting (PMF) using MALDI-TOF MS followed by SDS-PAGE and 2-DE analysis, as described by Shin *et al.* (2006b).

Production of monoclonal antibodies (MAbs)

BALB/c mice (Hyochang Science Inc., Korea) were immunized with Igs purified immunoaffinity and MBP affinity column from immunized sera, according to the method described by Al-Harbi *et al.* (2000). Briefly, 50 μ g of olive flounder Igs mixed 1:1 with Freund's complete (first) or incomplete (second and third) adjuvants were injected intraperitoneally every 2 weeks for 6 weeks. After 1 week of the third injection, mice were boosted with 50 μ g of Igs without adjuvant through tail vein injection. Three days after the adjuvant-free booster shot, spleen cells were harvested from immunized mice and fused with Sp2/o myeloma cells (CRL-1581, ATCC, USA) using polyethylene glycol. The fused cells were cultivated on a feeder layer of mouse blood cells in 96-well plates. MAbs were screened by ELISA and immunoblotting assay.

2-DE immunoblotting for L. garvieae KG9408 with MAbs

MAbs were used in 2-DE immunoblot assays to determine whether the two different IgMs reacted with *L. garvieae* antigens. Briefly, 2-DE and its immunoblotting assays for *L. garvieae* was performed as described by Shin *et al.* (2006b) and Kang *et al.* (2006), respectively. 2-DE gels were stained with silver nitrate for visualization of the protein spots, or transferred to a PVDF membrane (0.45 μ m; Millipore, USA). The membranes were blocked with 5% skim milk in PBS-T for 1 h (R.T.), washed three times with PBS-T, and then incubated for 4 h (R.T.) with antisera (1:20) collected from olive flounder on 6th week of post-infection with *L. garvieae* KG9408 (10^7 CFU/fish, intraperitoneal injection). The membranes were washed 3 times with PBS-T and separately incubated with M11A1-4 and G7G4-6 clones for each Ig for 4 h (R.T.). *L. garvieae* antigenic spots were visualized by incubation with goat HRP-conjugated anti-mouse IgG (Jackson, USA; 1:4,000) for 1 h at R.T.. The both membranes were then washed four times with PBS-T for 15 min, developed using an Enhanced Chemiluminescent (ECL) kit (Amersham Biosciences) and exposed to x-ray film for visualization of the antigenic proteins. The stained gels and immunoblotted membranes were digitized with an Agfa Arcus 1200™ image scanner (Agfa-Gevaert, Belgium) and the acquired images were analyzed using the Phoretix 2D software (Ver. 5.01; NonLinear Dynamics, UK). Antigenic spots were identified by comparison to a partial 2-DE reference map of *L. garvieae* KG9408 previously constructed in our laboratory (Shin *et al.*, 2006b) and the 2-DE immunoblot maps generated during the present study.

Results

Affinity column purification of olive flounder Igs

The MBP affinity and immunoaffinity columns were able to successfully purify Igs from serum samples taken from goat IgG-immunized olive flounders. The concentrations of purified Igs were as follows: immunoaffinity columns, 1.2 ± 0.2 mg/mL; MBP affinity columns, 0.9 ± 0.2 mg/mL. Goat IgG- and MBP-positive Igs were named as IMMIg and MBPIg, respectively. In contrast, the protein A affinity column was not able to purify any protein from immunized or non-immunized olive flounders.

SDS-PAGE and 2-DE of purified Igs

Igs purified using the two different columns were subjected to SDS-PAGE and 2-DE analysis (Fig. 1 and 2). In SDS-PAGE analysis, IMMIg yielded three bands of 66, 27 and 25 kDa and MBPIg showed three bands of approximately 68, 27 and 25 kDa (Fig. 1). In 2-DE analysis, the spot distribution in the 2-DE profile of IMMIg could be divided into two groups: (a) those in the range of *pI* 5.08–6.41 and MW 66 kDa, believed to be

Fig. 1. SDS-PAGE analysis of olive flounder IgS purified from immunized sera using protein A, immunoaffinity and MBP columns. Lane 1, whole sera of olive flounder; lane 2, Ig purified with protein A affinity column; lane 3, Ig purified with immunoaffinity column; lane 4, Ig purified with MBP affinity column.

the heavy chain; and (b) those broadly distributed across the range of *pI* 4–7 and MW 25–28 kDa, representing the light chain (Fig. 2 A). In the case of MBPIg (Fig. 2 B), 2-DE profile showed five groups: (c) those in the range of *pI* 5.30–5.73 and MW 68 kDa, (a') those in the range of *pI* 5.35–6.30 and MW 66 kDa, (d) those in the range of *pI* 5.03–5.32 and M.W 47–49 kDa, (e) those in the range of *pI* 4.59–4.92 and MW 47–45 kDa and (b') those in the range of and *pI* 4–7 and MW 25–28 kDa. In these groups, group (a') and (b') were found to correspond with group (a) and (b) on 2-DE profile of IMMIg in computational image analysis.

Identification of IgS

After image analysis of the SDS-PAGE and 2-DE

separations of IMMIg and MBPIg, the respective bands and spots were identified by PMF followed by MALDI-TOF MS (data not shown). The SDS-PAGE bands seen at 66 and 68 kDa were identified as the immunoglobulin M (Accession No. 14475599) or IgM precursors (Accession No. 7025429) of the olive flounder. These proteins were also identified from all spots of groups (a), (c), (a') and (d) in the 2-DE profiles of IMMIg and MBPIg. On the other hand, the bands of 25 and 27 kDa of SDS-PAGE and groups of (b), (b') and (e) on 2-DE profiles could not be identified from the olive flounder protein database.

Production of MAbs for IMMIg and MBPIg

Antibodies against IMMIg and MBPIg were generated in mice. ELISA screening followed by SDS-PAGE immunoblot assays allowed selection of 30 MAb clones for IMMIg and 25 MAb clones for MBPIg. Of the MAbs for IMMIg, 24 clones including G7G4-6 were specific for the heavy chain and 1 clone (G11B10-1) was specific for the 27 kDa light chain (Fig. 3A). Of the MAbs for MBPIg, 29 clones including M11A1-4 were specific for the heavy chain and 1 clone (M7C3-4) was specific for the 27 kDa light chain (Fig. 3B). Immunoblot assays of IMMIg and MBPIg revealed that the MAbs strongly recognized their homologous antigens, but reacted only weakly with the heterologous antigen (Fig. 3C). Of the tested MAbs, M11A1-4 and G7G4-6 were selected and used to compare antigenic spots reacting with MBPIg and IMMIg in 2-DE immunoblot assays of *L. garvieae*.

Comparisons of 2-DE immunoblot profiles of *L. garvieae* KG9408

2-DE immunoblot assays using the generated MAbs were used to compare *L. garvieae* antigen recognition by the two different types of IgM purified from olive flounder. The detected antigenic spots were identified by comparison to a partial 2-DE reference map of *L.*

Fig. 2. 2-DE analysis of the purified immunoaffinity-positive Ig (IMMIg; A) and MBP-positive Ig (MBPIg; B). 2-DE was performed using pH 4–7 IPG strips (7 cm) for the first dimension (IEF) and 12.5% SDS-PAGE for the second dimension.

Fig. 3. Specificity and cross-reactivity of MAbs raised against olive flounder IMMIg and MBPIg. Panel A and B were immunoblot assays using specific MAbs to heavy and light chains of IMMIg and MBPIg, respectively. Lane 1, SDS-PAGE profiles of IMMIg; Lane 2, MAb (G7G4-6) to IMMIg heavy chain; Lane 3, MAb (G11B10-1) to IMMIg light chain; Lane 4, SDS-PAGE profiles of MBPIg; Lane 5, MAb (M11A1-4) to MBPIg heavy chain; Lane 6, MAb (M7C3-4) to MBPIg light chain. Panel C was immunoblot assays for cross-reactivity of IMMIg (I) and MBPIg (M) using MAbs (G7G4-6 and M11A1-4) for heavy chain of respective IgM.

Fig. 4. 2-DE immunoblot profiles of *L. garvieae* KG9408 using specific MAbs to IMMIg (A) and MBPIg (B). Numbering of antigenic spots was performed by phoretix 2D software (Ver. 5.01; NonLinear Dynamics, UK)

garvieae KG9408 previously constructed in our laboratory (Shin *et al.* 2006b). An average of 85 and 87 antigenic spots ($n=3$ per MAb) were observed in 2-DE immunoblot profiles of *L. garvieae* KG9408 detected with G7G4-6 and M11A1-4, respectively (Fig. 4). Most of the antigenic spots were distributed at pI 4.5–5.7 and MW 20–60 kDa.

There were 56 common antigenic spots between both 2-DE immunoblot profiles. These antigenic spots included elongation factor Tu (EF-Tu), arginine

deiminase (AD), ATP synthase alpha subunit (ATP synthase), dTDP-glucose 4,6-dehydratase (4,6-dehydratase), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), phosphoglycerate kinase (PGK), DNA-directed RNA polymerase alpha chain (RNA polymerase), L-lactate dehydrogenase (LDH), UDP-galactose 4-epimerase (4-epimerase), 6-phosphofructokinase (PFK), fructose-bisphosphate aldolase (FBA), NADH-dependent enoyl-ACP reductase (ACP reductase), phosphoglycerate mutase (PGM), ornithine carbamoyltransferase

Table 1. List of common antigenic spots reacted with IMMIg and MBPIg on 2-DE immunoblot profiles of *L. garvieae* KG9408

IMMIg	MBPIg	Name of antigen	pI	MW	IMMIg	MBPIg	Name of antigen	pI	MW
3	4, 5, 6	—*	4.9	78.7	46	41	LDH	5.1	31.1
4	3	—	5.1	78.3	47	43	—	5.5	30.4
5	2	—	5.0	78.1	48	44	—	5.4	30.3
7	8	—	5.2	60.7	50	45	—	4.8	30.3
9	9	IMPD	6.1	57.1	51	46	—	4.7	30.2
13	10	—	5.2	54.9	53	47	4-epimerase	5.1	30.0
17	13	—	5.5	50.9	54	48	—	5.2	29.7
18	16	—	5.1	49.1	55	49	PFK	6.0	29.3
16, 20, 24	17	EF-Tu	4.9	47.0	60	53	—	5.4	26.1
21	20	AD	5.4	46.2	61	55	—	5.1	26.0
22	22	ATP synthase	4.7	45.2	62	56	FBA	5.2	26.0
23	21	AD	5.5	45.1	64	57	ACP reductase	6.7	24.8
26	25	—	5.5	41.2	65	59	—	5.5	24.4
27	26	4,6-dehydratase	5.4	37.9	66	58	—	4.7	24.3
28	32	PGK	4.9	37.3	69	65	PGM	5.4	23.3
29	27	—	5.3	37.3	70	66	—	5.8	23.1
30	31	—	5.0	36.9	71	68	—	5.7	23.1
31	28	GAPDH	5.2	36.9	73	69	—	5.1	22.4
32	29	GAPDH	5.1	36.3	74	70	—	4.9	22.0
33	30	—	5.0	36.0	77	74	—	5.0	20.6
36	33	—	5.5	34.3	78	77	—	5.3	18.0
38	35	OCT	5.0	32.4	79	78	—	5.7	17.4
39	34	—	5.1	32.4	80	79	—	5.5	17.3
42	37	RNA polymerase	4.9	32.0	81	81	—	5.0	14.6
43	40	—	4.9	31.4	83	82	—	4.6	14.3
44	38	—	4.8	31.3	84	83	—	4.4	14.3
45	39	—	4.9	31.3	85	84	—	5.0	13.5

*: non-identified protein; Identification of antigenic proteins was performed by comparing of 2-DE reference map of *L. garvieae* KG9408.

(OCT) and inosine-5'-monophosphate dehydrogenase (IMPD). In addition, we detected 31 IMMIg-specific spots including phosphomannomutase (PMM) and enolase and 29 MBPIg-specific spots including 30S ribosomal protein S2 (ribosomal S2) and UDP-glucose pyrophosphorylase (pyrophosphorylase). These common and specific antigenic spots were summarized in Table 1 and 2, respectively.

Discussion

IgMs were successfully purified from olive flounder sera by affinity columns with goat IgG and MBP, but not protein A. Protein A is a cell wall component of *Staphylococcus aureus* that binds to a domain in the Fc region of Ig (Sasso *et al.*, 1991; Harada *et al.*, 1994). MBP is an animal lectin that acts in pathogen clearance and possesses a high specificity for the mannose regions of IgM (Koppel *et al.*, 1994). These two molecules have been used for affinity column-based IgM purification from fish sera (Suzuki *et al.*, 1990; Al-Harbi *et al.*, 2000; Watts *et al.*, 2001; Crosbie and Nowak, 2002; Bromage *et al.*, 2004), as has goat IgG (Palenzuela *et al.*, 1996; Watts *et al.*, 2001). In the present work, we found that protein A possessed a low affinity for olive flounder IgM versus the other capture ligands. This is consistent with other

studies reporting that protein A had variable affinities for fish IgM; for example, protein A had low affinity for the IgM of rainbow trout and Atlantic salmon *Salmo salar* (Bromage *et al.*, 2004), higher affinity for the IgM of striped bass *Morone saxatilis*, Mozambique tilapia *Oreochromis mossambicus*, Nile tilapia *O. niloticus*, barramundi *Lates calcarifer* (Bromage *et al.*, 2004) and black rockfish *Sebastes schlegeli* (Kang *et al.*, 2006) in affinity columns, and no affinity for the IgM of Atlantic halibut *Hippoglossus hippoglossus* in immunoblotting and dot blotting assays (Grove *et al.*, 2006). These differences in affinity might be due to variations in the specificity of the protein A binding sites located in fish IgM.

SDS-PAGE analysis previously revealed that teleost IgM was comprised of a 70–81 kDa heavy chain and a 22–32 kDa light chain. On the other hand, previous 2-DE experiments showed that the spots corresponding to the heavy chain of human IgM were found within a pI range of 5.6 to 6.4 at a molecular weight (MW) of 80–82 kDa (Al-Harbi *et al.*, 2000; Watts *et al.*, 2001; Crosbie and Nowak, 2002; Bromage *et al.*, 2004; Kang *et al.*, 2006; Palenzuela *et al.*, 1996; Kaattari and Piganelli, 1996). The pI ranges and molecular weights of the heavy chain of IgM differed slightly in the emerald rockcod *Trematomus bernacchii* (pI range 4.0–6.0, MW

Table 2. List of specific antigenic spots reacted with IMMlg and MBPIg on 2-DE immunoblot profiles of *L. garvieae* KG9408

Specific antigenic spots for IMMlg				Specific antigenic spots for MBPIg			
Spot No.	Name of antigen	pI	MW	Spot No.	Name of antigen	pI	MW
1	—	5.6	117.9	1	—	4.9	78.9
2	—	5.7	116.1	4	—	4.9	75.8
6	—	5.1	61.1	6	—	4.8	75.4
8	—	4.4	57.6	7	—	6.1	62.0
10	—	6.3	56.9	11	—	6.2	53.8
11	—	4.9	56.3	12	—	5.7	52.6
12	—	5.1	55.3	14	—	5.4	51.3
14	—	5.9	54.8	15	—	5.3	50.5
15	—	5.2	51.8	18	—	5.6	47.5
16	—	4.9	51.7	19	—	5.3	46.9
19	enolase	4.6	48.7	23	—	4.5	44.3
24	—	4.9	43.7	24	—	5.7	43.9
25	—	5.3	41.4	36	—	4.7	32.3
34	—	5.6	36.0	42	—	4.9	31.0
35	PMM	4.8	34.9	50	—	4.7	28.7
37	—	5.3	33.3	51	ribosomal S2	5.6	28.6
40	—	4.8	32.3	52	—	5.5	28.4
41	—	6.4	32.1	54	—	5.5	26.0
49	—	4.6	30.3	60	—	5.9	24.4
52	—	5.1	30.1	61	—	4.9	24.3
56	—	5.0	26.9	62	—	5.0	23.9
57	—	6.1	26.7	63	—	5.5	23.5
58	—	5.0	26.6	64	pyrophospholylase	6.1	23.5
59	—	6.2	26.6	67	—	6.6	23.3
63	—	5.3	24.8	71	—	5.2	22.1
67	—	4.6	24.3	72	—	5.3	22.1
68	—	5.2	23.7	73	—	5.1	22.0
72	—	5.2	22.4	75	—	4.7	21.1
75	—	4.9	20.7	76	—	4.7	20.0
76	—	5.2	20.7	80	—	5.9	17.2
82	—	4.7	14.4	85	—	5.0	13.4
86	—	4.7	13.5				
87	—	4.7	11.8				

*: non-identified protein; Identification of antigenic proteins was performed by comparing of 2-DE reference map of *L. garvieae* KG9408.

78 kDa) (Pucci *et al.*, 2003), rainbow trout (pI 4.8–6.0, MW 76 kDa) and Arctic char *Salvelinus alpinus* (pI 5.0–6.5, MW 76 kDa) (Hordvik *et al.*, 2002), whereas the light chain spots in all three fish species were broadly distributed across a pI range of 4 to 7 (Hordvik *et al.*, 2002; Pucci *et al.*, 2003). Consistent with these findings, the purified Igs from olive flounder showed bands of 68 kDa and of 25–27 kDa in SDS-PAGE profile, and major spot groups at the ranges of pI 5.0–6.4 and MW 65–68 kDa, and pI 4–7 and MW 25–28 kDa on 2-DE profile. Based on the results of SDS-PAGE and 2-DE, bands and spots of approximately 66 and 68 kDa, and 25–27 kDa were believed to be the heavy chain and light chain, respectively. Furthermore, PMF followed by NCBI database searching identified the heavy chains of Ig proteins as IgM or IgM precursor.

Previously, 2-DE analysis was used to divide human IgM cryoglobulin (proteins precipitated from plasma or serum at low temperatures and resolubilized by warming) into 5 groups: μ chain of IgM (pI 5.6–6.4, 80 kDa), μ

chain fragments of IgM, SP α , J-chain and light chain (Tissot *et al.*, 1994, 2002). On the other hand, degradation of IgM due to freezing shock was observed in hybrid bass (*Morone saxatilis* $\times$ *M. chrysops*) by SDS-PAGE analysis (Nitzan *et al.*, 2003). Here, we found that MBPIg was consisted with 5 groups in 2-DE profiles. PMF analysis allowed us to identify groups (c), (a') and (d) as IgM or IgM precursor. Similarly, comparison of our 2-DE results to previously reported 2-DE maps of emerald rockcod, brown trout *Salmo trutta* and human IgMs (Hordvik *et al.*, 2002; Pucci *et al.*, 2003; Tissot *et al.*, 1994, 2002) suggested that the spots found in group (b) and (b') represent the light chain of olive flounder IgM. In addition, the spots found in group (d) were thought to be μ chain fragments of IgM induced by freezing, as previously seen in IgM cryoglobulin from human and hybrid bass (Tissot *et al.*, 1994, 2002; Nitzan *et al.*, 2003). However, group (e) could not match with any groups on 2-DE profiles for IgM. On the other hand, computational image and PMF analysis showed

that group (a') corresponded with group (a) on 2-DE profile of IMMlg. Therefore, MBP affinity column was thought to possess higher affinity to IgM consisted with heavy chain of 68 kDa. It might be difference in the degree of mannose glycosylation in the Fc regions of two different IgM types of olive flounder.

In a previous work, serum samples from olive flounder immunized with formalin-killed *E. tarda* were fractionated by gel filtration, which yielded two different proteins, high molecular protein (HMW) Ig and low molecular protein (LMW) Ig, both capable of agglutinating *E. tarda*. The heavy chain of the LMW Ig actually had a higher molecular weight than that of HMW Ig, as shown by SDS-PAGE analysis under reducing conditions (Bang *et al.*, 1996). Similarly, LMW Igs were identified in fish such as the giant grouper *Epinephelus itaira* (Clem, 1971), rainbow trout (Elcombe *et al.*, 1985), channel catfish *Ictalurus punctatus* (Lobb and Olson, 1988) and carp *Cyprinus carpio* (Rombout *et al.*, 1993). In these fishes, the LMW Igs had different structures and antigenicities versus the tetramer IgM, equivalent to the HMW Ig and various authors have suggested that the LMW Ig might be a distinct monomer formed as a degradative/cleavage product of tetramer IgM (Clem, 1971; Elcombe *et al.*, 1985; Lobb and Olson, 1988; Rombout *et al.*, 1993; Bang *et al.*, 1996). On the other hand, two isotypes of IgM could be purified from serum of Atlantic salmon and brown trout with anion exchange chromatography followed by gel filtration. Furthermore, 2-DE analysis showed difference in *pI* of heavy chains of respective IgM (Hordvik *et al.*, 2002). In the present study, we noted that the heavy chain band from MBPIg was larger than that from IMMlg in SDS-PAGE analysis under reducing conditions and MAbs raised against IMMlg and MBPIg showed cross-reaction but different reactivity between both Igs. In light of the previous reports on LMW and HMW Igs in other fishes (Clem, 1971; Elcombe *et al.*, 1985; Lobb and Olson, 1988; Rombout *et al.*, 1993), it might be thought that MBPIg is likely to be LMW Ig different from a degradative/cleavage product, while IMMlg is likely to be HMW Ig, which exists as a tetramer in IgM.

The previous studies were reported to be differed with respect to function of both IgM types; for example, LMW and HMW IgM purified from rainbow trout anti-haptn sera were distinguished from activating complement to lyse haptenated trout erythrocytes (Elcombe *et al.*, 1985) and LMW Ig purified from olive flounder immunized with *E. tarda* exhibited lower antibody titers than the corresponding HMW Ig in an agglutination test against *E. tarda* (Bang *et al.*, 1996). For suggesting more concrete evidence than previous studies, proteomic tools were applied for analyzing function of both Igs by comparing *L. garvieae* antigens reacted with respective IgM types in the present study. Computational image analysis to 2-DE immunoblot profiles

showed 56 common, 31 IMMlg-specific and 29 MBPIg-specific antigenic spots. Therefore, IMMlg and MBPIg were thought to play similar but different roles in recognition of *L. garvieae* antigens.

Kang *et al.* (2006) reported 22 antigenic spots of *L. garvieae* KG9408 recognized by black rockfish using 2-DE immunoblot assays. There were differences between 2-DE immunoblot profiles in previous and present study, in terms of antigenic profiles and the number of detected antigenic spots. These might be due to differences of protein loading volume for 2-DE immunoblot assays and humoral immune response between both fish species. On the other hand, novel finding of present study was 19 antigenic proteins identified from 22 antigenic spots on 2-DE immunoblot profile for *L. garvieae*. Functional analysis allowed us to divide these 19 antigens into 6 functional groups: translation, ribosomal structure and biogenesis (EF-Tu and 30S ribosomal protein S2), transcription (DNA-directed RNA polymerase alpha chain), nucleotide transport and metabolism (IMPD), carbohydrate transport and energy production (PGK, GAPDH, LDH, PFK, FBA, enolase, phosphomannomutase, phosphoglycerate mutase and ATP synthase), amino acid transport and metabolism (AD and OCT), cell wall/membrane/envelope biogenesis (dTDP-glucose 4,6-dehydratase, UDP-galactose 4-epimerase and glucose-1-phosphate thymidyltransferase) and lipid transport and metabolism (NADH-dependent enoyl-ACP reductase). Of these antigens, GAPDH has been reported as a vaccine candidate against *Schistosoma mansoni* and *E. tarda* (Argiro *et al.*, 2000; Liu *et al.*, 2005). In addition, PGK and OCT have been recognized as a protective antigen against various serotypes of *Streptococcus agalactiae* (Hughes *et al.* 2002). AD is a putative virulence factor observed on the capsular surface of *S. suis* and *S. pyogenes* (Winterhoff *et al.* 2002). These findings in association with two different antibody types might provide useful information in both studying antigens of *L. garvieae* and developing diagnostic marker and vaccine for this bacterium.

To sum up, we herein purified IMM- and MBP positive-IgM from olive flounder sera. The antigenic heterogeneity was noted between the IgMs, and proved that each IgM type was able to respond to specific antigens during immune response. These findings suggest that IMM- and MBP positive-IgM might play important roles during humoral immune response of olive flounder to *L. garvieae* infection.

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