Fish & Shellfish Immunology (2008) **24**, 779–783

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SHORT COMMUNICATION

Isolation and characterization of immunoglobulin of the Indian major carp, rohu [*Labeo rohita* (Ham.)]

P.P. Suresh Babu [a], K.M. Shankar [a,*], B.R. Honnananda [a], H.V. Vijaya Kumara Swamy [b], K. Prasanna Shama [a], V.V.S. Suryanarayana [c], H.J. Dechamma [c]

[a] *Fish Pathology and Biotechnology Laboratory, Department of Aquaculture, College of Fisheries, Karnataka Veterinary, Animal and Fisheries Sciences University, Mangalore 575002, Karnataka, India*
[b] *Department of Biotechnology, University of Agricultural Science, Bangalore 65, Karnataka, India*
[c] *Indian Veterinary Research Institute, Ganganagar, Bangalore, Karnataka, India*

Received 20 September 2007; revised 29 January 2008; accepted 31 January 2008
Available online 8 February 2008

KEYWORDS
Immunogloblin;
Indian major carp;
Rohu;
Labeo rohita

Abstract Information on the structure and character of immunoglobulin of fishes is essential in health management. A study was carried out to characterize the serum immunoglobulin (IgM) of the Indian major carp, rohu *Labeo rohita* (Ham.). Rohu (500 g) were immunised with bovine serum albumin (BSA) and the anti-BSA antibody was purified employing BSA–CL agarose affinity column chromatography. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of the purified Ig in a 3% gel under non-reduced conditions revealed a single protein having a molecular weight of 850 kDa. Analysis of the purified serum in 10% SDS-PAGE under reduced conditions revealed that the immunoglobulin contained heavy and light chains with molecular weights of 85 and 23 kDa, respectively. A polyclonal mouse anti-rohu IgM was prepared and used in an immunodot test which showed a specific reaction of the crude rohu anti-BSA antiserum and the purified anti-BSA IgM with BSA. Results indicate that the immunoglobulin of *L. rohita* is tetrameric IgM, similar to that of other fishes.

Indian major carps (IMC) form the mainstay of culture fishery in India, supported by a strong traditional knowledge base and scientific inputs in management. Intensification of culture of IMC in recent years has led to outbreaks of several diseases caused by fungal, bacterial and protozoan agents [1–6]. In this context, adequate knowledge about the structure and character of the immunoglobulin of IMC provides a strong base for development of diagnostics and vaccines. There are several studies on characterization of the immunoglobulin (Ig) of commercial fishes such as Japanese eel [7], Snapper [8], Rainbow trout [9],

* Corresponding author. Tel.: +91 824 2244356; fax: +91 824 2248366.
E-mail address: kalkulishankar@gmail.com (K.M. Shankar).

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doi:10.1016/j.fsi.2008.01.013

European Sea bass [10], Common carp [11,12], Turbot [13], African catfish [14], Cod [15], Atlantic salmon [16], Tilapia [17], Channel catfish [18] and Brown bullhead [19]. The predominant antibody type in fish is high molecular weight Ig often referred to as IgM or IgM like because this molecule has a heavy chain isotype similar to the mammalian μ chain [20]. Previously, it was believed that fish Ig is of only one class, i.e., IgM [21]. However, a truncated form of Ig from *Epinephelus itaira* [22] and Australian lung fish *Neoceratodus forsteri* [23] has been reported which is termed as IgY. An Ig heavy chain (IgH) with some sequence homology to be of IgD has been identified in Channel catfish [24] and Atlantic salmon [25]. Recently, based on the variation in the heavy chain genes, a few more isotypes of IgM such as IgZ in Zebra fish [26], IgT in trout [27] and an IgH chimera, IgM–IgT, in Common carp [28] have been reported. However, there is a dearth of published data on the characters of immunoglobulin of IMC. Here we present data on isolation, purification and characterization of Ig of the most widely cultured IMC, rohu, *Labeo rohita*.

Three rohu weighing about 500 g each, maintained in a 25 m^2 cement cistern at the freshwater fish farm of the College of Fisheries, Mangalore, were injected intraperitoneally (ip) each with 1 mg bovine serum albumin (BSA, Merck, India) dissolved in 250 μl of phosphate buffered saline (PBS, pH 7.4) emulsified with an equal volume of Freund's Complete Adjuvant (FCA). Similar doses of BSA emulsified with an equal volume of Freund's Incomplete Adjuvant (FIA) were given 15 and 22 days later. Two rohu injected with PBS (pH 7.4) alone were maintained as control for collection of unimmunised serum. On the 30th day, fish were anaesthetized with 10 ppm Benzocain for 3 min followed by collection of blood by puncturing the caudal vein. The blood was allowed to clot for 1 h at room temperature and overnight at 4 °C. Serum was collected after centrifuging at 6000 rpm for 10 min, pooled and stored at −20 °C.

Affinity purification of the rohu Ig was carried out according to Rathore et al. [14] using a 5 ml BSA–CL agarose column (Bangalore Genei, India). The column was equilibrated by passing 5 column volumes of PBS (pH 7.2). Two milliliters of serum from immunised fish, mixed with an equal volume of PBS (pH 7.2), was filtered through 0.45 μm syringe filter (MDI, India) and loaded onto the column. The serum was allowed to interact with the BSA for 45 min. Unbound serum protein was washed out with 10 column volumes of PBS (pH 7.2). The bound IgM was then eluted with 10 ml of elution buffer (0.1 M citric acid buffer, pH 3.0). Approximately 10 fractions of 0.55 ml each were collected and neutralized with 0.45 ml of 0.5 M Tris buffer (pH 11). Absorbance at 280 nm of each fraction was measured spectrophotometrically. The eluted samples were pooled and subjected to dialysis at 4 °C against PBS using a low molecular weight (12 kDa cut off) dialysis membrane (Sigma, USA). The dialysis membrane was shifted to a tray containing sucrose to concentrate the contents to 2 ml and the concentrated IgM was stored at −20 °C for further use.

Purified IgM was characterised by a non-reducing SDS-PAGE. Briefly, 10 μl of the 4× native PAGE sample buffer (10% SDS, 0.5 M Tris–HCl (pH 6.8), glycerol 0.8 ml, 0.01% bromophenol blue (W/V)) was added to 30 μl of the purified IgM. The purified IgM along with molecular weight markers (Sigma, USA) and native human IgM (Sigma, USA) were loaded onto the gel containing 3% acrylamide and 0.5% agarose according to Kobayashi et al. [29] and subjected to electrophoresis at 25 mA till the dye front reached the bottom of the gel. The gel was stained with Coomassie blue (10% acetic acid, 40% methanol, 0.1% Coomassie blue) for 8 h, destained with destaining solution (10% acetic acid, 40% methanol) and the molecular weight of the IgM was estimated. Further characterization of the IgM was carried out by a 10% SDS-PAGE according to Laemmli [30]. Ten microliters of the 4× sample buffer (10% SDS, 2-β-mercaptoethanol 0.4 ml, 0.5 M Tris–HCl (pH 6.8), glycerol 0.8 ml, 0.01% bromophenol blue (W/V)) was added to 30 μl of the purified IgM and boiled at 100 °C for 5 min. For comparison, crude serum from immunised fish was prepared in a similar way. The samples along with molecular weight markers (Sigma, USA) were loaded onto 4.5% stacking gel and subjected to electrophoresis at 30 mA in 10% separating gel till the dye front reached the bottom of the gel. The gel was stained with Coomassie blue and the molecular weight of heavy and light chains of Ig determined.

A polyclonal antibody (PAb) based immunodot assay was adopted for the detection of rohu antibodies. Ten-week-old Swiss albino mice were immunised (ip) with purified rohu IgM (50 μg in 250 μl PBS) mixed 1:1 with FCA. A similar second dose (ip) was given with the purified IgM in FIA (1:1) on the 15th day. On the 27th day, a booster dose of 10 μg purified IgM in 0.05 ml PBS was given intravenously. Blood was collected on the 30th day from the tail vein using a hypodermic syringe and clotted for 1 h at room temperature. Serum was collected by centrifuging the clotted blood at 6000 rpm for 10 min and used as PAb.

An immunodot assay was carried out by dotting BSA onto nitrocellulose (NC) papers cut into 2 cm^2 size. Purified IgM was dotted on the paper as positive control and *Aeromonas hydrophila* and *Vibrio* sp. bacterins were dotted as negative controls. Three such dotted membranes were prepared separately and used to detect antibodies in the affinity purified IgM, crude immunised and unimmunised sera. Free sites on the nitrocellulose membranes were blocked with 5% skimmed milk powder in PBS for 2 h and washed 3 times with PBS (pH 7.4). One of the membranes was treated overnight with 1 ml of the affinity purified IgM at a concentration of 0.1 mg/ml, and the other with crude immunised fish serum diluted 10 times with PBS (1 ml). The third membrane was treated overnight with the unimmunised fish serum diluted 10 times with PBS (1 ml). After washing three times with PBS–Tween 20 (0.05%), all the three membranes were treated with mouse anti-rohu IgM (PAb) diluted with PBS (1 ml, 1:1000 dilution) for 2 h. After washing with PBS–Tween 20, rabbit anti-mouse IgG horse-radish peroxidase (Bangalore Genei, India) in 3% BSA in PBS (1 ml, 1:1000 dilution) was added and incubated for 90 min. After washing with PBS–Tween 20 three times, 1 ml of substrate (0.3 mg of 4-chloro-1-naphthol in 10 μl ethanol, 1 μl of 30% H_2O_2, 1 ml of Tris buffer pH 7.6) was added. Clear purple blue dot development on the nitrocellulose paper was considered positive.

Although BSA is reported as a poor immunogen in fish [14,31], it was selected in the present study because of the availability of the ready made BSA ligated affinity column.