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SHORT COMMUNICATION

Isolation and characterization of IgM from Bengal goat blood serum

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ABSTRACT

Isolation of IgM from Bengal goat blood serum was carried out by centrifugation of the collected serum to eliminate the blood corpuscles and purity of serum was affirmed by the absence of pellets. Purified serum was obtained by ammonium sulphate precipitation. The isolated IgM obtained through dialysis, was quantified through silica gel chromatography using phosphate buffer saline (PBS) as a solvent with varied pH and obtained different fractions (namely I, II, III, IV and V). Quantification of protein was carried out by Lowry method and the molecular weight was determined by SDS-PAGE with a standard marker. The presence of IgM was confirmed by Immunodiffusion and Immuno Dot Blot. The results of the experiment suggest that Fractions I, II and III contain more stressed protein which has some similarity with ovalbumin. The resulting colour intensity obtained on performing Immuno Dot Blot using IgM as primary antibody, demonstrates that Fraction II contains maximum concentration of stressed protein.

Key Words: Blood serum, IgM, immuno dot blot, immunodiffusion, SDS-PAGE.
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INTRODUCTION

Immunoglobulin M (IgM) is a major class of immunoglobulin. It is the first antibody produced in an immune response and is later replaced by other types of antibodies. IgM is produced by B cells and forms polymers where multiple immunoglobulins are covalently linked together with disulfide bonds, mostly as a pentamer but sometimes also as a hexamer (Cambier *et al.*, 1974.). IgM has a molecular mass of approximately 900 kDa (in its pentamer form). Because each monomer has two antigen binding sites, a pentameric IgM has 10 binding sites. Typically, however, IgM cannot bind 10 antigens at the same time because the large size of most antigens hinders binding to nearby sites.

The J chain is found in pentameric IgM but not in the hexameric form. At present, it is still uncertain whether a J chain-containing pentamer contains one or more than one J chain. Because IgM is a large molecule, it cannot diffuse well, and is found in the interstitium only in very low quantities. IgM is primarily found in serum; however, because of the J chain, it is also important as a secretory immunoglobulin. Due to its polymeric nature, IgM possesses high avidity, and is particularly effective at complement activation. By itself, IgM is an ineffective opsonin; however it contributes greatly to opsonization by activating complement and causing C3b to bind to the antigen (Ohta *et al.*, 1990). IgM antibodies appear early in the course of an infection and usually reappear, to a lesser extent, after further exposure. These two biological properties of IgM make it useful in the diagnosis of infectious diseases. Demonstrating IgM antibodies in a patient's serum indicates recent infection, or in a neonate's serum indicates intrauterine infection (e.g. congenital rubella). IgM in normal serum is often found to bind to specific antigens, even in the absence of prior immunization. For this reason IgM has sometimes been called a "natural antibody". This phenomenon is probably due to the high avidity of IgM that allow it to bind detectably even to weakly cross-reacting antigens that are naturally occurring. For example the IgM antibodies that bind to the red blood cell A and B antigens might be formed in early life as a result of exposure to A- and B-like substances that are present on bacteria or perhaps also on plant materials (Richman *et al.*, 1982).

IgM antibodies are mainly responsible for the clumping (agglutination) of red blood cells if the recipient of a blood transfusion receives blood that is not compatible with their blood type. IgM is more sensitive to denaturation by 2-mercaptoethanol than IgG. This technique was historically used to distinguish between these isotypes before specific anti-IgG and anti-IgM secondary antibodies for immunoassays became commercially available. Serum samples would be tested for reactivity with an antigen before or after 2-mercaptoethanol treatment to determine whether the activity was due to IgM or IgG (Paster *et al.*, 1989).

MATERIALS AND METHODS

Isolation and characterization of IgM antibodies from Bengal goat serum:

The blood sample of Bengal Goat was collected and was kept in room temperature (upto 37°C) for clotting. The collected serum was flocked together in sterile vials and stored at -20°C.Latera pool of serum was prepared and centrifuged at 5000 rpm for 5 minutes to remove residual RBC and the collected supernatant was stored at -20°C.

Ammonium sulfate precipitation:

Immunoglobulin was saturated to 50% by adding 3.13g ammonium sulphate to 10ml serum and constantly stirred for 2 h. The serum was then centrifuged at 10000 rpm for 15 mins and the pellet was dissolved in phosphate buffer saline (PBS, pH 7.0). After addition of PBS, again and kept for 2 h, it was centrifuged in the same way and the collected pellet was dissolved it in PBS.

Dialysis:

The ammonium sulphate precipitated IgM was taken in a dialysis bag and dialysed against several changes of PBS for 2 days at 25°C to obtain crude IgM and ammonium sulphate.

Silica gel column chromatography:

The distilled water and the silica powder were poured in the silica gel tube up to aforesaid length taking care to avoid the occurrence of any crack in the silica gel. Then the dialyzed protein sample was poured in the silica gel column (Abdullah *et al.*, 1985).After pouring the dialyzed protein sample, the first fraction obtained is called the crude protein. PBS (pH 6.5) is then deluged in the silica gel column. The collected elute was named as fraction I. Again PBS (pH 6.8) is poured into the silica gel column to collect fraction II. PBS (pH 7) is poured in the silica gel column to collect fraction III. Again PBS (pH 7.2) is poured in the silica gel column to obtain fraction IV. Finally, PBS (pH 7.5) was deluged in the silica gel column. The fifth and the final elute, named as fraction V was obtained (Nethery *et al.*, 1990).

SDS gel electrophoresis:

Sodium Dodecyl Sulphate based gel electrophoresis was done. (Abdullah *et al.*, 1985)

Immunodiffusion assay:

1% Agarose was prepared in 0.9% normal saline (NaCl).After the solidification of the gel, 5 holes in 3 petri-plates and 6 holes in 2 petri-plates in the Agarose plate was made. In the centre of the 3 petri-plates, antibodies (fraction 1, fraction 2, and fraction 3) were added and in the remaining holes antigens (BSA and egg ovalblumin) and then primary Ab and secondary Ab were added. In another 2 sets, antigens (BSA and egg ovalblumin) were added in the centre hole and antibodies in the remaining holes (fraction 1, fraction 2, fraction 3, primary Ab and secondary Ab).The petri-plates were then incubated for overnight and observed.

Immunoblot or dot blot:

For the immuneblotting, the sample was spotted on the nitrocellulose membrane and it was immersed in the blocking buffer for few minutes, as the blocking buffer avoids the unnecessary binding. Solution was discarded and washing buffer was added and kept for 10mins and then it was washed thrice with the washing buffer. Primary antibody was added and incubated for 1hour with washing buffer. After that, secondary antibody was added and kept for 1 h. It was then dipped in reaction buffer and kept for 1h for color blue colour development (Paster *et al.*, 1989).

RESULTS AND DISCUSSION

Lowry estimation:

Standard curve was plotted against the different concentrations of bovine serum albumin (BSA) taking X axis as concentration in µg/ml and Y axis as OD values taken at 720 nm (figure 1). Then the observations were taken particularly at 720 nm in Lowry estimation, as Follin Ciocalteu (F.C.) reagent after reacting with the protein has the minimum absorbance at 720 nm. Then it was plotted the OD values of the protein samples viz. crude fraction I, II, III, IV, V (Abdullah *et al.*, 1985) on the standard curve plot, and obtained the unknown concentrations of the aforesaid samples. Now as the protein samples were concentrated so we diluted it 100 times with distilled water, thus multiplied the values of the concentrations by a factor 100 thus the final concentration values of the aforesaid protein samples are presented in table 1.

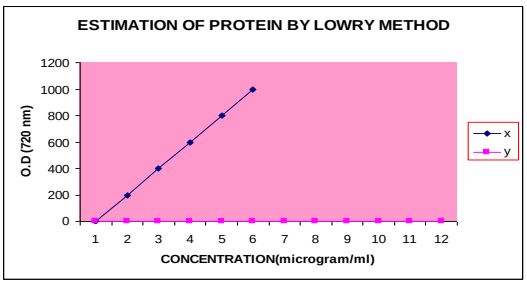

Figure 1. Lowry estimation standard curve

Table 1. Concentration of protein sample

Fraction	Calculation	Concentration
Fraction I	290*100	29 mg/ml
Fraction II	320*100	32 mg/ml
Fraction III	50*100	5 mg/ml
Fraction IV	0.00*100	0 mg/ml
Fraction V	0.00* 100	0 mg/ml

SDS-PAGE gel electrophoresis:

After SDS-PAGE running, the protein bands were obtained only in four lanes which infer that there were no proteins in fraction IV, fraction V and crude protein samples.

Table 2. Example of standard low molecular weight protein markers

PROTEIN	MOLECULAR WEIGHT(DALTONS)
Ovalbumin(coloured)	43500
Carbonic anhydrase	29000
Trypsin inhibitor	20100
Lysozyme	14300
Insulin(coloured)	3500

Table 3. Example of standard medium molecular weight protein markers

PROTEIN	MOLECULAR WEIGHT(KILODALTONS)
Phospholipase b	98
BSA	67
Ovalbumin	44
Glutathion S transferase	29

Lysozyme	16

Molecular weight determination:

To determine the size or molecular weight of unknown proteins, a series of standards (proteins of known molecular weight) treated similarly was electrophoresed along with the unknown proteins in adjacent lanes of the gel (figure 3). By measuring the distance travelled by each protein (in mm) from the loading well, it can be determined the relative mobility (R_f) of each protein. This value can then be plotted on semi-log paper to generate a calibration curve (figure 2) against which the molecular weight of unknown size proteins can be determined.

Table 4. Determination of molecular weight of bands on SDS-PAGE gel

Log of MW	**Rf values**	**Antilog MW (actual MW)**
1.99	0.160	97.94 (standard protein marker)
1.20	0.732	15.99 (standard protein marker)
1.81	0.290	64.50 (heavy chain, fraction I)
1.34	0.625	21.88 (light chain, fraction I)
1.67	0.393	46.77 (between heavy chain and light chain, fraction I)
1.54	0.482	34.67 (between heavy chain and light chain fraction II)
1.81	0.29	64.50 (heavy chain, fraction II)
1.34	0.625	21.88 (light chain fraction II)
1.72	0.357	52.48 (light chain fraction III)
1.82	0.286	66.07 (heavy chain fraction III)

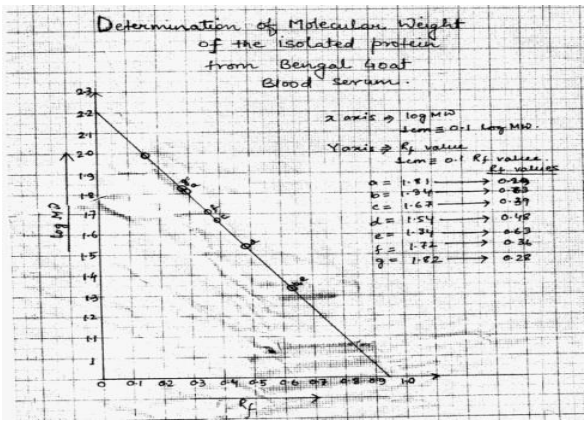

Figure 2. Determination of molecular weight of the isolated proteins using log table

Figure 3. SDS-PAGE in gel doc

Immunodiffusion:

Precipitation occurs with most antigens because the antigen is multivalent. Antibodies have at least two antigen binding sites (and in the case of IgM there is a 10 antigen binding sites), thus large aggregates or gel-like lattices of antigen and antibody are formed. Experimentally, an increasing amount of antigen is added to a constant amount of antibody in solution, initially at low antigen concentration, the entire antigen is contained in the precipitate. This is called the antibody-excess zone (i.e. prozone phenomenon). As more antigens are added, the amount protein precipitated increases until the antigen/antibody molecules are at an optimal ratio. This is known as the zone of equivalence or equivalence point. When the amount of antigen in solution exceeds the amount of antibody, the amount of precipitation will decrease. This is known as the antigen excess zone. In an antigen –antibody reaction, where BSA is the antigen used and fraction I, II, III at pH 6.5, 6.8, 7 are the antibodies. The bars appeared at the equivalent zones. Fig 4 shows the antibody-antigen reaction, where fraction I at pH 6.5 was the antibody and egg albumin and BSA were the antigens .The bars appeared against both the Ags at the equivalent zones. Another reaction contains the egg albumin that was the Ag and The Fraction I, II, III at pH 6.5, 6.8, 7 are the antibodies. The bars appeared at the equivalent zones. Fig 5 shows the antibody- antigen reaction, where fraction II at pH 6.8 was the antibody and egg albumin and BSA were the antigens. The bars appeared against both the Ags at the equivalent zones. In another antibody- antigen reaction, where fraction III at pH 7 was the antibody and egg albumin and BSA were the antigens. The bars appeared against both the Ags at the equivalent zones (Paster *et al.*, 1989).

Immunoblot or dot blot:

Here BSA, Fraction I (pH 6.5), Fraction II (pH 6.8) and Fraction III (pH 7) were used for making dots on Nitrocellulose membrane. Sigma IgM was used as the primary antibody against BSA, Fraction I, Fraction II, and Fraction III respectively. As the colour developed on the Nitrocellulose membrane, only Fraction I, Fraction II, Fraction III were spotted (figure 6). So it is obvious that the Ab-Ag complex formed in those parts and so the enzyme linked secondary Ab (goat anti rabbit Ab) binds to the Ab-Ag complex and colour formation occurred following the addition of NBT+BCIP. So it is confirmed that Fraction I, II, III contained some stressed protein which has some similarity with ovalbumin so that the

epitope of the Sigma IgM binds with the stressed protein in the 3 Fractions. The colour intensity was greater in Fraction II, than Fraction I and Fraction III. The colour intensity shows the concentration of the stressed proteins in the Fractions. Thus the Fraction II contains higher concentration of the stressed protein (an impurity in the isolated IgM from the Bengal goat blood serum).

Table 5. Lowry estimation of some known sample protein and the collected fraction

Sl. no.	**Stock Protein(µl)**	**Dist. water (µl)**	**Conc (µg/ml)**	**Reagent D (ml)**	WAIT FOR 30 MIN	**FC reagent (ml)**	WAIT FOR 10 MIN	**O.D (720nm)**
1	Blank	1000	0	4		0.4		0.00
2	200	800	200	4		0.4		0..31
3	400	600	400	4		0.4		0.84
4	600	400	600	4		0.4		0.92
5	800	200	800	4		0.4		1.22
6	1000	0	1000	4		0.4		1.53
7	Crude protein			4		0.4		0.00
8	Fraction I			4		0.4		0.44
9	Fraction II			4		0.4		0.49
10	Fraction III			4		0.4		0.08
11	Fraction IV			4		0.4		0.00
12	Fraction V			4		0.4		0.00

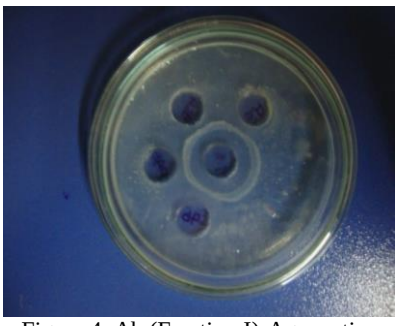

Figure 4. Ab (Fraction I)-Ag reaction

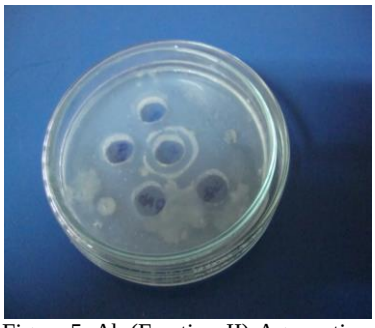

Figure 5. Ab (Fraction-II)-Ag reaction

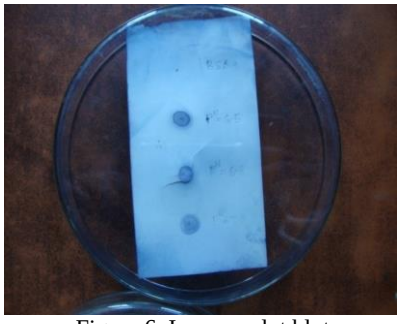

Figure 6. Immuno dot blot

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