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PURIFICATION AND CHARACTERISATION OF IMMUNOGLOBULINS FROM THE SERUM OF A CATFISH, *CLARIAS GARIEPINUS (BURCHELL, 1822)*

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ABSTRACT

Attempts have been made to characterize and purify immunoglobulins from the serum of Clarias gariepinus, which has been immunized with bovine serum albumen. Initially, the proteins in the serum were chromatographed successively by affinity chromatography column. The affinity-purified fraction was concentrated and checked in SDS-PAGE, two bands of heavy chain and two bands of light chain were observed. Since teleost immunoglobulins have been shown to belong to a single class, the extra bands in light and heavy chains in the present study might be the breakdown of immunoglobulin or some unpurified contaminants. The affinity-purified fraction was also subjected to gel filtration chromatography column.

Keywords: *Clarias gariepinus*, Immunoglobulin, Serum.

INTRODUCTION

Immunoglobulins play a major role in the humoral immune response of fish. Teleosts, like mammals possess the capability to produce specific immunoglobulins (Ig) in response to antigenic stimulation. However, they produce only one class of immunoglobulin having the characteristics in common with the IgM class in mammals. Purification and characterization of the immunoglobulin proteins have been widely investigated in freshwater teleosts (Ingram and Alexander, 1979, Clerx *et al.,* 1980, Kobayashi *et al.,* 1982, Isbell and Pauley, 1983, Ourth, 1986, Atanassov and Botev, 1988, Mohanty *et al.,* 1998). However, no data is available till date for the immunoglobulin of African catfish (Clarias gariepinus). So, attempts were made in this study to purify the serum immunoglobulins of *Clarias gariepinus,* which would facilitate further studies on structural analysis of these immunoglobulin proteins.

MATERIAL AND METHODS

A total of five number of fish, *Clarias gariepinus* (500-700 gms) were collected from the local fish market of Lucknow i.e., Telibagh and maintained in 500 1

fibre glass tanks with continuous aeration. These were fed with a good quality pelleted feed twice in a day.

The fish were immunized sub-cutaneously with BSA (100 mg/ml PBS) + FCA (100mg/ml PBS) (bovine serum albumin + Freund's complete adjuvant) at a dose rate of 0.2 ml volume per fish. These were boostered after 7 days of injection with the same dose without FCA and were bled after 14 days of booster by puncturing the caudal vein. Serum was separated by centrifugation, pooled and stored at - 20°C until use.

IHA test was carried out in standard microfibre plates by mixing 50µl of PBS (Phosphate buffered saline) with 50 µl of pooled sera in serial dilution. After this, 50µl of sensitized sheep RBC's (SSRBC's) were poured in each well. The control wells contained only PBS and SSRBC's. The plates were read after half an hour at room temperature.

For this purpose, 10ml of sheep blood was poured in 10ml alsviers solution. Then the blood was centrifuged twice with PBS at 2000 rpm for 5 minutes. Then, 2.5% of RBC's was taken in PBS. Incubated for 5-10 minutes and shook in between. Again centrifuged three times with PBS. After centrifugation, resuspended in 2.5% concentration of PBS. Now the RBC's get fixed. Pipette out 5ml of 2.5% RBC's in a glass vial and add to it 50ml of PBS + 12.5ml of BSA antigen (10mg/ml). Incubated for 5-10 minutes while shaking in between. Further centrifuged three times with PBS and resuspended in 2.5% concentration of PBS.

The purification of *Clarias gariepinus* immunoglobulins (Ig) was carried out using Pharmacia - 5ml BSA column. One ml of serum was applied to affinity chromatography column and the buffer used for its binding was phosphate buffer (pH 7.0).

The anti-BSA antibodies present in the serum bind to affinity column. The unbound material present in the column was washed by phosphate buffer pH 7.0. The fish anti-BSA antibodies were eluted from the column with 5 ml of elution buffer- citric acid (0.1M pH 3.0), at a flow rate of 1.5 ml per minute with automatic collection of 1.5 ml fractions by fraction collector. The collected fractions from affinity chromatography column were neutralized by 0.1M Tris-HCI (pH-11.0). The absorbance of the fractions was monitored at 280 mm and immunoglobulin specificity was checked by IHA test.

Repeated trials were made for 5 ml of serum and consequently 8 ml of elutant was collected keeping the flow rate of 1.5 ml/min. The fractions were checked by IHA test and concentrated to 4 ml by refrigerated vacuum condenser and stored in methanol beaker at - 20°C until use.

The desalting of the affinity purified concentrated fraction was done in commercially available Pharmacia Hitrap-5ml desalting column. Approximately 1.5ml of sample was applied to the column and the fraction was collected in a test tube keeping the flow rate in the range of 2ml/min. The buffer used for this procedure was Phosphate buffer + 0.2 M NaCl (pH 7.0). A total of 7.5 ml elutant was collected

from 4 ml of affinity purified concentrated sample.

The whole elutant was taken in a small beaker, frozen in methanol and finally condensed for about 26 hours so that the volume of sample reaches to approximately, 2.5ml. The more the condensation more will be the concentration of immunoglobulins in the solution. The sample after its concentration was stored at - 20°C.

Gel filtration was carried out on sephacryl S-300 column with Po_4 buffer + 0.2 M NaCl (pH - 7.0). The gel was packed using downward elution at a constant flow rate of 40 ml/cm^2/hr. The known molecular weight markers used were thyroglobulin, apoferritin and alcohol dehydrogenase. The known markers were loaded in the column to obtain the graph of their molecular weights thereby showing Kav. values for each marker. Before loading the markers, Blue dextran-2000 (10mg/ml) was loaded to know the Void volume (Vo) of the Column.

Therefore, Vo=52ml

Then the elution volume (Ve) of known markers was calculated.

Ve of thyroglobulin = 70ml

Ve of apoferritin = 80ml

Ve of alcohol dehydrogenase = 88ml

The total bed volume (Vt) of the gel filtration column was calculated by using the formula 22/7r2h.

Hence, bed volume (Vt) = 114ml.

After knowning the values of Vo, Ve and Vt;

Kav of thyroglobulin = Ve-Vo/Vt-Vo=70-52/114-52=0.29.

Kav of apoferritin = Ve-Vo/Vt-Vo = 80-52/114-52 = 0.45.

Kav of alcohol dehydrogenase = Ve-Vo/Vt-Vo = 88-52/114-52 = 0.58.

Then, the affinity purified, concentrated, desalted fraction (Ig) was loaded in the gel filtration column to know its elution volume (Ve).

Ve of Ig = 72ml.

The graph thus obtained was compared and finally the Kav value of the sample (fraction) was evaluated. Therefore Kav of Ig = Ve-Vo/Vt-Vo = 72-52/114-52 = 0.32.

SDS - PAGE is a discontinous buffer system in which the molecules (proteins) are degraded according to their molecular weights. Molecular weight determination and the purity of the sample recovered from gel filtration chromatography was assessed by SDS-PAGE. Electrophoresis was performed using a discontinuous buffer system at 5% stacking gel, pH 6.8 and 8% resolving gel, pH 8.8 as described by Laemmli (1970). Samples were processed with sample buffer (50 m M Tris Cl pH 6.8, 2% SDS, 10% glycerol, 0.1% bromophenol blue) with a reducing agent 100mM dithiotheritol. After electrophoresis, gel was stained with 0.25% of Coomassie Brilliant Blue R 250 (Fig. 4).

RESULTS AND DISCUSSION

A combination of affinity chromatography with gel filtration chromatography and SDS-PAGE has been successfully used by several workers to purify and characterize the serum immunoglobulins from fish species (Bradshaw et al., 1971 and Mohanty *et*

al., 1998). A similar approach was adopted in the present study to purify the serum immunoglobulins from *Clarias gariepinus.* Anti-BSA activity detected by IHA served as serological market for these immunoglobulins.

The initial pooled sera of single fish showed an IHA-titre of 1:128. Then the IHA titre of all the other fishes was taken together and the pooled sera was subjected to affinity chromatography column and revealed 2 peaks, the first peak was not accompanied by antibodies while the 2nd peak that eluted at 0.1M citric acid (pH-3.0) gave an antibody titre of 1:512. The affinity-purified fraction was loaded in desalting column and there the IHA was 1:1024. (Fig.1)

The purified and desalted sample was subjected to gel filtration chromatogrphy column. Its graph was compared with that of the known markers previously loaded in the column. The known molecular weight markers loaded were thyroglobulin, apoferritin and alcohol dehydrogenase whose Kav values were 0.29, 0.45 and 0.58, respectively. The Kav. value of the anti-BSA antibodies was found to be 0.32.

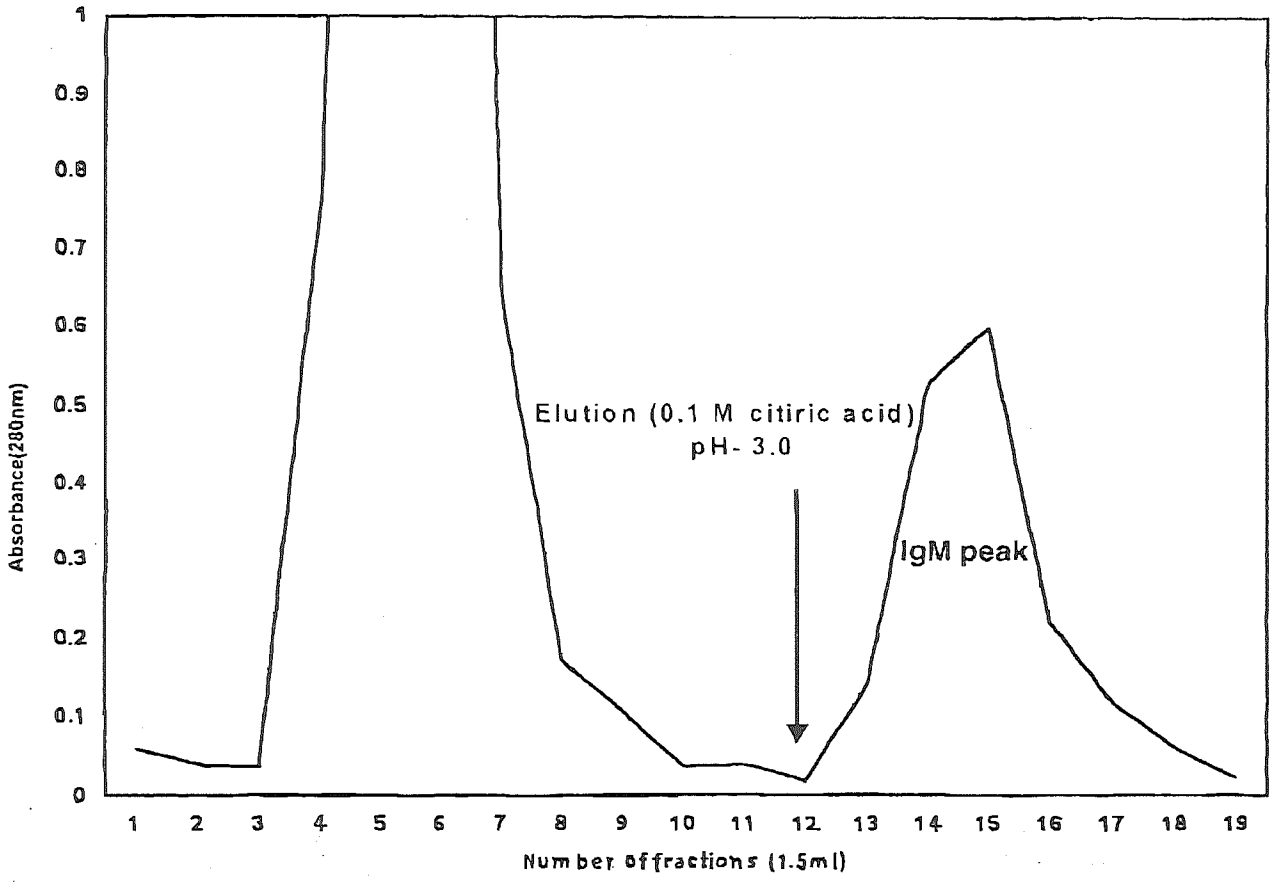

Fig.1 : Purification of IgM from hyperimmune serum of Clarias gariepinus *by BSA-agarose affinity chromatography*

Thus, plotting a graph between the Kav values and the molecular weights, the molecular weights of IgM by gel filtration chromatography obtained was approximately 620 KDa. (Figs. 2 & 3).

When 10µl of protein sample from gel filtration chromatography was analyzed by SDS-PAGE under reduced conditions, two

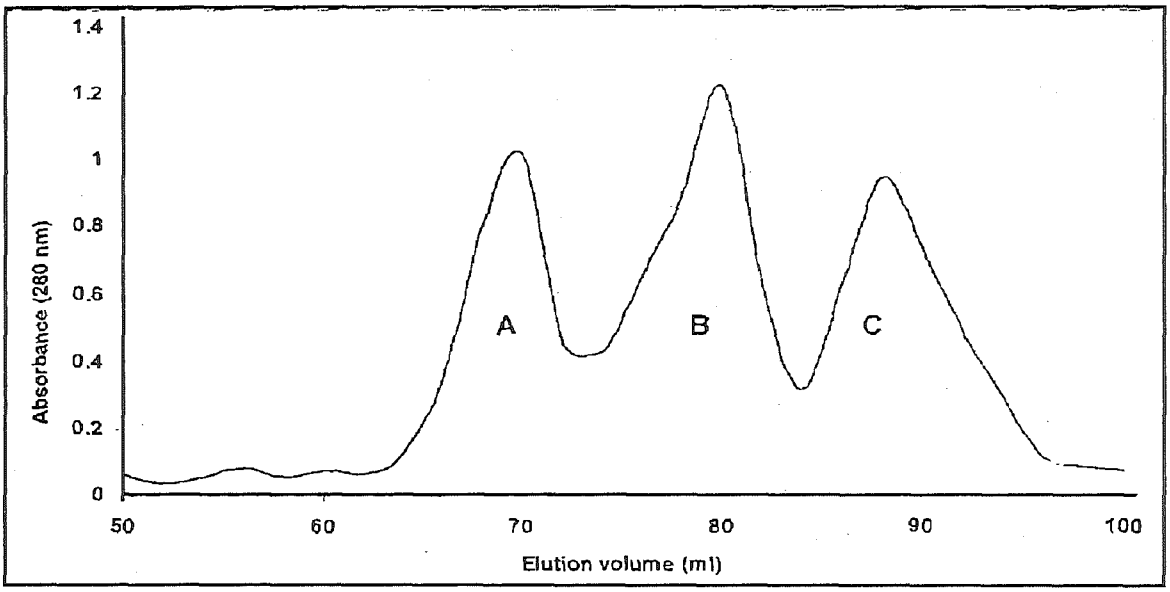

Fig. 2 : Elution profile for calibration kit proteins on Sephacryl S-300 Elution Volume (Ve) : Thyroglobulin (A)-70 ml; Apoferritin (B)-80 ml; Alcohol Dehydrogenase (C)-88 ml

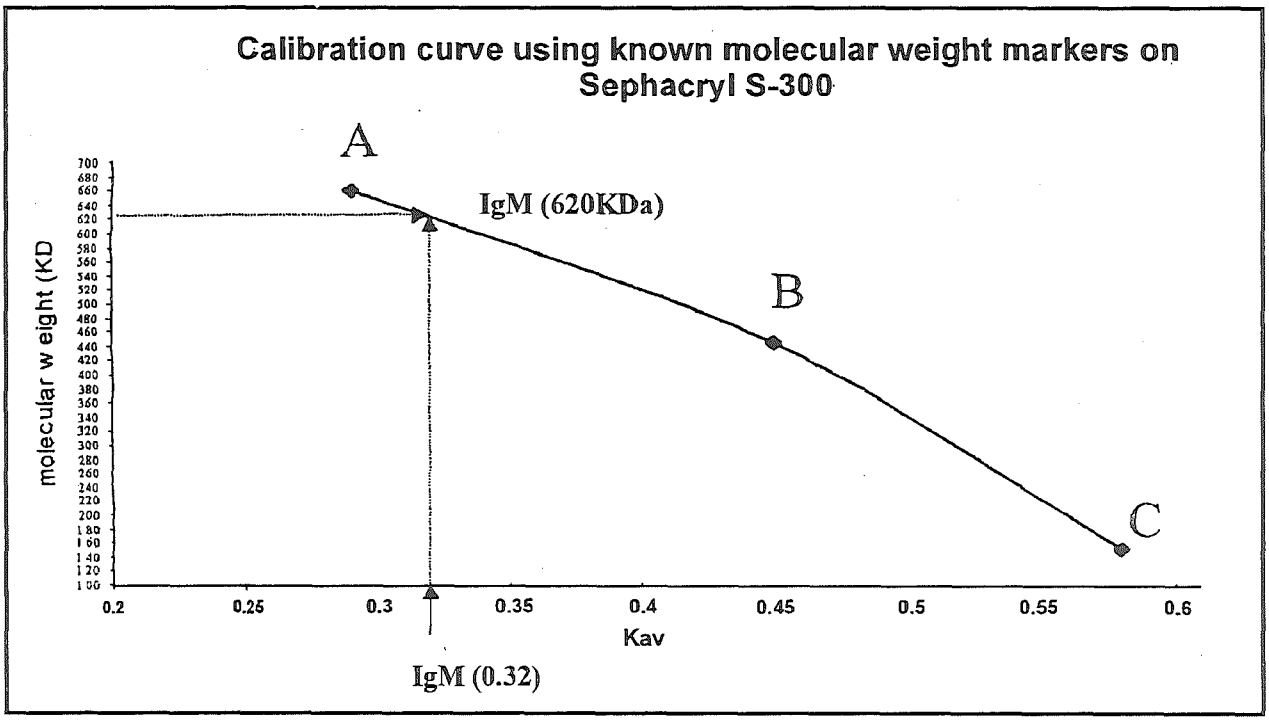

Fig. 3 :Molecular weight determination of affinity purified Clarias gariepinus *IgM by Gel Filteration chromatography.*
A-Thyroglobulin (669KDa); B- Apoferritin (443KDa); C-Alcohol Dehydrogenase (150KDa)

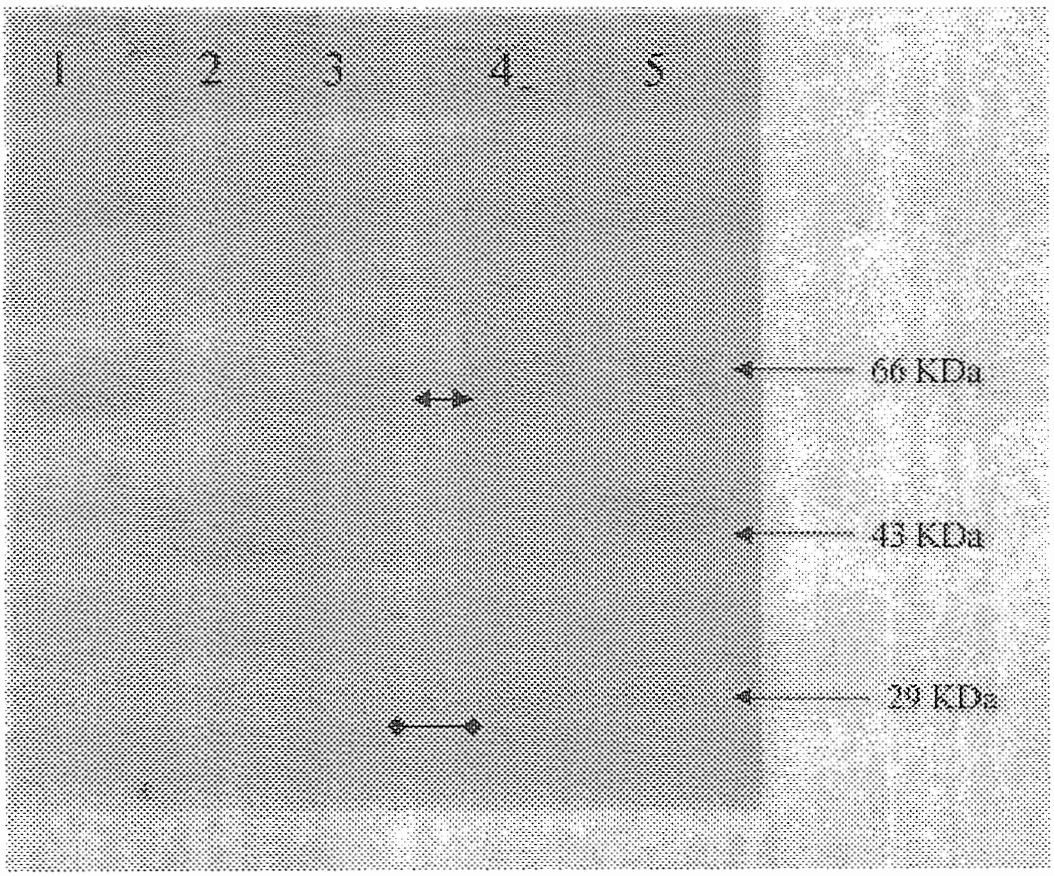

Fig.4 : SDS-PAGE of affinity purified Clarias gariepinus *IgM; Lane 1: Bovine Serum albumin; Lane 2: Ovalbumin; Lane 3 & 4 : Purified IgM (◀——▶ Heavy chain, ◆——◆ Light chain); Lane 5 : Molecular Weight Markers*

successive bands of H-chain and 2-successive bands of L-chain were observed (Fig. 4). Thus far, serum immunoglobulins from all studied fish species of order teleosti have been characterized as single tetrameric IgM like macroglobulins (Pilstrom and Bengten, 1996, Dorson 1981). So, the extra bands in H-chain and L-chain in our experiment could be due to breakdown of proteins of due to some proteolytic degradative products of Ig (Mohanty *et al.* 1998). Since, the pooled IHA positive fractions from affinity column belonged to single peak (2nd peak) but there might be some contamination by 1st peak or during collection process. Otherwise, there might be certain degree of degradation of Ig during our attempt to concentrate these proteins from the dilute fractions. Lim (1987) has also not been able to concentrate the eluted proteins from weak solutions (< 1 µg/ml), without gross denaturation. Further studies are being carried out in this aspect to eliminate the anomaly and to purify the Ig to homogeneity.

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