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Isolation and characterization of serum immunoglobulins of *Cyprinus carpio*

NEERAJ SOOD[1], GAURAV RATHORE[2], T RAJA SWAMINATHAN[3], REHANA ABIDI[4], B N MISHRA[5] and W S LAKRA[6]

National Bureau of Fish Genetic Resources, Lucknow, Uttar Pradesh 226 002 India

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ABSTRACT

Common carp (*Cyprinus carpio*) immunoglobulin (Ig) was purified from serum by affinity chromatography using bovine serum albumin as capture ligand. The purified Ig had a molecular weight (MW) of 820 kDa as determined by gel filtration chromatography. The MW of heavy and light chain of common carp Ig was 73.7 and 25.3 kDa, respectively, in SDS-PAGE. In non-reducing SDS-PAGE, 3 bands of different MW were observed, which were presumed to be of different forms of Ig.

Key words: Chromatography, *Cyprinus carpio*, Immunoglobulin, SDS-PAGE

A better understanding of the structure and function of fish Ig has become all the more important in recent years due to the need of the fish farming industry for effective prevention and control of various fish diseases. Fish Ig characterization has received special attention in the recent past (Swain *et al.* 2004, Grove *et al.* 2006, Rathore *et al.* 2006). There are a few reports on purification and characterization of common carp Ig from other countries (Kusuda *et al.* 1987, Zhong *et al.* 1999). Though, *Cyprinus carpio* is one of the important culture fish in India, however, there are no reports on Ig purification and availability of anti-fish Ig of this species for application in immunoassays. The broader objective of our study is to develop monoclonal antibodies to purified common carp Ig that can serve as diagnostic reagent in immunoassays. This work describes the isolation and characterization of serum Ig from *C. carpio* as a part of above objective.

MATERIALS AND METHODS

Fish: Apparently healthy *C. carpio* (n=15), weighing 400–500 g, procured from fish farm, were divided in test and control groups. The test group comprised 10 fish and control had 5 fish. Both the groups were kept separately in fiber reinforced plastic tanks and provided pelleted fish feed. The fish were acclimatized for 1 week before immunization.

Raising of hyper-immune serum: Bovine serum albumin (BSA) was used as an immunogen to induce anti-BSA antibodies in fishes. BSA (1 mg) in 0.1 ml phosphate buffer saline (PBS), emulsified with equal volume of Freund's complete adjuvant, was injected intra-peritoneally to each fish of the test group. This was followed by 3 injections of similar emulsion in Freund's incomplete adjuvant at 2, 4 and 6 weeks interval. The fish in control group were injected with PBS emulsified with adjuvant, similarly. Pre-and post-immunization (7 days after fourth injection) blood samples were collected from the fish via caudal vein, and serum was stored at 4°C.

Indirect haemagglutination (IHA) test: IHA was used to assess the humoral immune response in immunized fish. Washed sheep erythrocytes were fixed in glutaraldehyde and sensitized with BSA following Cho *et al.* (1976). For the determination of anti-BSA titre, 50 µl of fish serum was serially diluted in 50 µl of PBS in a 96 well microtitre plate, except in RBC control wells. Thereafter, 50 µl of BSA-sensitized RBCs was added to each well and incubated for half an hour at 37°C. The highest dilution of serum showing agglutination was considered as titre.

Purification of Ig by affinity chromatography: BSA-CL agarose column was used to purify anti-BSA Ig from immunized fish having an IHA titre of 1: 64 and above. Hyper-immune serum (2 ml) was mixed with equal volume of PBS, filtered through 0.4µ filter and loaded in the affinity column. The column was washed with PBS and anti-BSA Ig 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 by UV-spectrophotometer (280 nm) to determine the protein concentration. Fractions having an OD of >0.1 were pooled and concentrated with centriplus YM–100 filter. The concentrated Ig was exchanged with PBS and again

Present address: [1-4]Scientist, [6]Director.

[5]Institute of Engineering and Technology, Sitapur Road, Lucknow 226 002.

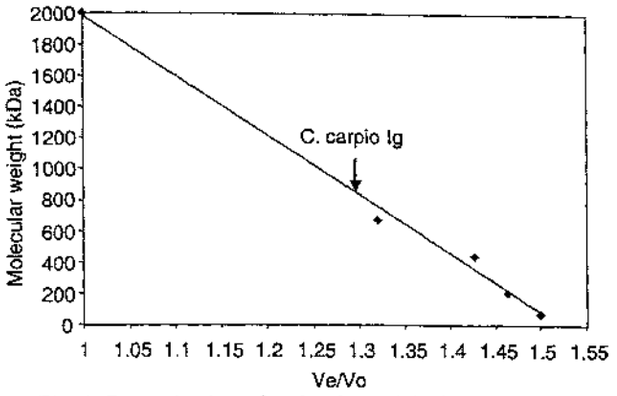

Fig. 1. Determination of molecular weight (MW) of common carp Ig from standard curve of known protein markers through gel filtration chromatography.

concentrated to 1/10th of eluted volume and stored at 4°C.

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 having a total volume (V_t) of 114.6 ml. The column was run @ 5.7 ml/h using 0.1 M PBS (pH 7.2) and fractions of 1.9 ml were collected using automatic fraction collector. The OD of elutes was measured at 280 nm and void volume (V_o) of the column bed was 53.2 ml as determined with blue dextran. The column was calibrated with protein MW markers, viz. thyroglobulin (669 kDa), apoferritin (443 kDa), amylase (200 kDa) and bovine serum albumin (66 kDa). The elution volume (V_e) for each marker was determined from the peak OD at 280 nm. Thereafter, 1 ml of concentrated common carp Ig containing 1.5 mg total protein was applied to the column. The MW of purified Ig molecule was estimated from a standard curve of the markers against their V_e divided by V_o of blue dextran (Fig. 1).

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE): Purified Ig was characterized by polyacrylamide gel electrophoresis under both reducing and non-reducing conditions to determine purity. The MW of the Ig in reduced form was determined by SDS-PAGE using Labworks Version 4.5 software. Similarly, Ig was analyzed under non-reducing conditions in a 4% gel, with samples diluted in loading buffer that did not contain ß-mercaptoethanol, to determine the different forms of Ig.

RESULTS AND DISCUSSION

The post-immunization antibody titre in the test group ranged from 1: 4 to 1: 128, whereas, the titre was <1: 2 in pre-immunization serum and control group. Three fish in the test group showed an IHA titre of 1: 128, 2 each had a titre of 1: 64 and 1: 16. The remaining three fish in the test group showed an antibody titre of 1: 4. In the present experiment, BSA was used to immunize common carp for raising anti-BSA antibodies. However, the response of fishes to BSA was quite inconsistent. Poor response to BSA, similar to that seen in the present study has also been documented earlier (Bryant *et al.* 1999, Rathore *et al.* 2006).

In affinity chromatography, a single peak was observed on elution with glycine buffer. The concentrated Ig fraction showed an IHA titre of 1: 128. Affinity chromatography using BSA-CL agarose column was quite effective in purification of serum Ig and the yield was dependent on IHA titre. The sera with IHA titre of 1: 16 or less resulted in insignificant yield of Ig. Previously, affinity chromatography using BSA as capture ligands has been applied for purification of fish Igs (Bryant *et al.* 1999, Swain *et al.* 2004). Mannan binding protein (MBP) and staphylococcal protein A (SpA) have also been used as capture ligand in affinity chromatography (Watts *et al.* 2001). However, SpA has variable capacity to bind with Igs from different fish genera (Estevez *et al.*1993) and MBP affinity column is reported to be of limited use in isolating fish Igs (Crosbie and Nowak 2002).

By gel filtration chromatography, the MW of common carp Ig was determined to be approximately 820 kDa. Variations in MW of common carp Ig have been reported earlier. Shelton and Smith (1970) and Richter *et al.* (1973) found common carp Ig to be of 740 and 608 kDa, respectively. However, Kusuda *et al.* (1987) concluded that *C. carpio* Ig had a MW of approximately 800 kDa through gel filtration chromatography. Zhong *et al.* (1999) reported a MW of 760–768 kDa based on estimation of MW of H and L chains of Ig by SDS-PAGE. Variations in MW of common carp Ig may be due to use of different techniques for estimation of MW. Besides common carp, variation in MW of Ig has been reported in *Lates calcarifer* also. Crosbie and Nowak (2002) indicated that native *L. calcarifer* Ig molecule had a MW of 929 kDa while Bryant *et al.* (1999) reported a MW of 768 kDa.

Molecular weights for most of the teleost H and L chains are generally in the range of 70–81 and 23–28 kDa, respectively. In our study, the affinity purified Ig revealed 2 bands of 73.7 kDa and 25.3 kDa by SDS-PAGE under reducing conditions, corresponding to the heavy and light chain, respectively. Similarly, Zhong *et al.* (1999) observed H and L chain of 71 and 24–26 kDa for *C. carpio* Ig. In tilapia Ig, the MW of H and L chain was 90 and 30 kDa, respectively (Rajavarthini *et al.* 2000). Swain *et al.* (2004) reported that MW of H chain variants in *Clarias batrachus* was 66 and 59 kDa while that of L chain variants was 28 and 26 kDa. Similarly, in barramundi, MW of H and L chains were 70 and 27 kDa (Bryant *et al.* 1999). As an Ig molecule in its monomeric form is composed of two heavy and two light chains, the calculated MW of this molecule would be 198 kDa. Therefore, a molecule of 820 kDa would have a tetrameric structure in accordance with observations from other teleost fish.

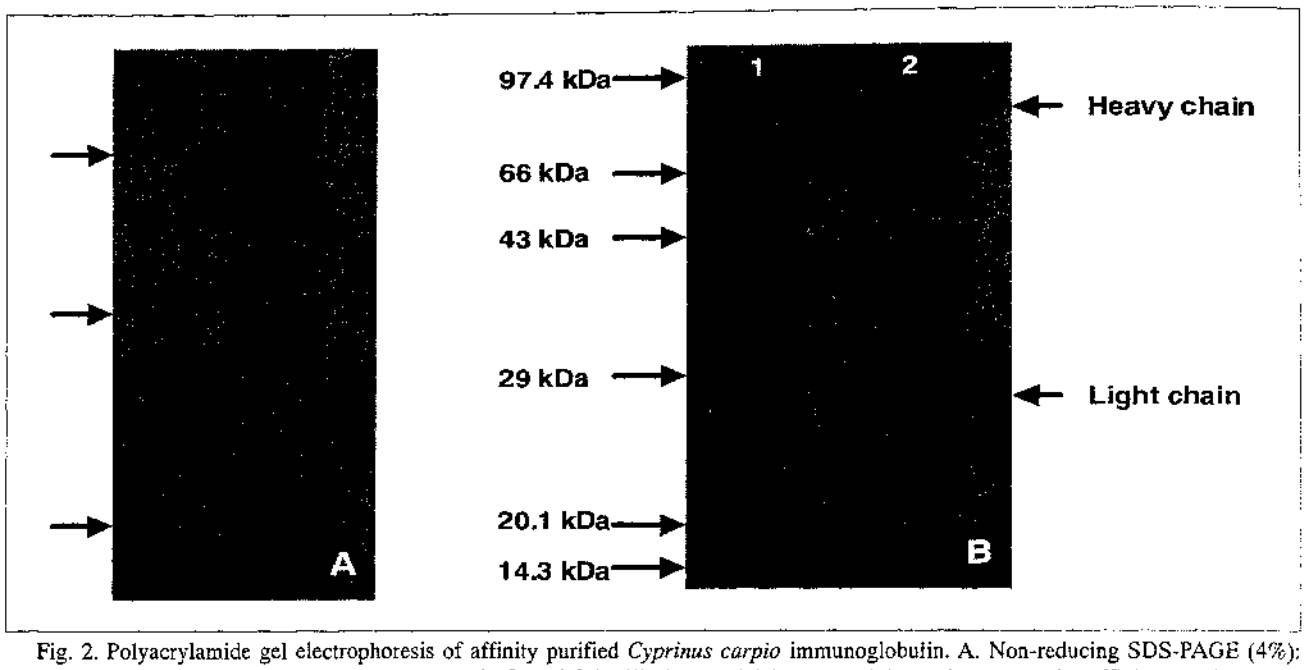

Fig. 2. Polyacrylamide gel electrophoresis of affinity purified *Cyprinus carpio* immunoglobulin. A. Non-reducing SDS-PAGE (4%): Arrows indicate likely forms of Ig; B, Denaturing SDS-PAGE (12%): Lane 1, Molecular weight markers, Lane 2– Affinity purified Ig.

Under non-reducing conditions, the affinity purified Ig was shown to have 3 bands of different molecular weights (Fig. 2). Earlier also, 3 forms of common carp Ig viz. tetramer, dimer and monomer have been recorded by Rombout *et al.* (1993) in serum as well as mucus. The native form of IgM is predominantly tetrameric in fishes (Whittington 1993, Grove *et al.* 2006). Watts *et al.* (2001) have reported that disulphide bonding between adjacent H chains is not uniform in teleost Ig and non-covalent bonding is a feature of the association of subunits to form a complete tetramer. These non-covalent bonds are disrupted by denaturation with sodium dodecyl sulphate and a portion of the tetrameric teleost IgM has been shown to split into mono-, di- and trimers depending on the species (Grove *et al.* 2006). Therefore, 3 bands observed in non-reducing SDS-PAGE in this study are most likely different forms of Ig.

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