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Isolation and Characterization of IgG from Human Serum, a Comparative Study on Different IgG Purification Methods

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***Abstract*—In the present investigation IgG from serum sample was purified by Ammonium sulfate ($(NH_4)_2SO_4$) fractionation, Protein A and Thiophilic affinity chromatography in order to select a method that can be used for further studies. Among the three methods employed, Protein A affinity chromatography was the best method both in terms of purity and protein concentration. Presence of IgG in $(NH_4)_2SO_4$) fractionation, Protein A and Thiophilic affinity chromatography fraction were confirmed by direct ELISA using goat anti human IgG-HRP conjugate. The molecular weights of $(NH_4)_2SO_4$) fractionated IgG, Protein A and Thiophilic affinity chromatography purified IgG were found to be 28 and 52 kDa respectively.**

***Index Terms*— IgG, $(NH_4)_2SO_4$) fractionation, Protein A affinity chromatography, Thiophilic affinity chromatography, ELISA, SDS-PAGE**

I. Introduction

Immunoglobulins also called as antibodies are glycoproteins that are produced by B cells in response to an antigenic challenge. Once there are produced, they either neutralize the antigen or mark them for elimination (e.g., Opsonization or complement activation). In contrast, membrane bound immunoglobulins (IgM monomer and IgD) confer antigen specificity on B cells. There are five different classes of immunoglobulins, namely, IgG, IgA, IgM, IgE and IgD. Each of these differ in their, molecular size, structure and function. . Most abundant immunoglobulin in serum are in the IgG class (constitute about 80% of total immunoglobulins in serum). IgG is present in blood plasma and tissue fluids. This class of immunoglobulin plays important functions like (i) opsonization, (ii) toxin neutralization. (iii) Complement activation by the classical pathway. It is the only immunoglobulin molecule that has the ability to cross the placenta and hence provides natural immunity in utero and to the neonate at birth (Lansing M. Prescott., 2002).

Immunoglobulins have proven to be very useful in diagnostic (as an in vitro diagnostic reagent) e.g. Secondary antibody conjugates are used in the detection of various diseases including HIV (ELISA), western blotting, blood typing and in clinical medicine. In addition, immunoglobulins are also used for passive immunization, treatment of immunodeficiency disorders and protection against the anticipated exposure to infectious agents against which one does not have immunity (Goldsby RA et al., 2003). Since immunoglobulins have several important applications, the present investigation was carried out to compare different purification methods for purification of human IgG (Ammonium sulphate fractionation, Protein A affinity chromatography and Thiophilic Affinity Column).

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II. Materials and Methods

A. Serum Collection

Human blood sample was obtained from a healthy donor.

B. Purification of IgG

Three different methods were selected for isolation of IgG in order to select a suitable method for future use. These methods include, (NH4)2SO4 fractionation, Protein A affinity chromatography and Thiophilic affinity chromatography. Purification of human serum immunoglobulin G (IgG) fraction by $(NH_4)_2SO_4$) precipitation (micro-method) was carried according to the method of Javois, L (Javois, L., 1994). Most commercially available affinity chromatography resins are protein A based (Hahn et al., 2003, Hahn et al., 2005, Hahn et al., 2006). IgG from serum samples were purified by the method described in Protein A Agarose affinity chromatography and Thiophilic affinity chromatography kit, Bangalore Genie Pvt Ltd Bangalore India. All experiments were carried in duplicates.

C. Estimation of protein

Protein concentration in serum sample,$(NH_4)_2SO_4$ fractionated IgG, Protein A affinity chromatography and Thiophilic affinity chromatography purified IgG before and after dialysis were determined in triplicate by the method of Lowry et al. (1951) using bovine serum albumin as a standard.

D. Enzyme linked immunosorbent assay (ELISA)

Serum sample, $(NH_4)_2SO_4$ fractionated IgG, Protein A and Thiophilic affinity chromatography purified IgG were analyzed by ELISA essentially as described by Ramesh Kumar K, et al (2014)

E. SDS-PAGE analysis

SDS-PAGE was essentially carried out as described by Laemmli (1970). Sample (serum sample, $(NH_4)_2SO_4$) fractioned IgG, Protein A and Thiophilic affinity chromatography purified IgG to be analyzed on SDS-PAGE were mixed with sample buffer in a 1:1 ratio. After mixing, the samples were boiled for 10 min. 25-30 µg of the protein samples were loaded in the wells and electrophoresis was carried out at 100V along with standard protein markers. After completion, the gel was subjected to Coomassie Brilliant Blue R 250 staining.

III. Results and Discussion

IgG from human serum sample was fractionated by (NH4)2SO4 precipitation. After fractionation, immunoglobulins were dialyzed overnight against 0.05M phosphate buffer pH 8.0 to remove $(NH_4)_2SO_4$. The protein content in the serum sample and $(NH_4)_2SO_4$ fractionated IgG before and after dialysis was estimated and results are summarized in the Table I. The concentration of protein in serum sample, $(NH_4)_2SO_4$ fractionated IgG before and after dialysis were found out to be 141 mg/mL in serum sample, 29.25 mg/mL and 12.13 mg/mL respectively. After dialysis, there was a reduction in the concentration of IgG by about 50% (Table 1 and Fig 1).

Table 1: Protein content in serum sample and $(NH_4)_2SO_4$ fractionated IgG before and after dialysis

Serum sample	Before dialysis	After dialysis
141 mg/mL	29.25 mg/mL	12.13 mg/mL

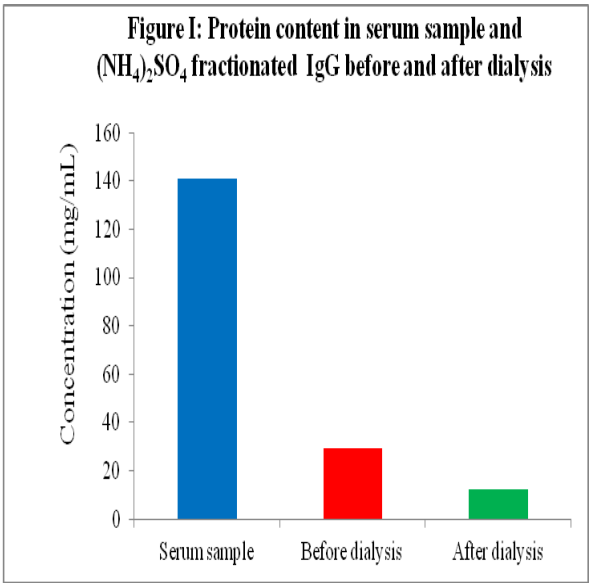

Fig 1: Protein content in serum sample and $(NH_4)_2SO_4$ fractionated IgG before and after dialysis

Purification of IgG by Protein A affinity chromatography

Staphylococcal protein A is found in bacterial cell wall of Staphylococcus aureus. It binds to most mammalian IgG and can therefore, be used for detecting or purifying such antibodies (i.e., for purifying Monoclonal and polyclonal antibodies)

The absorbance values and elution profile for Protein A affinity purified IgG are given in Table II and Fig 2. Fractions showing absorbance values greater than 0.2 were pooled and the sample were subjected to dialysis. Protein concentration in the pooled protein A fraction before and after dialysis were estimated

The protein concentration in Protein A fractionated IgG was found out to be 14 mg/mL before dialysis and 6.58 mg/mL after dialysis (Table III & Fig 3). The protein concentration in the pooled protein A fraction was almost 50% lower than that observed for $(NH_4)_2SO_4$) fractionated proteins. IgG from serum sample eluted as a single peak (Fig 2).

Table II: Absorbance values for Protein A affinity chromatography eluted fractions

Fraction number	A_{280}
1	0.5
2	1.5
3	2.0
4	1.6
5	0.6

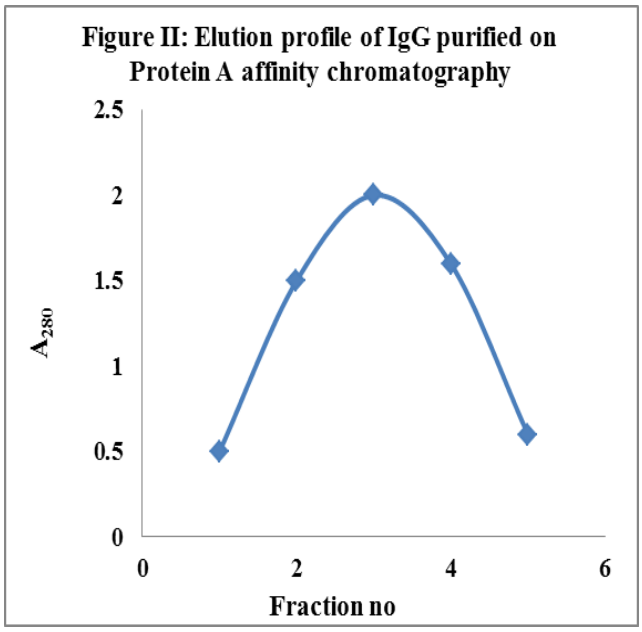

Fig 2: Elution profile of IgG Purified on Protein A affinity chromatography

Table III: Protein content in serum sample and Protein A purified IgG before and after dialysis

Serum sample	Before dialysis	After dialysis
141 mg/ml	14 mg/ml	6.58 mg/ml

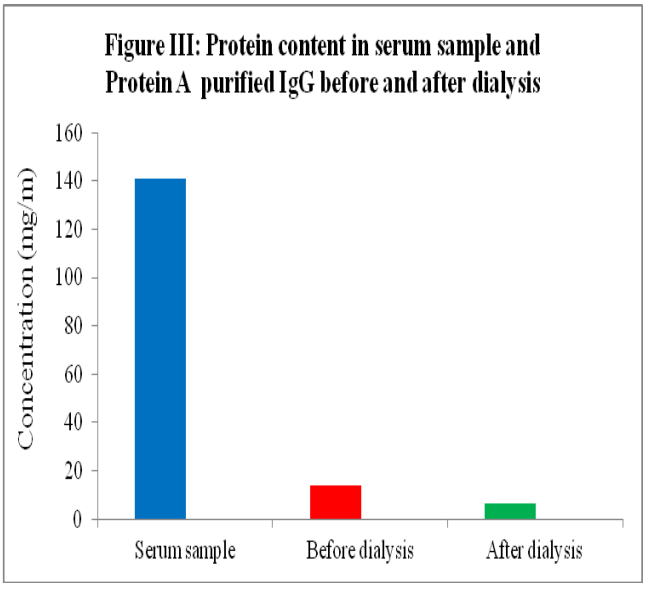

Fig 3: Protein content in serum sample and Protein A purified IgG before and after dialysis

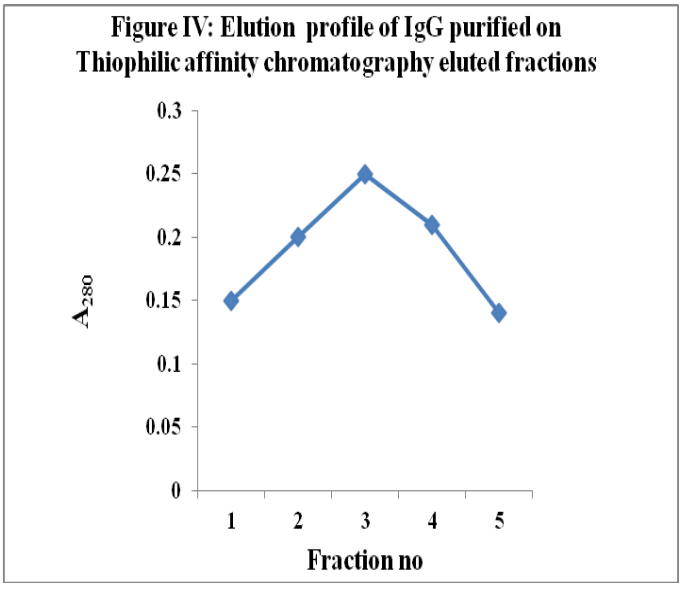

Fig 4: Elution profile of IgG purified on Thiophilic affinity chromatography eluted fractions

A. Purification of IgG by Thiophilic affinity chromatography

Thiophilic adsorption chromatography (TAC) developed by Porath et al. (1984) is a group-specific, salt-dependent purification technique with distinct adsorption affinity towards immunoglobulins and α2-macroglobulins.

The absorbance values and elution profile for Thiophilic affinity purified IgG are given in Table IV and Fig IV. Fractions showing absorbance values greater than 0.2 were pooled and the sample were subjected to dialysis. Protein concentration in the pooled protein A fraction before and after dialysis were estimated.

The protein concentration in Thiophilic affinity purified IgG was found out to be 8 mg/mL before dialysis and 4 mg/mL after dialysis. The protein concentration in the pooled Thiophilic affinity purified IgG was almost 50% lower than that observed for $(NH_4)_2SO_4$ fractionated proteins. IgG from serum sample eluted as a single peak (Fig 4).

Table IV: Absorbance values for Thiophilic affinity chromatography eluted fractions

Fraction number	A_{280}
1	0.15
2	0.20
3	0.25
4	0.21
5	0.14

Protein content in serum sample and Thiophilic purified IgG before and after dialysis is given in Table V and Fig 5.

Table V: Protein content in serum sample and Thiophilic purified IgG before and after dialysis

Serum sample	Before dialysis	After dialysis
141mg/ml	8mg/ml	4mg/ml

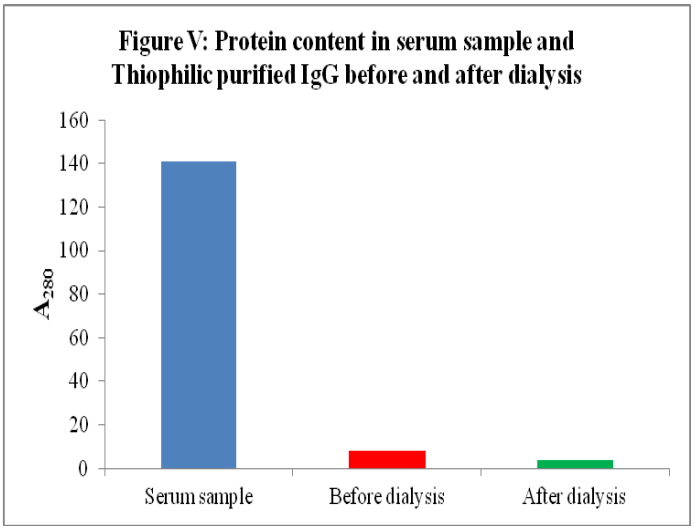

Fig 5: Protein content in serum sample and Thiophilic purified IgG before and after dialysis

B. Enzyme linked immunosorbent assay (ELISA)

Pure human IgG, Protein A purified and Thiophilic affinity purified IgG, serum sample and $(NH_4)_2SO_4$ fractioned IgG, were analysed by direct ELISA. It was observed that goat anti human IgG HRP conjugate reacted specifically with wells that were coated with pure human IgG, Protein A purified IgG, Thiophilic purified IgG, serum sample and $(NH_4)_2SO_4$ fractioned IgG (Fig 6) indicating the presence of IgG in these samples.Goat anti human IgG-HRP conjugate failed to react with wells that were coated with rabbit IgG (Fig 6). These results not only confirm for the presence of IgG but also

indicated the specificities of goat anti human IgG-HRP conjugate for human IgG (Fig 6). Pure IgG (positive) BSA and Egg albumin (Negative) were used as controls.

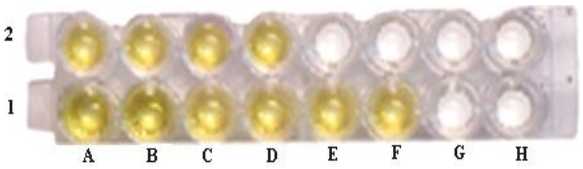

Fig 6: Enzyme-Linked Immunosorbent Assay

A1B1: Pure IgG; C1D1: Protein A purified IgG;
E1F1: Thiophilic purified IgG
G1H1: Rabbit IgG;
A2B2: Serum sample;
C2D2: Ammonium sulphate fraction
E2F2: BSA; G2H2 Egg albumin.

C. SDS-PAGE: Determination of purity and molecular weight of purified IgG

Proteins from Protein A purified IgG, Thiophilic affinity purified IgG and $(NH_4)_2SO_4$ fractioned IgG were analyzed on a 12% separating gel and 6% stacking gel. Protein samples were electrophoresed along with standard protein markers. Coomassie Brilliant Blue R-250 stained gel is shown in Fig 7. As shown in Figure 7 several proteins with molecular weight ranging between 15 to 88 kDa were found in the serum sample (Fig 7: lane 4). Several proteins with molecular weight ranging from 15 to 70 kDa were also found in the case of ammonium sulphate fractionated immunoglobulins but the concentrations were lower as compared to Thiophilic affinity purified IgG (Figs 7: lane 7). In case of Thiophilic affinity purified IgG three prominent bands corresponding to molecular weights ranging between 66 to 26 kDa were observed (Fig 7 : lane 5). In contrast, Protein A purified IgG showed to bands corresponding to molecular weights 25 and 50 kDa (Fig 7 : lane 6).

The molecular weights of Thiophilic affinity purified IgG, Protein A affinity purified IgG and $(NH_4)_2SO_4$ fractionated IgG were found out to 28 and 52 kDa respectively (Fig 7). The molecular weight of proteins 28 and 52 kDa corresponds to light chain (25 kDa) and heavy chain (52 kDa) of immunoglobulin molecules. .

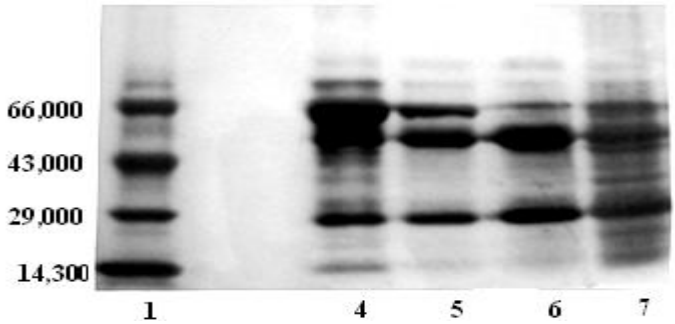

Fig 7: SDS-PAGE Analysis (Determination and purity of IgG)

Lane-1: Standard protein molecular weight markers
4: Serum sample
5: Immunoglobulins purified by Thiophilic column
6: IgG purified by protein A column
7: Ammonium sulphate fractionated immunoglobulins

IV. Conclusion

Among the three methods employed for IgG purification, the choice of purification method would be Protein A affinity chromatography because IgG purified by this method was fairly pure. In addition the concentration of protein was also higher in the case of protein A purified IgG. Although the protein concentration was higher in the case of $(NH_4)_2SO_4$ fractionated IgG this does not represent IgG only. As evident from the SDS-PAGE analysis several proteins were present in $(NH_4)_2SO_4$ fractionated IgG, this may account for the higher protein content in this fraction.

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