Provided for non-commercial research and education use.
Not for reproduction, distribution or commercial use.

This article appeared in a journal published by Elsevier. The attached copy is furnished to the author for internal non-commercial research and education use, including for instruction at the authors institution and sharing with colleagues.

Other uses, including reproduction and distribution, or selling or licensing copies, or posting to personal, institutional or third party websites are prohibited.

In most cases authors are permitted to post their version of the article (e.g. in Word or Tex form) to their personal website or institutional repository. Authors requiring further information regarding Elsevier's archiving and manuscript policies are encouraged to visit:

http://www.elsevier.com/copyright

Author's personal copy

Fish & Shellfish Immunology (2008) **25**, 761–774

available at www.sciencedirect.com

ScienceDirect

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

Development of monoclonal antibodies to rohu [*Labeo rohita*] immunoglobulins for use in immunoassays

Gaurav Rathore*, Gokhlesh Kumar, Neeraj Sood, D. Kapoor, W.S. Lakra

National Bureau of Fish Genetic Resources, Canal Ring Road, P.O. Dilkusha, Lucknow 226 002, Uttar Pradesh, India

Received 15 July 2007; revised 16 February 2008; accepted 26 February 2008
Available online 4 March 2008

KEYWORDS
Chromatography;
Electrophoresis;
ELISA;
Flow cytometry;
Immunoglobulins;
Labeo rohita;
Monoclonal antibodies

Abstract Serum immunoglobulins [Ig] of rohu [*Labeo rohita*] were purified by affinity chromatography using bovine serum albumin as capture ligand. The purified rohu Ig [r-Ig] had a molecular weight [MW] of 880 kDa as determined with gel filtration chromatography. The heavy chain of r-Ig had an MW of 77.8 kDa and that of light chain was 26.4 kDa in SDS-PAGE. Purified r-Ig was used for the production of two anti-rohu Ig monoclonal antibodies [D7 and H4] that belonged to subclass IgG2b and IgG1, respectively. Both the MAbs were specific to heavy chain of r-Ig as seen in Western blotting.

Anti-rohu Ig MAb was used as a diagnostic reagent in ELISA and immunocytochemical assays to demonstrate its application for sero-surveillance and for immunological studies in rohu. A competitive ELISA was used to demonstrate the antigenic relatedness of r-Ig with whole serum Ig of other fish species. Cross reactivity of anti-rohu Ig MAb was observed with serum Ig of *Catla catla* and *Cirrihinus mrigala*. No reactivity to serum Ig of *Ophiocephalus striatus* and *Clarias gariepinus* was seen. Anti-rohu Ig MAb was found to be suitable for the detection of pathogen specific [*Edwardsiella tarda*] antibodies in serum of immunized rohu by an indirect ELISA. In flow cytometry using D7 MAb, the mean percentage [±SE] of Ig positive cells in spleen and blood of rohu were found to be 64.85% [±2.34] and 51.84% [±2.55] of gated lymphocytes, respectively. Similarly, D7 MAb also stained 52.84% [±1.30] and 10.5% of gated lymphocytes in kidney and thymus, respectively. The anti-rohu Ig MAbs also showed specific staining of Ig bearing cells in spleen sections by the indirect immunoperoxidase test.
© 2008 Elsevier Ltd. All rights reserved.

Introduction

Enhanced understanding of the fish immune system and rapid identification of pathogens are two key approaches in effective management of diseases in aquaculture [1]. The diverse applications of monoclonal antibodies [MAbs]

* Corresponding author. Tel.: +91 522 2442440, 522 2442441; fax: +91 522 2442403.
E-mail address: rathore69@rediffmail.com (G. Rathore).

1050-4648/$ - see front matter © 2008 Elsevier Ltd. All rights reserved.
doi:10.1016/j.fsi.2008.02.014

Author's personal copy

provide an effective answer for both the above-mentioned aspects i.e. study of fish immunology and detection of pathogens. More specifically, MAbs to serum immunoglobulins [Ig] of fish are of immense use in developing immunoassays for quantification of total Ig [2–4] and antigen-specific Ig [5,6]. Anti-Ig MAbs have also been used in immunological studies for detection and isolation of Ig+ cells to study ontogeny, distribution and functions of reactive lymphocyte populations [7]. Recently, anti-Ig MAbs have been also utilized in flow cytometry for measuring immune function in a number of fish species [8,9]. Flow cytometry has been helpful for identification of surface immunoglobin positive cell populations in blood, spleen and kidney from many fish species [8,10]. Presently, MAbs against serum Ig are available for several marine and freshwater fish species as reviewed by Scapigliati et al. [11] and have contributed significantly to studies in fish immunology. These cited studies have made known the utility of anti-Ig MAbs as a crucial diagnostic reagent for research on prophylaxis, therapy and diagnosis of fish diseases, all of which fulfill the practical needs of a fish farmer.

Rohu [*Labeo rohita*] is an important culture fish of India. Approximately 25% of the total aquaculture produce of India is contributed by rohu, and its total culture production in South East Asia was 0.7 million metric tones in 2003 [12]. India is the largest producer of rohu in the region and accounts for nearly 62% of the total production. Presently, several assays are in use for monitoring the immune response in rohu, particularly with respect to non-specific cellular immunity [13] and specific humoral immune response against bacterial fish pathogens using polyclonal anti-rohu-globulin-HRP conjugate [14,15]. However, polyclonal antibodies have their own limitations of specificity, for detection of immune response as seen in vaccination after immersion [16]. In view of these, the development of MAbs to serum Ig of rohu was a long felt need, as MAbs have not been developed against the Ig of any of the native fishes found in the Indian subcontinent. Taking into account the commercial importance of this fish and future prospects, the MAbs to rohu Ig [r-Ig] would be instantly useful in sero-monitoring and aid in verifying the absence of specific pathogens in culture systems through immunoassays.

The main objectives of this investigation were development of MAbs to r-Ig and demonstration of their use in ELISA and immunocytochemical methods. In the present study, we report [i] Purification and characterization of r-Ig; [ii] Development and characterization of MAbs against purified r-Ig; [iii] Application of anti-rohu Ig MAb for measurement of humoral immune response to *Edwardsiella tarda*, quantification and demonstration of Ig bearing cells by flow cytometry and immunohistochemistry, respectively; [iv] Demonstration of antigenic relatedness of r-Ig molecule with whole serum Ig of other members of the Indian major carps i.e. *Catla catla* and *Cirrhinus mrigala*, and with African catfish *Clarias gariepinus* and murrel *Ophiocephalus striatus*.

Materials and methods

Fish immunization

Fifteen apparently healthy *L. rohita*, weighing 200–250 g, were divided into test and control groups. The test group comprised 10 fish, whereas five fish served as control. Both the groups were kept separately in fibre reinforced plastic tanks and provided pelleted fish feed. The fish were acclimatized for 1 week before immunization. Bovine serum albumin [BSA] was used as an immunogen to induce anti-BSA antibodies in fish. One milligram of BSA in 0.1 ml phosphate buffer saline [PBS], emulsified with an equal volume of Freund's complete adjuvant [FCA], was injected intra-peritoneally in each fish of the test group. This was followed by three injections of a similar emulsion in Freund's incomplete adjuvant [FIA] at 2, 4 and 6 weeks' interval. The fish in the control group were injected with only PBS emulsified with adjuvant. Pre- and post-immunization [7 days after the 4th injection] blood samples were collected from the fish via the caudal vein and serum was stored at −20 °C.

Indirect haemagglutination [IHA] test

IHA was used to assess the humoral immune response in immunized fish. Washed sheep red blood cells [sRBC] were fixed in glutaraldehyde and coated with BSA following Cho et al. [17]. The highest dilution of fish serum showing agglutination was considered as IHA titre for anti-BSA antibodies.

Purification of r-Ig by affinity chromatography

A BSA-CL agarose column [Genei, India] was used to purify anti-BSA antibodies from immunized fish sera having an IHA titre of 1:64 and above. Two millilitres of hyper-immune serum was mixed with an equal volume of PBS, filtered through a 0.4 μm filter and loaded on to the affinity column. Anti-BSA antibodies were eluted from the column with 0.1 M-glycine buffer [pH 11.0] and collected as 2 ml fractions. The optical density [OD] of each fraction was measured through a UV-spectrophotometer at 280 nm to determine the protein concentration. All the fractions having an OD of >0.1 were pooled and concentrated with a Centriplus YM-100 filter [Millipore]. The concentrated fraction was buffer exchanged with PBS and again concentrated to 1/10th of the eluted volume. These purified immunoglobulins of rohu were termed as r-Ig and stored at −20 °C in aliquots. Protein concentration of r-Ig was determined by a commercial protein estimation kit [Genei, India] with BSA as standard.

Gel filtration chromatography

The purity and molecular weight [MW] of eluted r-Ig was determined by gel filtration chromatography on a 1.6 × 57 cm column bed of Sephacryl S-300 having a total volume [V_t] of 114.6 ml and void volume [V_0] of 53.2 ml. The column was run at a flow rate of 5.7 ml per hour using 0.1 M PBS [pH 7.2] and fractions of 1.9 ml were collected using an automatic fraction collector [Pharmacia]. The column was calibrated with protein MW markers [Sigma] viz. thyroglobulin [669 kDa], apoferritin [443 kDa], amylase [200 kDa] and BSA [66 kDa]. The elution volume [V_e] for each marker was determined from the peak OD at 280 nm. Thereafter, 0.8 ml of concentrated r-Ig containing 1.5 mg total protein was applied to the column and eluted using the above conditions. The MW of r-Ig was estimated

Author's personal copy

from a standard curve prepared by plotting the known MW of each marker against its [V_e/V_0] value.

Sodium dodecyl sulphate polyacrylamide gel electrophoresis [SDS-PAGE]

Purified r-Ig was characterized by polyacrylamide gel electrophoresis under reducing and non-reducing conditions to determine its MW. The MW of the reduced r-Ig was determined on a 7–15% gradient gel by LabWorks image acquisition & analysis software, version 4.5 [UVP, Inc., U.K]. To determine the different forms of r-Ig, samples were diluted in the gel loading dye that did not contain β-mercaptoethanol and run on a gradient 3–10% gel. Pooled and concentrated fractions of individual peaks of r-Ig obtained in gel filtration chromatography were also analyzed under reducing conditions on a 7–15% gradient gel.

Fish serum samples

Standard procedures were used for blood collection, processing and storage of fish serum in this study. Thus, blood was collected from the caudal vein of the fish and allowed to clot overnight at 4 °C. It was then centrifuged at low speed to collect the serum. The harvested serum was stored at −20 °C prior to use. Twenty-five serum samples (5/species) from adult *L. rohita*, *C. catla*, *C. mrigala*, *O. striatus*, and *C. gariepinus* were collected and used subsequently in the competitive ELISA as field serum samples.

Production and characterization of monoclonal antibodies

Monoclonal antibodies were produced as per the standard procedures described earlier [18] with some modifications i.e. use of affinity purified r-Ig antigen for immunization of mice and feeder cells [peritoneal macrophages] for hybridoma production. Two female Balb/c mice, weighing 18–20 g, were inoculated intra-peritoneally [I/P] with 50 μg of affinity purified r-Ig emulsified in an equal volume of FCA and boosted [I/P] 2 weeks later with the same dose of antigen mixed with an equal volume of FIA. The second booster was administered [I/P] with the same dose of antigen in FIA, 2 weeks after the previous booster. The mice were bled for testing immune response on the 45th day of immunization. Three days before the fusion, a final prefusion boost of 25 μg antigen in PBS was given intravenously to mice producing r-Ig specific antibody of the higher titre. The detection of anti-r-Ig antibodies in the serum of immunized mice was done by an indirect ELISA, on r-Ig coated plates as described below. To determine the background antigen reactivity of mouse serum, Balb/c mice were bled on day 0, prior to the initial injection and their serum stored at −20 °C for further use.

In brief, spleen cells were collected from the immunized mice and fused with myeloma cells [SP-2/0] at a ratio of 4:1, respectively, using polyethylene glycol [MW 1450, Sigma]. Cells were seeded in 96 well microtitre plates and the growth of hybridoma was observed regularly under a selection medium [HAT] containing aminopterin. Well-to-well screening of hybridoma clones, employing an indirect ELISA, was performed at an appropriate stage of growth [25% of the well surface covered by cells]. Positive clones were amplified, followed by single-cell cloning and subcloning using the limiting dilution method. Single-cell cloned hybrids were screened by the indirect ELISA at the appropriate stage of growth. Single-cell cloning was repeated at least twice so as to prove the monoclonality of the hybridoma culture supernatant. Isotyping of MAbs was carried out on selected clone supernatants using a self-indicating dipstick kit [Sigma].

Indirect ELISA

An indirect ELISA was performed for titration of mouse serum for r-Ig antibodies and screening of the wells containing r-Ig positive hybridoma. The ELISA plates [Nunc, Maxisorp] were coated with 1 μg ml^{-1} of purified r-Ig [50 μl/well]. The plates were incubated at 37 °C for 1 h under constant shaking. Unbound antigen was washed thrice with PBS-T [PBS with 0.1% Tween-20] in each well. Further, either serial dilutions of mouse serum [50 μl] were added to each well or 50 μl of hybridoma culture supernatants were transferred from the cell culture plate to ELISA plate wells in the same format. The plates were incubated for 1 h at 37 °C and washed. Anti-mouse HRP conjugate [Sigma] diluted 1:10 000 in dilution buffer [PBS-T with 1% BSA] was added at the rate of 50 μl to each well. Plates were further incubated for 1 h at 37 °C under constant shaking. Plates were washed again and 1 mg ml^{-1} of *ortho*-phenylenediamine [OPD] containing H_2O_2 [50 μl/well] was added. The colour reaction was allowed to develop for 15 min followed by stopping of the reaction with an equal volume of 2 N H_2SO_4. The absorbance of the wells was read at 492 nm [A_{492}] in an ELISA reader [Tecan]. The hybridoma culture supernatants that gave at least five times the A_{492} values of day 0 mouse serum were considered positive for anti-r-Ig antibodies or hybridomas measuring an absorbance [A_{492}] of 0.5 or more were selected. This absorbance corresponded to approximately five times the absorbance of the background [day 0 mouse serum].

Western blotting

Standard procedures of Western blotting were used to determine the reactivity of the anti-rohu Ig MAbs in hybridoma culture supernatants to reduced r-Ig on a 7–15% gradient gel.

Application of anti-rohu Ig MAbs in Immunoassays

One of the strongly reacting MAb [D7] was selected for application in immunoassays as it gave a consistent reaction with both purified r-Ig and also with whole rohu serum. Optimization of the concentrations of antigen, MAb and anti-mouse Ig peroxidase conjugate was done through a standard checkerboard titration using doubling dilutions. Four types of immunoassays were used to test the various applications of selected MAb D7: [i] Competitive ELISA for demonstration of antigenic relatedness of r-Ig with whole serum of other fish species, [ii] Indirect ELISA for detection of rohu anti-*E. tarda* antibodies in immunized fish, [iii] Flow cytometry for quantification of Ig bearing mononuclear cells in blood, spleen, kidney and thymus and [iv] Indirect Immunoperoxidase test for demonstration of Ig bearing cells in spleen sections.

Author's personal copy

Competitive ELISA [c-ELISA]: Demonstration of antigenic relatedness of r-Ig

ELISA plates were coated with 50 μl/well of optimal concentration of the antigen [r-Ig] at 37 °C for 1 h. Serial dilutions [1/400 to 1/51 200] of field rohu serum samples as test species or field sera of other test fish species [*C. catla*, *C. mrigala*, *O. striatus* and *C. gariepinus*], in dilution buffer were used as competitor. Competition was carried out by incubating the washed plates with 50 μl/well of serially diluted competitor along with 50 μl of optimal dilution of D7 MAb (1:100) in dilution buffer, at 4 °C overnight. Subsequently, the plates were washed and 50 μl of goat anti-mouse Ig-HRP conjugate [1:10 000] was added to the wells. After incubation for 1 h at 37 °C, plates were again washed and 50 μl substrate (OPD) was added. Wells were observed for the development of color and the reaction was stopped using 2 N H_2SO_4 to obtain an expected OD range of 1.0 ± 0.2 in MAb control wells. Percentage competition was calculated using the following formula:

$$\%\text{Competition} = 100 - \{[\text{OD of field serum sample}/\text{OD of MAb control}] \times 100\}.$$

Indirect ELISA: Detection of rohu anti-*E. tarda* antibodies

Five test rohu were immunized intra-peritoneally with 10^8 cells of formalin killed *E. tarda* in 100 μl PBS, emulsified in an equal volume of FCA. Simultaneously, three control fish received 100 μl PBS emulsified in an equal volume of FCA. After 21 days, the test fish were boosted with a similar dose of antigen emulsified with FIA, while controls with PBS. One week later, all the fish were bled, serum collected and stored at −20 °C for further use. The detection of specific anti-*E. tarda* Ig in the rohu serum was done by means of an indirect ELISA, using D7 MAb as detector antibody. For this, ELISA plates were coated with 50 μl of 10^6 sonicated cells/ml of formalin killed *E. tarda* diluted in carbonate-bicarbonate buffer as per the method described by Magnadottir and Gudmundsdottir [19]. After washing, 50 μl of test rohu serum was serially diluted [in dilution buffer] in the coated plate. The plates were incubated overnight at 4 °C. Optimal dilution of D7 MAb was added to washed plates and incubated at 37 °C for 2 h. The binding of MAb to rohu Ig was detected with goat anti-mouse-IgG conjugated with HRP. Colour reaction was achieved by the addition of OPD substrate as described above.

Flow cytometry: Quantification of Ig bearing cells in blood and spleen

Peripheral blood mononuclear cells [PBMC] were isolated from each adult rohu [$n = 3$, mean weight 505 g] by density gradient centrifugation. Heparinized rohu blood [5 ml] was layered 1:1 on Histopaque-1077 [Sigma] and centrifuged at 400 g for 30 min at room temperature. PBMC were collected from the upper layer of the opaque interface and washed by mixing with PBS [1:4] and centrifuged at 250 *g* for 10 min. The cell pellet was resuspended to 10^7 cells ml^{-1} in Dulbecco's Minimum Essential Medium [DMEM] containing 5% fetal calf serum, and kept on ice for further use.

Individual cell suspensions of adult rohu (450—600 g) spleen, kidney and thymus were prepared in DMEM, by squeezing the individual tissues sequentially through a coarse mesh and then a fine (40 μm) nylon gauge cell strainer (BD falcon). Cells were washed twice in PBS and the pellet was incubated for 5 min in 0.15 M NH_4Cl at room temperature for lysis of erythrocytes. The cells were again washed and resuspended in DMEM [10^7 cells ml^{-1}], and layered 1:1 on Histopaque-1077 as described above for separation of mononuclear cells. Isolated mononuclear cells of individual tissues were kept on ice for flow cytometry.

Analysis by flow cytometry

For flow cytometry, PBMC, spleen, kidney or thymus mononuclear cells [10^6] were individually washed in DMEM and resuspended in 250 μl of D7 MAb [1:100 dilution in DMEM] and incubated for 30 min on ice. Again, the cells were washed twice and incubated for 30 min on ice with rabbit anti-mouse fluorescein isothiocyanate labeled conjugate [1:100 in DMEM]. Thereafter, cells were washed twice, resuspended in 500 μl DMEM and analyzed with a flow cytometer. In control cells, D7 MAb was replaced with myeloma culture supernatant so as to determine the non-specific reaction of MAb. In all, blood, spleen and kidney samples from three fish were analyzed in duplicate by flow cytometry. In the case of the thymus, tissue samples from four fish were pooled and analyzed in duplicate. Analysis was carried out on a FACS Caliber [Becton Dickinson] equipped with an argon-ion laser tuned to 480 nm. Ten thousand events were acquired from each sample and data were analyzed using software. Putative lymphocytes were characterized and gated by their Forward Scatter (FSC) and Side Scatter (SSC) properties. Surface Ig+ cells of FSC vs. FL1 dot plot were enumerated as percent of total events.

Indirect immunoperoxidase technique [IIPT]: Demonstration of Ig bearing cells in spleen

Anti-rohu Ig MAbs were used to demonstrate Ig bearing cells in rohu spleen sections by IIPT [20]. Briefly, formalin-fixed paraffin-embedded spleen sections were deparaffinised in two changes of xylene for 15 min each, followed by rehydration in descending grades of alcohol and washing in tap water. These tissue sections were then conditioned in 0.01 M PBS for 1 h at 37 °C, followed by blocking of endogenous peroxidase activity with 3% H_2O_2 in methanol for 15 min. Heat mediated antigen retrieval was carried out in 0.01 M citrate buffer [pH 6.0] at 750 W for 20 min using a microwave oven. Cold water was added slowly to the slide container. Thereafter, the slides were laid flat and blocking of sections was done with the blocking buffer for 20 min. The buffer was then drained and sections were then overlaid with two different anti-rohu Ig MAbs [1:100 dilution of D7 MAb or undiluted culture supernatant of H4 MAb] at 37 °C for 1 h in a humidified chamber. The sections were washed with PBS thrice and again overlaid with 100 μl of goat anti-mouse IgG-peroxidase conjugate [Sigma] diluted 1:200 for 30 min at 37 °C. The sections were again washed as above and covered with substrate 3-amino-9-ethyl carbazole [AEC], and left at 37 °C for 10 min. The slides were rinsed in tap water and counterstained with Mayer's

Author's personal copy

haematoxylin. The slides were again washed and mounted in an aqueous mountant and observed under a microscope.

Results

Purification and characterization of r-Ig

The IHA titre of the BSA immunized rohu fish in the test group ranged from 1:4 to 1:128, whereas, the titre was <1:2 in pre-immunization serum and control group. In affinity chromatography, a single peak was observed on elution with glycine buffer. The purified and concentrated r-Ig showed an IHA titre of 1:128. Our results indicate that affinity chromatography using a BSA-CL agarose column was quite successful in purification of rohu serum Ig and the yield was dependent on the IHA titre of the serum. The sera with an IHA titre of 1:16 or less resulted in an insignificant yield of Ig. Purified r-Ig was loaded on a Sephacryl S-300 column in order to determine its MW. A peak was observed at an elution volume of 66.5 ml corresponding to an MW of approximately 880 kDa. Two more peaks were observed at an elution volume of 74.1 and 77.9 ml, respectively [Fig. 1] denoting different forms of r-Ig. On SDS-PAGE gel under reducing conditions, the purified r-Ig resolved into two major bands of 77.8 and 26.4 kDa, corresponding to the heavy chain [HC] and light chain [LC], respectively [Fig. 2(a)]. To determine if the different peaks of r-Ig obtained in gel filtration chromatography actually represented several forms of r-Ig or contaminants, concentrated fractions of all peaks were electrophoresed under reducing conditions. Each of the three peaks yielded HC and LC similar to purified r-Ig, thereby suggesting different forms of Ig [Fig. 3(a)]. The whole molecule of purified r-Ig electrophoresed under non-reducing conditions showed one prominent band with an MW little lower than bovine IgM [900 kDa] [Fig. 3(b)]. This is suggestive of a tetrameric structure in common with other teleost Ig. Some faint bands of lower MW were also seen below the tetrameric form.

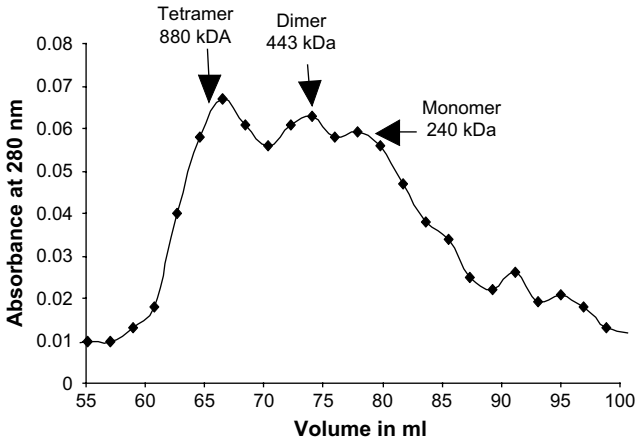

Figure 1 Elution profile of rohu Ig (r-Ig) on the Sephacryl S-300 column suggesting different forms of immunoglobulins. A total of 0.8 ml of r-Ig (1.6 mg ml^{-1}) was loaded on a 1.6 × 57 cm column bed of Sephacryl S-300 having a total volume (V_t) of 114.6 ml and void volume (V_o) of 53.2 ml.

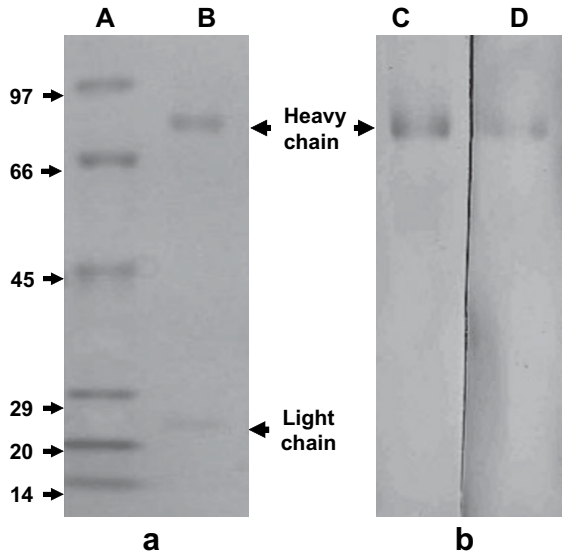

Figure 2 (a) Gradient SDS-Polyacrylamide gel electrophoresis (7.5—15%) and (b) Western blots of purified rohu immunoglobulin (r-Ig) with MAbs. Lane A — Molecular weight marker (kilodaltons); Lane B — Purified r-Ig; Lane C — Western blot of r-Ig with H4 MAb (undiluted hybridoma supernatant); Lane D — Western blot of r-Ig with D7 MAb (1/100 dilution). Both MAbs were found to be specific to the heavy chain of r-Ig.

Production and characterization of monoclonal antibodies

Testing of mice sera by indirect ELISA on 45th day of the immunization schedule showed a considerable increase in antibody titre to r-Ig, as compared to day 0 mouse serum [Fig. 4], thereby suggesting the immunogenic nature of r-Ig. Hybridoma culture supernatants, at appropriate stage of

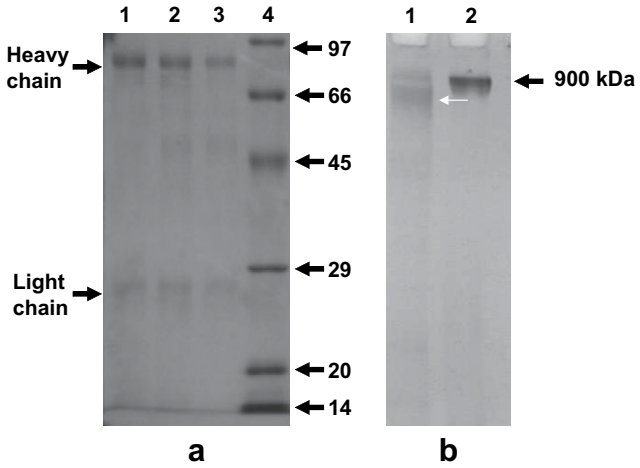

Figure 3 (a) SDS-PAGE analysis (7.5—15% gradient gel) of r-Ig obtained from Gel filtration chromatography under reducing conditions; Lane 1 — 1st peak; Lane 2 — 2nd peak; Lane 3 — 3rd peak; Lane 4 — Molecular weight marker (kDa). Three major fractions of each peak were pooled and concentrated to 1/10th volume for analysis. (b) Non-Reducing SDS-PAGE analysis of affinity purified rohu Ig (r-Ig) on the 3—10% gradient gel. Lane 1 — r-Ig (white arrow); Lane 2 — Bovine IgM (Sigma).

Author's personal copy

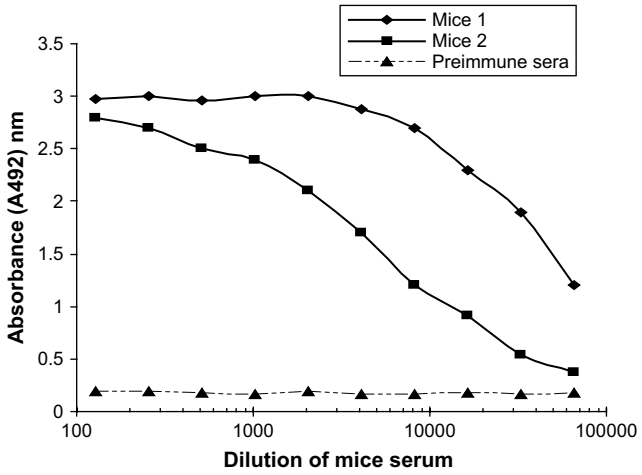

Figure 4 Titration curves of mouse serum on the 45th day of immunization against purified rohu Ig in an indirect ELISA. Mouse 1 was boosted with r-Ig antigen on the 49th day for subsequent fusion after 3 days.

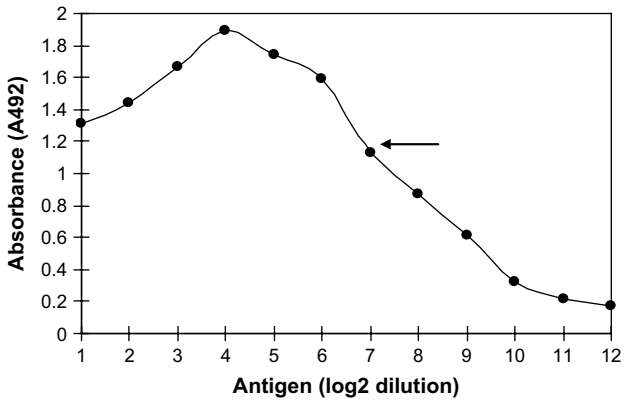

Figure 5 Titration of antigen in an indirect enzyme linked immunosorbent assay (ELISA), using the selected monoclonal antibody (MAb). Serial twofold dilutions of the antigen were coated on ELISA plates and bound MAb D7 was detected with rabbit anti-mouse horseradish peroxidase conjugate. The arrow indicates the dilution of antigen giving an absorbance of nearly 1—1.2.

screening, showed significantly high absorbance [A_{492}] values in contrast to day 0 mouse serum dilution [1/100]. The reactive clones were selected for further investigations. A total of 14 positive clones consistently specific to r-Ig were obtained. Out of these, five strong positive primary hybridoma clones were initially selected on the basis of reactivity in the indirect ELISA. The polypeptide specificity of these clones to reduced r-Ig was determined by Western blotting. Two of the clones [2D7 and 3H4] were specific to HC of r-Ig [Fig. 2(b)], while the other three clones failed to react with either the HC or LC and hence were not subjected to single-cell cloning. Both the positive clones [2D7 and 3H4] were subjected to single-cell cloning and subcloning. The limiting dilution method of hybridoma clones was adopted for this purpose. Monoclonality of a clone was accepted only when all the wells of a microtitre plate with growing cells gave a positive reaction in the indirect ELISA after repeated subcloning. The MAbs secreted by these clones were designated as D7 and H4 and belonged to subclasses IgG2b and IgG1, respectively.

Applications of anti-rohu Ig MAb in Immunoassays

The optimal concentration of antigen and dilution of MAb to be used in the ELISA were determined through standard checkerboard titration using doubling dilutions to obtain an expected OD range of 1.0 ± 0.2 (Figs. 5—6).

Demonstration of antigenic relatedness of r-Ig molecule with other fish Ig

Antigenic relatedness of r-Ig molecule was demonstrated indirectly as the cross reactivity pattern of D7 MAb with whole serum Ig of different species of fish i.e. *C. catla*, *C. mrigala*, *O. striatus* and *C. gariepinus* through the indirect competitive ELISA [Fig. 7(a)]. The analyzed results indicate only 36% reactivity to *C. catla* serum at log[−3.8] serum dilution, as compared to rohu serum. No cross reactivity was seen with *C. mrigala*, *O. striatus* and *C. gariepinus* at this serum dilution [Fig. 7(b)].

Detection of rohu anti-*E. tarda* antibodies

D7 MAb was used as detector antibody in the indirect ELISA for measuring the humoral antibody response in rohu. An elevated and measurable immune response to *E. tarda* was observed in the sera of immunized fish (Fig. 8). An almost linear decline in specific antibody level was observed with an increase in serum dilution. The non-immunized control fishes had almost five times lower ELISA values at similar serum dilutions.

Distribution of Ig+ cells in lymphoid tissues of rohu

Rohu PBMC, spleen, kidney and thymus mononuclear cells were individually analyzed for FSC and SSC patterns representing size and granularity of cells, respectively. Lymphocytes were presumed to be represented by medium sized cells with less granularity, in blood as well as other analyzed lymphoid tissues. The mononuclear cell population in blood had relatively homogeneous FSC [150—300] and SSC [100—200] properties [Fig. 9(a)]. Mononuclear cells

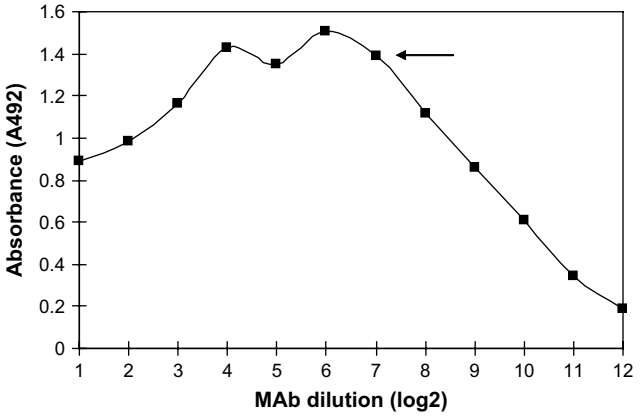

Figure 6 Titration of anti-rohu Ig MAb (culture supernatant) in an indirect enzyme linked immunosorbent assay (ELISA) using a fixed quantity of antigen. MAb (D7) dilution chosen for competitive ELISA is indicated by an arrow.

Author's personal copy

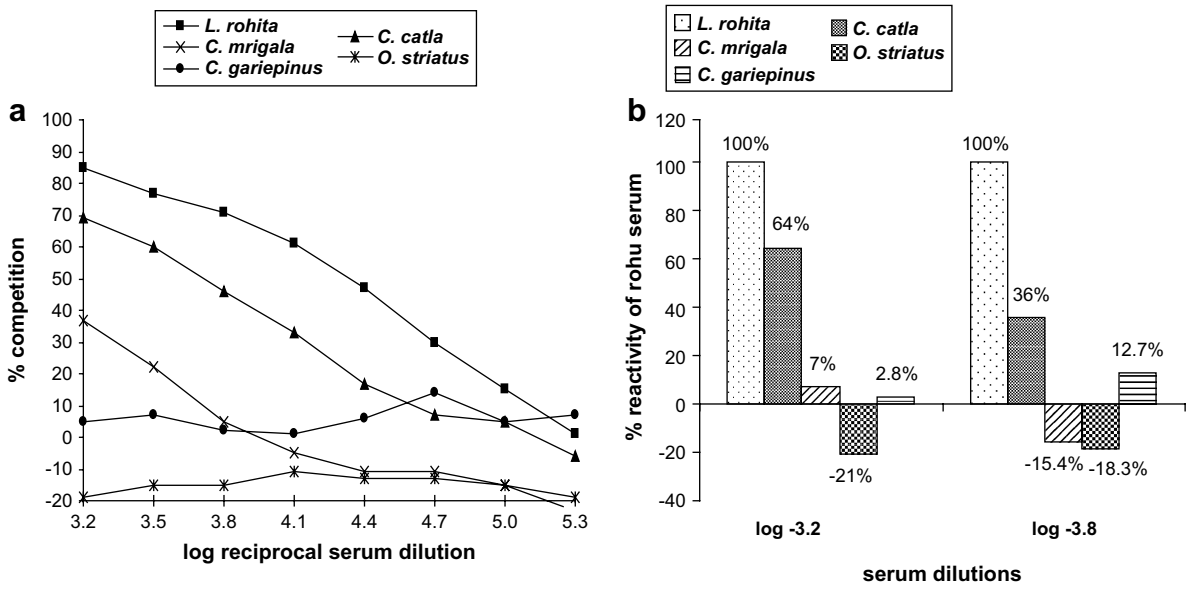

Figure 7 (a) Demonstration of antigenic relatedness of rohu Ig with serum Ig of heterologous fish species by the indirect competitive ELISA (b) Analyzed results shown as % reactivity to rohu serum. Competition was carried out on rohu Ig coated plates with serial dilutions of test fish serum along with a fixed quantity of D7 MAb.

in spleen, kidney and thymus also contained a homogeneous cell population with almost similar FSC and SSC properties to those of PBMC [Figs. 10, 11, 12(a)]. Dead cells and cell debris were seen in all lymphoid tissues at the extreme left with FSC values closer to 50, probably as a result of RBC lysis. The distribution of putative B-lymphocytes in individual lymphoid tissues of adult rohu was measured by incubating the cells with D7 MAb and subsequently staining with anti-mouse Ig-FITC conjugate. Representative flow cytometry histograms of D7 MAb stained lymphocytes in PBMC, spleen, kidney and thymus are shown in Figs. 9(c), 10(c), 11(c) and 12(c), respectively. Figs. 9(b), 10(b), 11(b) and 12(b) represent negative control samples, wherein D7 MAb was replaced with myeloma culture supernatant. The percentages [mean ± SE of three individual fish] of Ig positive cells in spleen and blood were found to be 64.85% [±2.34] and 51.84% [±2.55] of gated lymphocytes, respectively, suggesting that a large portion of this population consisted of B-lymphocytes. Similarly, D7 MAb also stained 52.84% [±1.30] and 10.5% of gated lymphocytes in the kidney and the thymus, respectively [Table 1].

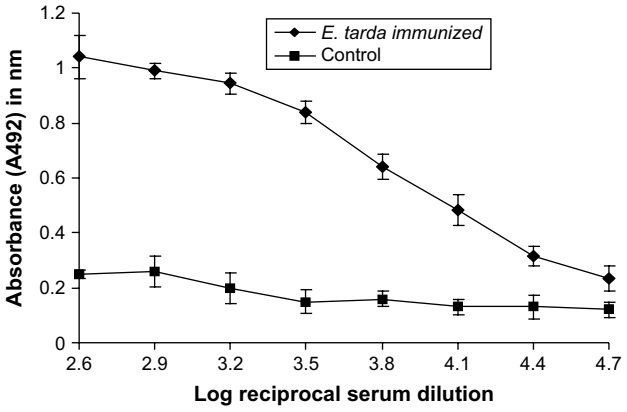

Figure 8 Sero-monitoring of immune response in rohu ($n = 5$) immunized against *E. tarda*, using D7 MAb as detector antibody through indirect ELISA. Each immunized fish was injected with 10(8) cells ml^{-1} of formalin killed *E. tarda*, boosted on day 21, and bled after 1 week. Controls ($n = 3$) received only phosphate buffered saline. Each point represents the mean ± SE for duplicates of test fishes.

The specificity of D7 MAb for rohu Ig was also established by the procedure as described previously by Milston et al. [8]. Rohu PBMC of three individual fish were incubated in the dark for 30 min at 4 °C with D7 MAb either in the presence of an excess of rohu serum or *O. striatus* serum. After incubation, the cells were washed twice, incubated with FITC labeled anti-mouse conjugate and were then analyzed by flow cytometry. Binding of D7 MAb to rohu PBMC was inhibited in the presence of rohu serum [data not shown]. However, in the presence of *O. striatus* serum, a positive peak was observed indicating the binding of D7 MAb to rohu PBMC.

Immunoperoxidase

Anti-rohu Ig D7 MAb showed specific staining of spleen cells by IIPT. The Ig bearing cells appeared mostly as round cells having brightly stained cytoplasm [red color], and a large nucleus [Fig. 13] suggestive of plasma cells. The stained cells were distributed throughout the splenic tissue, mostly in clusters and occasionally as single cells. The slides in which the MAbs were omitted and replaced with day 0 mouse serum did not show immuno-staining of cells.

Discussion

Previously, affinity chromatography using BSA as capture ligand has been applied for purification of fish Ig [21,22]. Mannan binding protein [MBP] and Staphylococcal protein

Author's personal copy

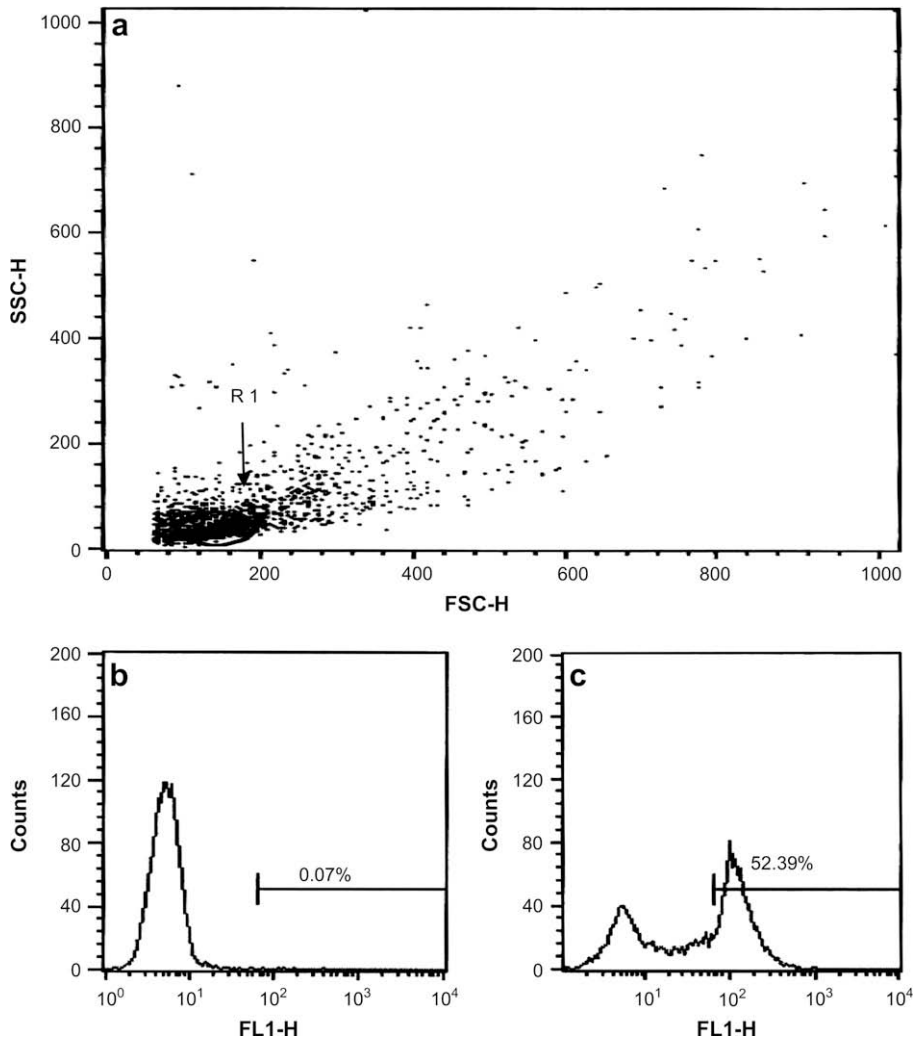

Figure 9 Representative flow cytometry generated forward/side scatter (FSC/SSC) profile and fluorescence histogram of rohu peripheral blood mononuclear cells (PBMC) by indirect immunofluorescence with anti-rohu Ig MAb. (a) FSC/SSC dot plot of PBMC with gated lymphocytes (R1); (b,c) Fluorescence histogram of gated lymphocytes stained without MAb and with D7 MAb, respectively.

A [SpA] have also been used as capture ligands in affinity chromatography [23]. SpA has variable capacity to bind with Ig from different fish genera [24], while MBP affinity column is reported to be of limited use in isolating fish Ig [25]. We were not able to purify the r-Ig through commercially available protein A and G columns, due to very poor binding as also reported for halibut Ig by Grove et al. [26]. The choice of affinity chromatography using BSA as capture ligand for purification was successful but time taking, due to erratic immune response to BSA in fish. In our study, the yield of rohu Ig from the BSA column was approximately 750 μg to 1.5 mg ml^{-1} of rohu serum. Yield differences between lots were probably due to differences in Ig levels in serum samples or a decrease in the binding efficiency owing to the repeated use of the same column.

MW analysis of r-Ig by SDS-PAGE under reducing conditions showed that, as with most teleost Ig studied to date, r-Ig appears to be made up of HC and LC. MWs of teleost Ig HC and LC are generally in the range of 71—81 and 23—28 kDa, respectively. Recently, halibut Ig was shown to consist of single HC [76 kDa] and six possible MW variants of LC in the range of 23—28.5 kDa [26]. Rohu Ig showed two dominant bands with an MW of 77.8 and 26.4 kDa, representing HC and LC, respectively. Additionally, multiple bands of variable intensity in different r-Ig batches were also occasionally recorded in the vicinity of 55 kDa. These could be interpreted as possible variants of HC or its degradation product. But variants of HC generally range within 5—10 kDa of the dominant band, thereby negating the argument. These could probably be considered as the product of the purified immunoglobulins, more specifically of the H chain and may be antigenically related. Similar findings were also reported in purified Ig of many fish species i.e. *Dicentrarchus labrax* and *Sparus aurata* [27], *Perva fluviatilis* [28], and also *Oncorhynchus mykiss* [29]. It may be also pertinent to mention here the observations on trypsinolysis of channel catfish Ig which resulted in a yield of a 50 kDa fragment, referred to as the Fab fragment [30]. These fragments contained some HC and LC antigenic determinants and hapten-binding sites almost similar to

Author's personal copy

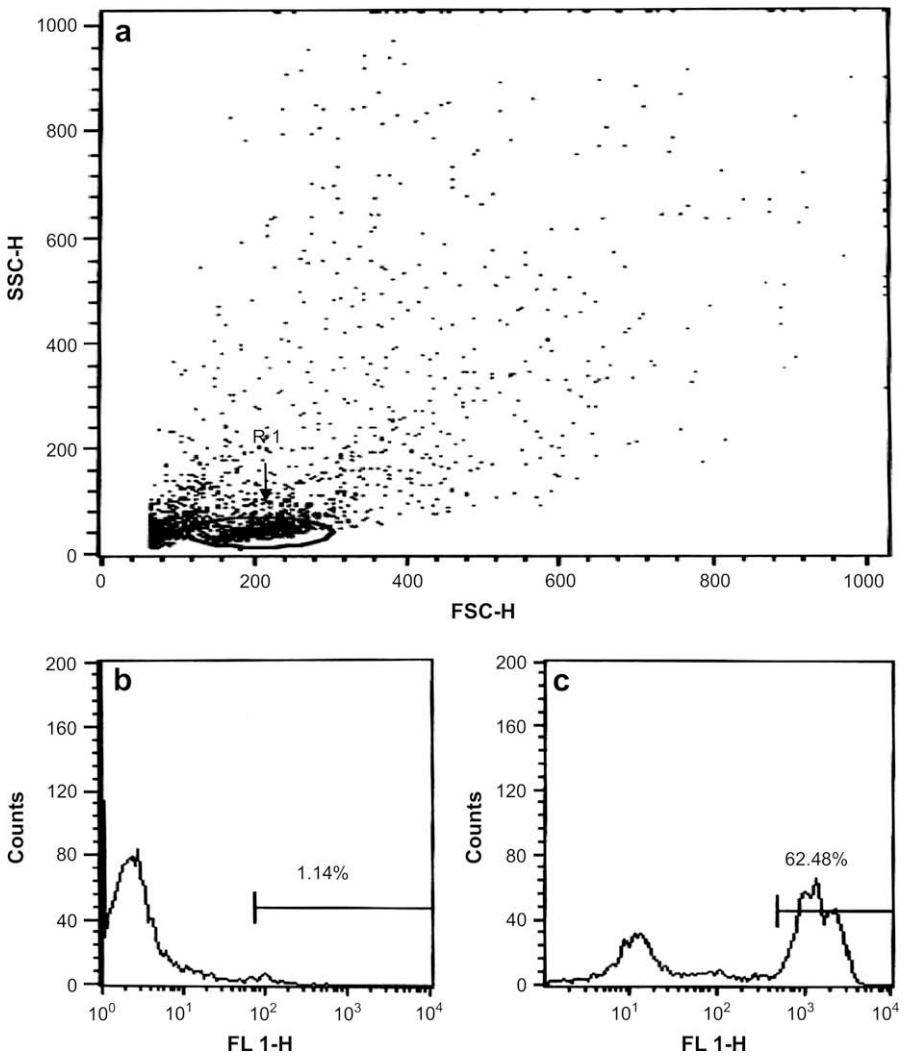

Figure 10 Representative flow cytometry generated forward/side scatter (FSC/SSC) profile and fluorescence histogram of rohu spleen mononuclear cells by indirect immunofluorescence with anti-rohu Ig MAb. (a) FSC/SSC dot plot of spleen mononuclear cells with gated lymphocytes (R1); (b,c) Fluorescence histogram of gated lymphocytes stained without MAb and with D7 MAb, respectively.

the intact molecule. In view of this, it is possible that 55-kDa band is a proteolytic fragment product of r-Ig, having antigenic similarity with HC. However, additional work is required to resolve the exact cause of proteolysis of Ig including the elution conditions in affinity chromatography, storage conditions or the intrinsic factors of Ig itself.

In non-reducing SDS-PAGE of r-Ig, several faint protein bands corresponding to different forms were seen in addition to the tetrameric form. It is presumed that the additional bands represent different redox forms of r-Ig, as already reported in fish [31]. To further substantiate the hypothesized forms, the three different peaks of r-Ig obtained in gel filtration chromatography representing molecular weight of 880, 443 and 220 kDa were analyzed by reducing SDS-PAGE. All the three peaks provided analogous products of HC and LC, in spite of variable molecular weights of all three whole molecules. This suggests that r-Ig has three forms i.e. tetramer, dimer and monomer. This result is in accordance with the study of Grove et al. [26], who also presented different forms of halibut fish Ig. The MW of 880 kDa for probable tetrameric form of r-Ig was further correlated with results of SDS-PAGE. It is well established that an Ig molecule in its monomeric form is composed of two HCs and two LCs, so the calculated MW of r-Ig molecule would be 206 kDa. Taking into consideration the values of the monomer, therefore, a molecule of approximately 880 kDa would have a tetrameric structure in accordance with observations from other teleost fish Ig. This was re-established by electrophoresis under non-reducing conditions, wherein r-Ig was shown to have one main band of high molecular weight.

This is the first report on development of monoclonal antibodies specific to Ig of rohu. In the present study, the hybridomas were obtained by immunizing Balb/c mice with purified r-Ig. The hybridomas were initially screened by the indirect ELISA, where r-Ig was coated as antigen. Five stable clones [1C8, 2D7, 3H4, 5A6 and 5B3] were selected for further characterization. It was later observed that three clones [1C8, 5A6, 5B3], although positive in the indirect ELISA, failed to react with r-Ig in the Western blotting. The

Author's personal copy

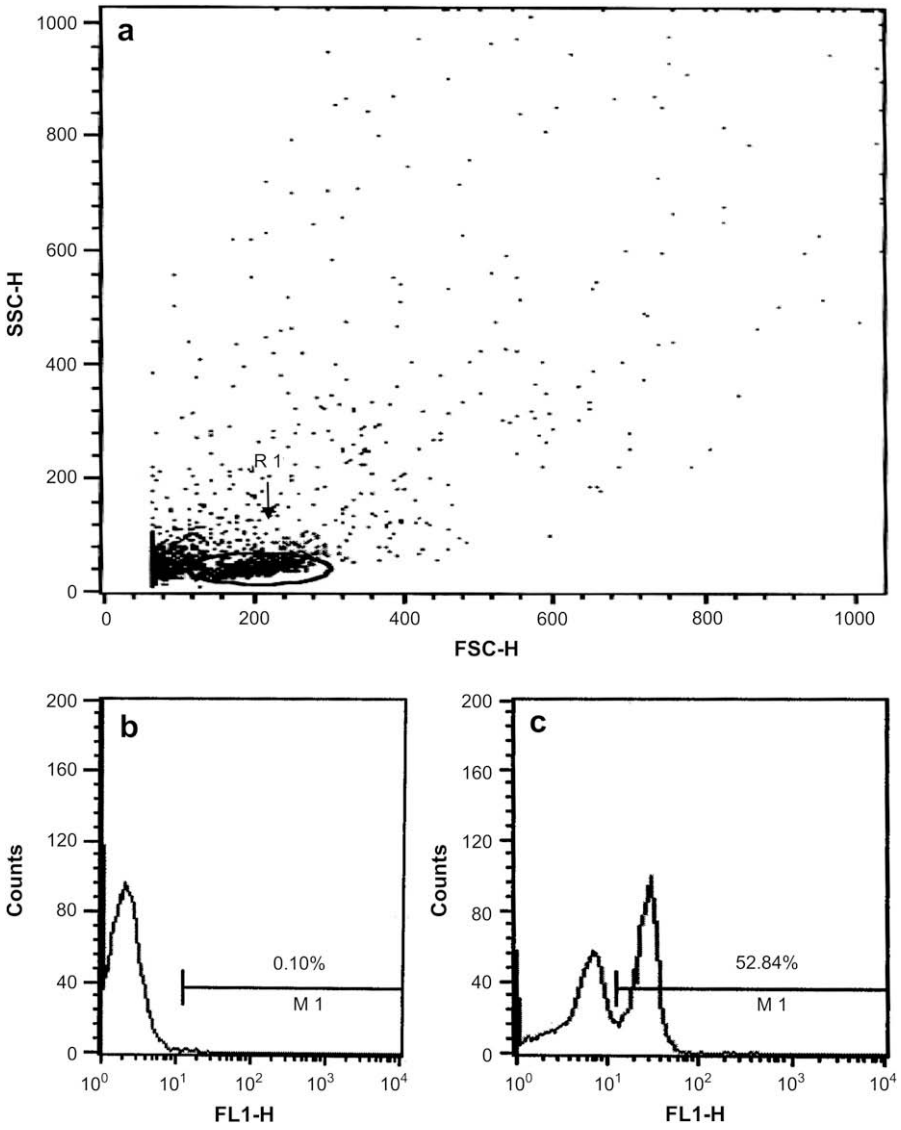

Figure 11 Representative flow cytometry generated forward/side scatter (FSC/SSC) profile and fluorescence histogram of rohu kidney mononuclear cells by indirect immunofluorescence with anti-rohu Ig MAb. (a) FSC/SSC dot plot of kidney mononuclear cells with gated lymphocytes (R1); (b,c) Fluorescence histogram of gated lymphocytes stained without MAb and with D7 MAb, respectively.

remaining two clones D7 and H4 recognized the HC of rohu Ig in Western blotting. Scapigliati et al. [32] have also observed that 47% of hybridomas to seabass Ig were unable to recognize the antigen when denatured and reduced. The three MAbs, which failed to react with reduced rohu Ig in Western blotting, suggest that they recognized a particular conformation of the epitope, which involves S—S bonding on the Ig molecule. This particular conformation of the molecule was altered in the presence of a reducing agent. The antigenic determinants recognized by D7 and H4 MAbs seem to be similar, since they were present in all the rohu serum tested. Another interesting observation was that no reactivity was seen with the LC even at higher concentrations of MAbs. Previous studies have also shown that HC is more antigenic in nature as compared to LC, as most of the MAbs against fish Ig, are specific for the Ig HC [11].

The criterion of MAb selection in the present study was to ensure an adequate and specific reaction in a broad range of immunoassays either for detection of pathogen specific Ig in serum or cell surface Ig. The specificity of selected MAbs was clearly demonstrated by the Western blot of the purified Ig and also rohu whole serum [data not shown]. In both cases, a definite reaction was observed, while it was exposed to a number of antigenic determinants. D7 MAb appeared to be suitable for the detection of pathogen specific antibodies by means of an indirect ELISA. A clear difference in the antibody titre of immunized and unimmunized fish could be observed, using D7 MAb as secondary antibody. This application of anti-rohu Ig MAb can be utilized in evaluating antibody production in response to infection or for investigating the possible effects of vaccination on antibody production.

Cross reactivity of D7 MAb with serum Ig of related fish species namely *C. catla* and *C. mrigala* was observed at a lower serum dilution, in the competitive ELISA. This MAb was not reactive to serum of other distantly related

Author's personal copy

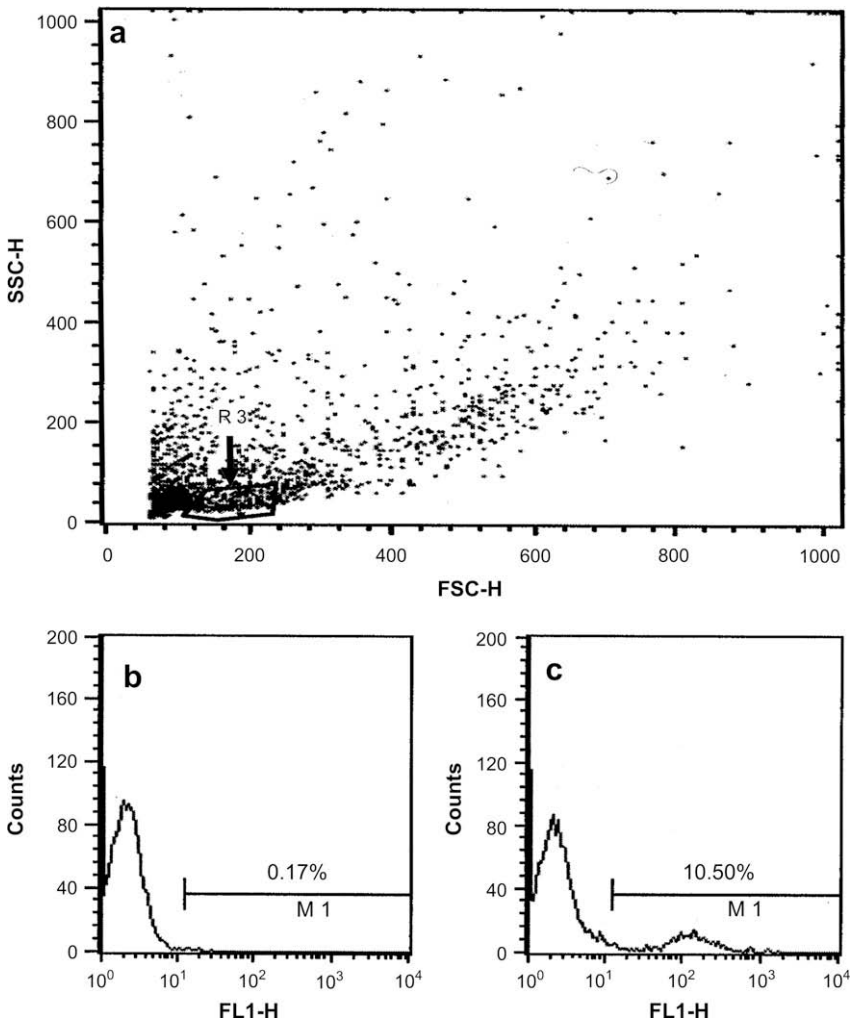

Figure 12 Representative flow cytometry generated forward/side scatter (FSC/SSC) profile and fluorescence histogram of rohu thymus mononuclear cells by indirect immunofluorescence with anti-rohu Ig MAb. (a) FSC/SSC dot plot of thymus mononuclear cells with gated lymphocytes (R3); (b,c) Fluorescence histogram of gated lymphocytes stained without MAb and with D7 MAb, respectively.

fish species belonging to *O. striatus* and *C. gariepinus*. Our results suggest that the epitope of rohu Ig recognized by D7 MAb is partially conserved among related fish species of same Cyprinidae family i.e. *C. catla* and *C. mrigala*, which leads to varying degrees of cross reactivity. However, no reactivity was seen in the species from different orders such as *O. striatus* (Order Perciformes) and *C. gariepinus* (Order Siluriformes). MAbs directed against Ig have shown cross-reaction to Ig of other phylogenetically related species of the same family or order [5,10,27]. So it is not unexpected to get a similarity between the Ig molecules of Indian major carps in our results, as they belong to one

Table 1 Percent Ig positive mononuclear cells stained by anti-rohu Ig D7 MAb in peripheral blood, spleen, kidney and thymus of rohu

Individual fish number	Blood			Spleen			Kidney			Thymus*		
	1[a]	2[a]	Mean	1[a]	2[a]	Mean	1[a]	2[a]	Mean	1[a]	2[a]	Mean
Rohu 1	52.39	50.63	51.51	62.48	58.12	60.3	48.12	54.65	51.38	10.36	10.65	10.50
Rohu 2	46.48	48.69	47.58	68.04	64.25	66.14	50.07	53.34	51.70	—	—	—
Rohu 3	54.65	58.21	56.43	65.78	70.45	68.11	52.95	57.95	55.45	—	—	—
Overall mean			51.84			64.85			52.84			10.50
Overall standard error (SE)			±2.55			±2.34			±1.30			—

[a] For each fish, duplicate samples [1 and 2] were run in the assay. * Pooled samples ($n = 4$, mean weight — 485 g).

Author's personal copy

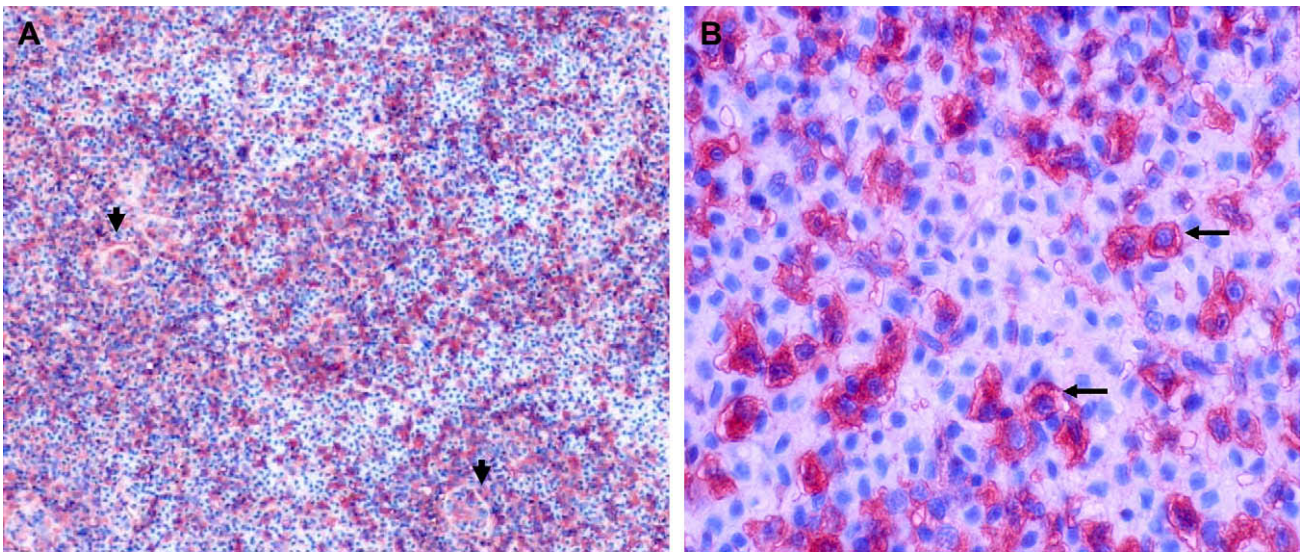

Figure 13 Demonstration of Ig bearing cells in rohu spleen tissue sections by indirect immunoperoxidase technique (IIPT), using D7 MAb. (A) Clusters of Ig positive cells in splenic corpuscles (100×). Arrowheads indicate ellipsoids. (B) Ig positive plasma cells (arrows) at a higher magnification (400×).

common family. Thus, anti-rohu Ig MAbs can have further application in detailed phylogenetic analysis within the family members in the future.

The distribution of Ig+ lymphocytes in blood, spleen, kidney and thymus was investigated by flow cytometry using anti-rohu Ig MAb. FACS analysis showed varying percentages of mononuclear cells from peripheral blood, spleen, kidney and thymus had Ig on their surface, which reacted with anti-rohu Ig MAb. These cells would be lymphocytes, judging from their low scores of FSC and SSC in the dot plot FACS analysis [8,10]. This indicates that cells reacting with MAb could be either plasma cells or B-lymphocytes, while non-reacting cells could be T lymphocytes or thrombocytes, as fish thrombocytes are nucleated and appear mixed with lymphocytes. The results of our FACS analysis revealed that 64.85% and 52.84% of mononuclear cells in rohu spleen and kidney were reactive with MAb against rohu immunoglobulin, respectively. The lowest reactivity of anti-Ig MAb was seen in gated mononuclear cells of the thymus, as also observed in earlier studies [10,32,33]. Wide variations in the mean percentage of Ig+ cells of spleen have been reported in different fish species i.e. 22.2% in seabass [32]; 25% in carp [33]; 25.3% in European eel [6]; 42.5% in torafugu [10] and 62.1% in chinook salmon [8]. These differences could be due to the amount of lymphoid tissue that varies with the degree of antigenic stimulation [34]. In view of this, it can be expected to obtain a wide variation in the number of Ig+ cells in different fish species and among individuals of the same species. Therefore, it can be assumed that elevated Ig+ cells in the spleen of tested rohu could be either due to greater antigenic stimulation in the culture environment or an inherent strong immune capability of the species itself. Further, it may also be interesting to determine the relationship between percentage of Ig positive cells in the spleen with culture conditions i.e. water temperature and total antigenic load, especially with reference to tropical warm water fishes and temperate cold water fishes. A related aspect that needs supplementary investigation is whether higher numbers of Ig+ cells in the spleen of rohu are actually translated into elevated serum Ig levels.

Similarly, FACS analysis revealed that 51.84% of gated PBMC in rohu were reactive with D7 MAb. This is comparable with the values of 45%, 50.2% and 56.7% obtained in trout [35], torafugu [10] and chinook salmon [8], respectively. In Atlantic salmon, mean percentage of different MAbs reacting to blood mononuclear leucocytes including lymphocytes ranged from 2.1% to 59.7% [7]. However, a lower percentage [25% and 33%] of Ig+ cells in peripheral blood leucocytes have been reported for European eel [6], and carp [36], respectively, using anti-Ig MAbs. Our data show that rohu has a high percentage of Ig producing lymphocytes in the peripheral blood, spleen and kidney, which could be suggestive of strong immunocompetence of the tested individuals. Interestingly, no significant changes in the number of B-cells in head kidney or peripheral blood leucocytes [PBL] were observed in virus-infected parr compared to control fish [9]. Even then, the quantification of the Ig secreting lymphocytes can be a useful tool in the assessment of the health status of fish, as B-lymphocytes eventually play a role in mounting an immune response to a pathogen.

In immunoperoxidase studies, D7 MAb reacted strongly with Ig+ cell population from spleen, as also reported in other fishes [26]. It is presumed that while most Ig+ cells probably were B-cells or plasma cells, macrophages, neutrophils and non-specific cytotoxic cells have been also reported to be Ig+, probably through binding of Ig to Fc receptors [26].

In conclusion, anti-rohu Ig MAb has proven to be a useful reagent for detection of serum Ig and cell surface Ig. They can be successfully employed for studies on fish immunology and sero-monitoring of pathogens in rohu and help in undertaking effective surveillance campaigns in fish farms, leading to a reduction in the spread of disease and eventually contribute to an increase in production.

Author's personal copy

Acknowledgements

The first author is also thankful to Dr R.P. Singh, IVRI, for providing support on production of monoclonal antibodies. The technical help provided by Mr. A.L. Vishwakarma, CDRI, Lucknow, on flow cytometry is duly acknowledged.

References

[1] Ellis AE. Innate host defense mechanisms of fish against viruses and bacteria. Dev Comp Immunol 2001;25:827—39.
[2] Estevez J, Leiro J, Santamarina MT, Ubeira FM. A sandwich immunoassay to quantify low levels of turbot (*Scophthalmus maximus*) immunoglobulins. Vet Immunol Immunopathol 1995;45:165—74.
[3] Scapigliati G, Scalia D, Marras A, Meloni S, Mazzini M. Immunoglobulin levels in the teleost sea bass *Dicentrarchus labrax* (L.) in relation to age, season, and water oxygenation. Aquaculture 1999;174:207—12.
[4] Huttenhuis HBT, Ribeiro ASP, Bowden TJ, Bavel CV, Taverne-Thiele AJ, Rombout JHWM. The effect of oral immuno-stimulation in juvenile carp (*Cyprinus carpio*). Fish Shellfish Immunol 2006;21:261—71.
[5] Estevez J, Leiro J, Santamarina MT, Dominguez J, Ubeira FM. Monoclonal antibodies to turbot (*Scophthalmus maximus*) immunoglobulins: characterization and applicability in immunoassays. Vet Immunol Immunopathol 1994;41: 353—66.
[6] Van der Heijden MHT, Rooijakkers JBMA, Booms GHR, Rombout JHWM, Boon JH. Production, characterization and applicability of monoclonal antibodies to European eel (*Anguilla anguilla* L. 1758) immunoglobulin. Vet Immunol Immunopathol 1995;45:151—64.
[7] Pettersen EF, Bjerknes R, Wergeland HI. Studies of Atlantic salmon (*Salmo salar* L.) blood, spleen and head kidney leucocytes using specific monoclonal antibodies, immunohistochemistry and flow cytometry. Fish Shellfish Immunol 2000; 10:695—710.
[8] Milston RH, Vella AT, Crippen TL, Fitzpatrick MS, Leong JAC, Schreck CB. In vitro detection of functional humoral immunocompetence in juvenile chinook salmon (*Oncorhynchus tshawytscha*) using flow cytometry. Fish Shellfish Immunol 2003; 15:145—58.
[9] Rønneseth A, Pettersen EF, Wergeland HI. Neutrophils and B-cells in blood and head kidney of Atlantic salmon (*Salmo salar* L.) challenged with infectious pancreatic necrosis virus (IPNV). Fish Shellfish Immunol 2006;20:610—20.
[10] Miyadai T, Ootani M, Tahara D, Aoki M, Saitoh K. Monoclonal antibodies recognising serum immunoglobulins and surface immunoglobulin-positive cells of puffer fish, torafugu (*Takifugu rubripes*). Fish Shellfish Immunol 2004;17: 211—22.
[11] Scapigliati G, Romano N, Abelli L. Monoclonal antibodies in fish immunology: identification, ontogeny and activity of T- and B-lymphocytes. Aquaculture 1999;172:3—28.
[12] FAO. Aquaculture production 2003. Yearbook of Food and Agriculture Organization of United Nations. Rome: Italy; 2003.
[13] Swain P, Dash S, Sahoo PK, Routray P, Sahoo SK, Gupta SD, et al. Non-specific immune parameters of brood Indian major carp *Labeo rohita* and their seasonal variations. Fish Shellfish Immunol 2007;22:38—43.
[14] Swain P, Nayak SK, Sahu A, Mohapatra BC, Meher PK. Bath immunisation of spawn, fry and fingerlings of Indian major carps using a particulate bacterial antigen. Fish Shellfish Immunol 2002;13:133—40.
[15] Swain P, Behura A, Dash S, Nayak SK. Serum antibody response of Indian major carp, *Labeo rohita* to three species of pathogenic bacteria; *Aeromonas hydrophila*, *Edwardsiella tarda* and *Pseudomonas fluorescens*. Vet Immunol Immunopathol 2007;117:137—41.
[16] Thuvander A, Hongslo T, Jansson E, Sundquist B. Duration of protective immunity and antibody titres measured by ELISA after vaccination of rainbow trout, *Salmo gairdneri* Richardson, against vibriosis. J Fish Dis 1987;10:486—97.
[17] Cho HJ, Tuhnke HL, Langford EV. The indirect haemagglutination test for the detection of antibodies in cattle infected with mycoplasmas. Can J Comp Med 1976;40:20—9.
[18] Harlow E, Lane D. Antibodies: a laboratory manual. New York: Cold Spring Harbor Laboratory Press; 1988.
[19] Magnadottir B, Gudmundsdottir BK. A comparison of total and specific immunoglobulin levels in healthy Atlantic salmon (*Salmo salar* L.) and in salmon naturally infected with *Aeromonas salmonicida* subsp. *achromogenes*. Vet Immunol Immunopathol 1992;32:179—89.
[20] Polak JM, Noorden SV. Immunocytochemistry. United Kingdom: BIOS Scientific Publishers Ltd; 2003.
[21] Bryant MS, Lee RP, Lester RJG, Whittington RJ. Anti-immunoglobulin antisera used in an ELISA to detect antibodies in barramundi *Lates calcarifer* to *Cryptocaryon irritans*. Dis Aquat Org 1999;36:21—8.
[22] Swain T, Mohanty J, Sahu AK. One step purification and partial characterization of serum immunoglobulin from Asiatic catfish (*Clarias batrachus* L.). Fish Shellfish Immunol 2004;17: 397—401.
[23] Watts M, Munday BL, Burke CM. Isolation and partial characterization of immunoglobulin from southern bluefin tuna *Thunnus maccoyii* Castelnau. Fish Shellfish Immunol 2001;11: 491—503.
[24] Estevez J, Leiro J, Sanmartin ML, Ubeira FM. Isolation and partial characterization of turbot (*Scophthalmus maximus*) immunoglobulins. Comp Biochem Physiol 1993;105A: 275—81.
[25] Crosbie PBB, Nowak BF. Production of polyclonal antisera against barramundi (*Lates calcarifer* Bloch) serum immunoglobulin derived from affinity columns containing mannan-binding protein or staphylococcal protein A. Aquaculture 2002;211:49—63.
[26] Grove S, Tryland M, Press CM, Reitan LJ. Serum immunoglobulin M in Atlantic halibut (*Hippoglossus hippoglossus*): characterization of the molecule and its immunoreactivity. Fish Shellfish Immunol 2006;20:97—112.
[27] Palenzuela O, Sitja-Bobadilla A, Alvarez-Pellitero P. Isolation and partial characterization of serum immunoglobulins from sea bass (*Dicentrarchus labrax* L.) and gilthead sea bream (*Sparus aurata* L.). Fish Shellfish Immunol 1996;6:81—94.
[28] Whittington RJ. Purification and partial characterization of serum immunoglobulin of the European perch (*Perca fluviatilis* L.). Fish Shellfish Immunol 1993;3:331—43.
[29] Sanchez C, Lopez-Fierro P, Zapata A, Dominguez J. Characterization of monoclonal antibodies against heavy and light chains of trout immunoglobulin. Fish Shellfish Immunol 1993; 3:237—51.
[30] Van Ginkel FW, Pascual DW, Clem LW. Proteolytic fragmentation of channel catfish antibodies. Dev Comp Immunol 1991; 15:41—51.
[31] Kaattari SL, Evans DA, Klemer JV. Varied redox forms of teleost IgM: an alternative to isotypic diversity? Immunol Rev 1998;166:133—42.
[32] Scapigliati G, Romano N, Picchietti S, Mazzini M, Mastrolia L, Scalia D. Monoclonal antibodies against sea bass *Dicentrarchus labrax* (L.) immunoglobulins: immunolocalisation of immunoglobulin-bearing cells and applicability in immunoassays. Fish Shellfish Immunol 1996;6:383—401.

Author's personal copy

[33] Koumans-van Diepen JCE, Taverne-Thiele AJ, van Rens BTTM, Rombout JHWM. Immunocytochemical and flow cytometric analysis of B cells and plasma cells in carp (*Cyprinus carpio* L): an ontogenic study. Fish Shellfish Immunol 1994;4:19—28.

[34] Zapata AG, Chiba A, Varas A. Cell and tissues of the immune system of fish. In: Iwama G, Nakanishi T, editors. The fish immune system. San Diego: Academic Press; 1996. p. 1—62.

[35] Thuvander A, Fossum C, Lorenzen N. Monoclonal antibodies to salmonid immunoglobulin: characterization and applicability in immunoassays. Dev Comp Immunol 1990;14:415—23.

[36] Koumans-van Diepen JCE, Egberts E, Peixoto BR, Taverne-Thiele AJ, Rombout JHWM. B-cell and immunoglobulin heterogeneity in carp (*Cyprinus carpio* L.): an immuno(cyto)-chemical study. Dev Comp Immunol 1995;19:97—108.