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Affinity purification and partial characterization of IgM-like immunoglobulins of African catfish, *Clarias gariepinus* (Burchell, 1822)

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IgM like macroglobulin from bovine serum albumin (BSA)-immunized African catfish *C. gariepinus* was purified by affinity chromatography and partially characterized. The molecular weight of this macroglobulin was 840 kDa, as estimated by gel filtration chromatography. Purified macroglobulin was analyzed using SDS-PAGE under reducing and non-reducing conditions. The molecular weight (MW) of heavy and light chain was 74.8 kDa and 27.2 kDa respectively, in presence of a reducing agent. In non-reducing SDS-PAGE, a single high MW band was observed representing tetrameric form.

Keywords: Chromatography, *Clarias gariepinus*, Fish, Immunoglobulin

Teleost fish possess an immune system similar in many respects to that of mammals[1]. The immunoglobulin (Ig) of the teleost immune system is B cell derived component[2]. Immunoglobulin molecules have been identified in the plasma, gut, skin, bile and mucus of fish[3]. Unlike other groups of vertebrates, teleost fish have only one class of Ig, which is tetrameric in structure and with a molecular weight (MW) of 600-800 kDa[4]. Fish IgM generally lack the J chain associated with mammalian IgM, however J-like chains have been isolated from channel catfish (*Ictalurus punctatus*)[5] and sheepshead (*Archosargus probatocephalus*)[6]. A chimeric gene has been sequenced from channel catfish (*Ictalurus punctatus*) that has partial sequence homology with the heavy (H) chain of mammalian IgD[7]. The specific structural characteristics of the Ig have been investigated in a few species of fish. These include channel catfish[5], white sturgeon[8], seabass[9], turbot[10], salmon[11], gilthead sea bream[12], tilapia[13], common carp[14], etc. In India, there are few reports on isolation and characterization of fish Ig[13,15].

The purpose of the study is to isolate, purify and characterize African catfish (*Clarias gariepinus*) Ig in order to develop polyclonal and monoclonal antisera against *C. gariepinus* Ig.

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Materials and Methods

Fish—Ten adult *C. gariepinus*, weighing 800-1000 g were procured from the local fish market. The fish were divided in two groups (test and control) of 5 each and maintained separately in 1000 litre outdoor fibre reinforced plastic tanks at ambient temperature with *ad libitum* feeding. The fish were acclimated for 2 weeks before immunization.

Immunization—Each fish in test group was injected intraperitoneally (ip) with 1 mg of BSA in 0.1 ml phosphate buffer saline emulsified in an equal volume of Freund's complete adjuvant. After every 14 days, a booster dose of BSA (1 mg) in Freund's incomplete adjuvant was administered, thrice. Similarly, the control fishes were injected with PBS emulsified in adjuvant as above. Pre and post immunization (7 days after 4th injection) blood samples were collected from the fish via caudal vein. Serum was stored in 0.5 ml aliquots at –20°C.

Determination of serum antibody titre by indirect haemagglutination (IHA)—The method described by Cho *et al.*[16] was followed. Briefly, for the sensitization of sheep red blood cells (RBCs), nine volumes of 2.5% sheep RBCs were mixed with one volume of 0.6% glutaraldehyde and incubated for 10 min. Washed RBCs were sensitized with BSA (10 mg/ml) for 30 min. The RBCs were washed again and suspended in PBS to a final concentration of 2.5%.

IHA test—Two-fold dilution of individual serum was done with PBS in a 96 well microtitre plate, except RBC control wells. Sensitized RBCs (50 µl) were added in each well, incubated for 30 min and the highest dilution of serum showing agglutination was considered as titre.

Purification of Ig by affinity chromatography—BSA-CL agarose column (5 ml; Genei Bangalore) was equilibrated with 50 ml PBS. Serum (2 ml) from immunized fish having an IHA titre of > 1:64 was mixed with equal volume of PBS, filtered through 0.4 µ filter and loaded in the affinity column. The serum was allowed to adsorb to the agarose beads for 30 min. After the adsorption of the serum, the column was again washed with PBS, so as to remove the unbound material present in the column. The anti-BSA antibodies were eluted from the column with elution buffer (0.1 *M* citric acid buffer, *p*H 3) and collected as 2 ml fractions. These Ig fractions were immediately neutralized with 0.6 ml of 0.1*M* Tris buffer, *p*H 11. The protein concentration of each fraction was estimated through UV-spectrophotometer at 280 nm.

Molecular weight determination by gel filtration chromatography—The purity and molecular weight (MW) of eluted Ig was determined by gel filtration chromatography on a 1.6 × 57 cm column bed of Sephacryl S-300 (Pharmacia, Sweden) having a total volume (Vt) of 114.6 ml. Void volume (Vo) of the column bed was 53.2 ml. The column was calibrated with standard protein MW markers (Sigma) viz. thyroglobulin (669 kDa), apoferritin (443 kDa), amylase (200 kDa) and bovine serum albumin (66 kDa). The elution volume (Ve) for each marker was determined from the peak optical density (OD) at 280 nm. One ml of concentrated Ig containing 2 mg total protein was applied to the column at a flow rate of 5.7 ml per hour using 0.1 *M* PBS (*p*H 7.2). Fractions of 1.9 ml were collected using automatic fraction collector (Pharmacia) and OD of elutes was measured at 280 nm. The MW of purified Ig molecule was estimated from a standard curve of the markers against their Ve divided by Vo of blue dextran (Fig. 1).

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)—Purified Ig was partially characterized by polyacrylamide gel electrophoresis under both reducing and non-reducing conditions to determine purity and MW of reduced Ig molecules. The MW of the Ig in reduced form was determined by SDS-PAGE as described by Laemmli *et al*.[17] using Labworks Version 4.5 software. Medium range MW marker (PMW-M, Genei Bangalore) was used as standard. Purified Ig was also analyzed under non reducing conditions in a 4% gel, with samples diluted in loading buffer that did not contain β-mercaptoethanol, so as to determine the different forms of Ig.

Results

The IHA titre of individual fish is given in Table 1. The antibody titres ranged from 1:4 to 1:128 in test group whereas, the titre was nil in control group.

Affinity chromatography—A single peak was observed on elution with citric acid buffer (*p*H 3). All the fractions having an optical density of >0.1 were pooled, desalted using Hitrap desalting column (Pharmacia, Sweden), concentrated and stored at 4°C. The concentrated Ig fraction showed an IHA titre of 1:128.

Gel filtration chromatography—The Ig sample was loaded to a gel filtration column containing Sephacryl S-300 in order to determine its MW. A single peak was observed at an elution volume of 68.4 ml corresponding to MW of approx. 840 kDa.

Table 1—Indirect haemagglutination titre of individual immunized *C. gariepinus*

Group	Fish number	Titre
Test	1	1:64
	2	1:4
	3	1:128
	4	1:128
	5	1:64
Control	1-5	Nil

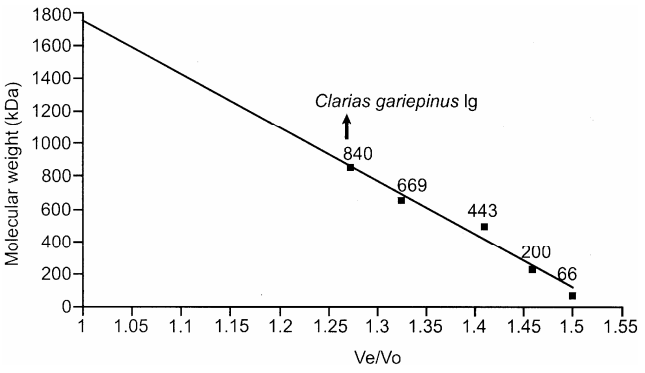

Fig. 1—Molecular weight determination of affinity purified *Clarias gariepinus* Ig by gel filtration chromatography

SDS-PAGE—The affinity purified Ig when analyzed by SDS-PAGE under reducing conditions, revealed two bands of 74.8 and 27.2 kDa corresponding to the heavy and light chain. Under non-reducing conditions, a single band of high molecular weight was observed (Fig. 2).

Discussion

The present study was carried out with an aim to isolate fish Ig of *C. gariepinus* in order to develop anti-fish Ig conjugate. This conjugate can be used for detection of fish antibodies in ELISA. This can serve as an important reagent in sero-surveillance of fish pathogens by non-lethal sampling. So to develop such immuno-reagents, isolation of Ig from fish serum is a prerequisite. Various techniques for the separation of Ig have been used including precipitation by saturated ammonium sulphate, molecular sieving, ion exchange chromatography and affinity chromatography[11,18]. In the present study, affinity chromatographic technique was used to isolate fish Ig. Immunization with BSA produced a variable response among five fish of the test group, with one fish showing very little response. Poor response to BSA was observed in some immunized barramundi fish also[19]. This indicates that BSA is a poor immunogen in fish and alternate high MW proteins may be used for immunization.

MW of the purified Ig from serum of *Clarias gariepinus* was estimated to be 840 kDa by gel filtration chromatography. Similarly, the estimated MW of Indian catfish *Clarias batrachus* was reported to be 863 kDa by native gradient PAGE[15]. Reported MW of the Ig has been estimated as 870 kDa in white sturgeon[8], 883 kDa in seabass[9], 820 kDa in turbot[10], 800 kDa in Atlantic salmon[11], 855 kDa for sea bass and 830 kDa for gilthead sea bream[12].

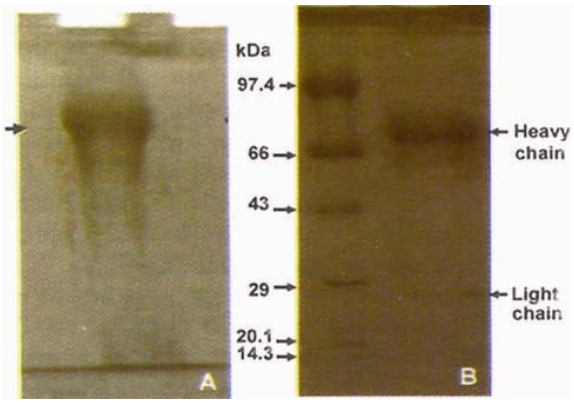

Fig. 2—Analysis of affinity purified *C. gariepinus* immunoglobulin by polyacrylamide gel electrophoresis [(A) Non-reducing SDS-PAGE (4%) (arrow indicates tetrameric form); (B) Denaturing SDS-PAGE (12%)- Lane 1: Molecular weight marker (Genei, Bangalore); Lane 2: *C. gariepinus* Ig]

SDS-PAGE analysis of the Ig showed that the affinity chromatography was very effective in purifying *C. gariepinus* Ig. MW of the two main bands present in purified Ig are indicative of putative H and L chains of IgM which is the predominant Ig in the serum of teleost fish. Apart from MW, the intensity of band staining was consistent with a characteristic of Ig in that the H chains being bigger than L chains, take up more stain. In the present study, the MW of H chain of the reduced Ig of *C. gariepinus* was estimated as 74.8 kDa and the L chain were estimated as 27.2 kDa. These values are in accordance to the MW reported for the H and L chain of *C. batrachus*[15]. In channel catfish Ig, the MW of H and L chain was 70 and 23 kDa, respectively[20]. H and L chains of Atlantic salmon were found to be of 72 and 27 kDa respectively[11], while in Atlantic cod, the H and L chains were of 81 and 27.5 kDa[21]. Similarly, in gilthead sea bream, MW of H and L chains were 70 and 25 kDa[22] and H and L chains of Ig of turbot had a MW of 78 and 27 kDa[23].

Considering that IgM like protein in its monomeric state has a 2H-2L structure, the fundamental structure of every monomeric Ig, the MW of monomeric IgM could be calculated as 204 kDa for *C. gariepinus* Ig by SDS-PAGE. Thus a molecule of 840 kDa should have a tetrameric structure ($204 \times 4 = 816$) which is almost similar to that obtained in gel filtration chromatography. Under non-reducing conditions, the affinity purified Ig was shown to have a single band which was also observed in Indian catfish[15]. As reported earlier, this may be indicative of tetrameric form of Ig.

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