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ISOLATION OF IMMUNOGLOBULIN G FROM CANINE SERUM USING PROTEIN A AFFINITY CHROMATOSRAPHY

BY

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SUMMARY

Affinity chromatography utilizing protein A covalently bound to sepharose beads was applied to isoalte pure immunoglobulin G from canine serum. Immunoglobulin M was eliminated from the sample by a single passage of serum on DEAE-cellulose prior to chromatography on protein A-sepharose. The technique is simple, inexpensive and does not require complicated equipments. The preparation proved to be highly purified when tested by immunoelectrophoresis against anti-dog whole serum. The fraction included two distinct subclasses of IgG of different electrophoretic mobility. Monospecific antiserum to purified IgG was prepared for the use in determining IgG levels in dog sera.

INTRODUCTION

Protein A is a major cell wall component of most strains of *Staphylococcus aureus*. It was characterized and studied by **Jensen (1959)**. The molecule is a single polypeptide chain of molecular weight 42.000 and contains little or no carbohydrate. It is covalently linked to the peptidoglycan portion of the cell wall and is secreted during the exponential growth phase of the organism **(Goding, 1978)**.

The major feature of protein A is its extraordinary affinity for immunoglobulins, in particular IgG. This pseudo-immune reaction was detected by an apparently high incidence of natural antibodies to Staph. *aureus* in normal human sera. Subsequent studies have revealed that Protein A reacts with the Fc portion of the H (heavy) chain of gamma-G globulin. One molecule of protein A bind two molecules of IgG (Forsgren and Sjoquist, 1966).

Furthermore, protein A was found to bind diferent immunoglobulin classes of several mammalian species including mice, rabbits, horses, pigs, cats, goats, sheep, and bovines (Goding, 1978; Goudswaard et al., 1978). Regarding dog, protein A can bind both serum IgG and IgM (Goudswaard et al., 1987). This useful property has been widely exploited as an immunologicai tool. Affinity chromatography on protein A covalently bound to sepharose beads has been utilized to isolate pure immunoglobulin isotypes from mammalian sera (Warr and Hart, 1979).

In this research we have utilized affinity chromatography on protein A to isoalte canine serum IgG through a series of simple techniques. This rapid method yielded adequate quantities of highly purified immunoglobulin G suitable for preparation of monospecific anti-IgG antiserum.

MATERIALS AND METHODS

Animals: Blood samples were collected from five

adult Alsatian dogs. Animals were apparently healthy and had no history of recent vaccination.

Elimination of Immunoglobulin M: Immunoglobulin G-rich peak of DEAE-cellulose was isolated according to the technique of Elsaid (1993). Pooled canine serum (4 ml. 8 mg protein/ml) was diluted in 0.01 M phosphate buffer pH 6.3 (1:4) with slow stirring overnight at 4°C. The sample was centrifuged at 6000 rpm for 30 min., then concentrated usimg Centricon-10 units (Amicon, USA). Five ml sample was applied on Diethyl Amino Ethyl (DEAE)-cellulose (Microgranular D 52, Whatman, UK) packed in 1.5 x 30 cm column (Biorad, USA). DEAE-cellulose was equilibrated with 0.01 M phosphate buffer pH 6.3. Elution was done at room temperature under hydrostatic pressure with flow rate of 2 ml/min. Five ml fractions were collected manually. Protein content of the eluted fractions was monitored by UV spectrophotometer at 280 nm. The IgG-rich wash peak was eluted using 0.01 M phosphate buffer.

Protein A Affinity Chromatography: Affinity chromatography was performed using protein A covalently bound to Sepharose, using modification of the technigue of Warr and Hart (1979). Protein A Sepharose CL-4B (Pharmacia, Sweden) was rehydrated, washed and equilibrated in 0.1 M phosphate buffer pH 7.5 according to the manufacturer instructions. The bed material (8.6 ml) was packed in 1x15 cm chromatographic column (Biorad, USA). Immunoglobulin G-rich wash peak of DEAE-cellulose was dialyzed against 0.05 M phosphate buffer pH 7.5. Ten ml of the fraction (2.6 mg protein / ml) were applied to oversaturate the column. Then the column was incubated at 34°C for 1 hour. The column was allowed to return to room temperature, then washed (ten times the bed volume) to remove the unbound proteins using 0.1 M phosphate buffer pH 7.5 Elution of bound IgG was done using 0.1 M glycine-Hcl pH 2.8 at room temperature. Two ml fractions were collected manually. Protein content of the eluted fractions was monitored by UV spectrophotometer at 280 nm.

Antisera: Polyvalent antiserum to dog whole serum. rabbit anti-dog IgG, anti-dog IgM and anti-dog IgA were obtained from Sigma (USA).

Immunodiffusion(ID) and Immunoelectrophoresis (IEF): The protein fractions were identified by immunodiffusion in 1% agar (Difco, USA) according to the method of Garvey et al., (1977). Electrophoresis was performed on ACI universal agarose films and processed in ACI-Corning electrophoresis chamber (Corning, USA) according to the instruction of the manufacturer.

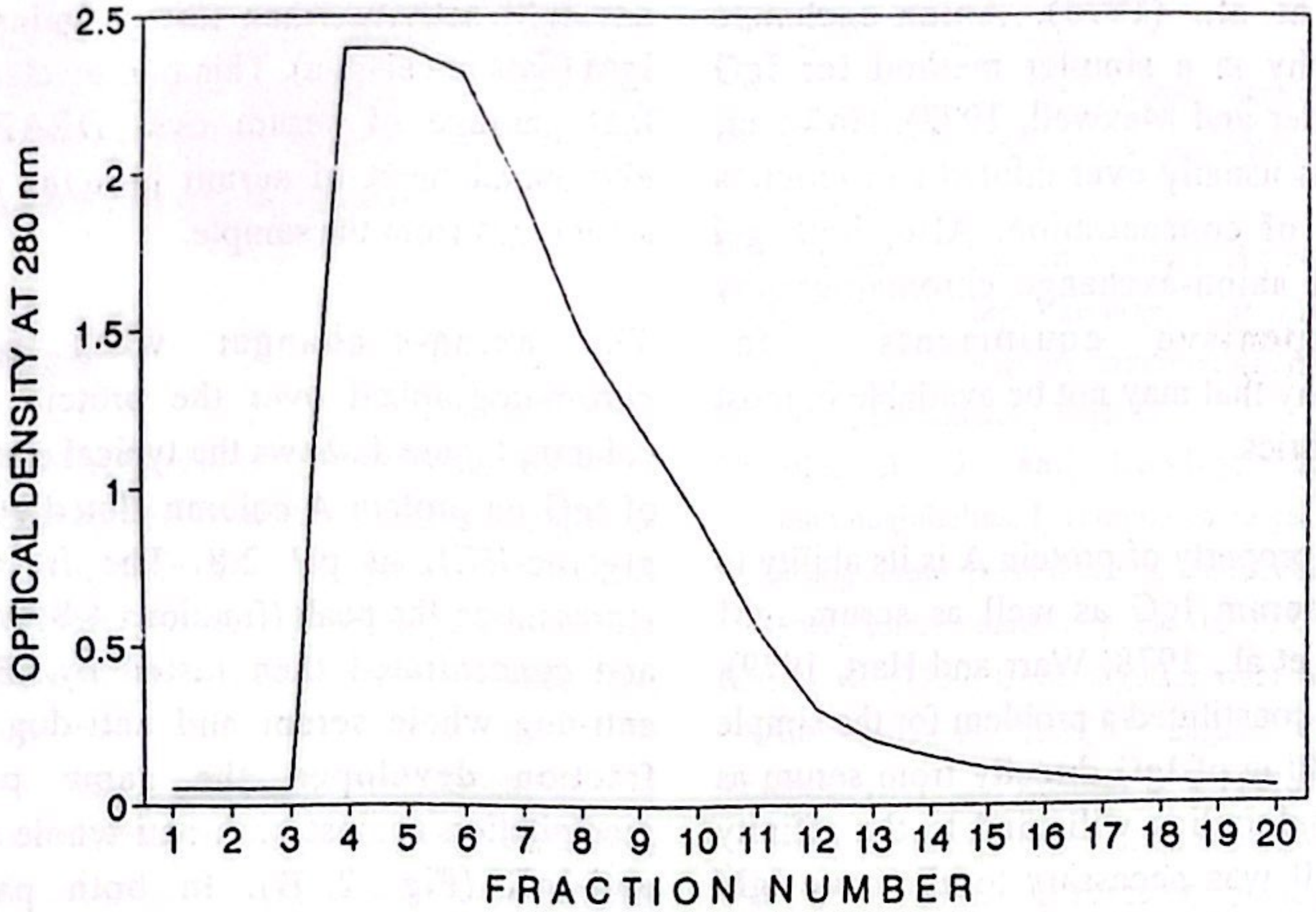
Concentration of Protein Fractions: Protein fractions were concentrated with Centricon-10 units (Amicon, USA).

RESULTS AND DISCUSSION

In spite of the importance of canine and feline infectious diseases, especially for public health, no serious sttempts have been made to study their immune response to infectious diseases of local importance. The problem, in part, is accentuated by the unavailability of elemental reagents e. g. purified immunoglobulins (Ig) and their respective monospecific antisera. The commercially available reagents are extremely expensive. Moreover, monospecific antisera to some critical Ig classes and subclasses are even not commercially available.

Monospecific antisera are essential reagents for the quantitation of serum Ig levels using radial immunodiffusion technique. Estimation of serum Ig levels is crucial for the diagnosis of gamopathies associated with chronic infections as well as hypogammaglobulinemia predisposing the susceptibility to infectious agents. Further

Fig. 1 ELUTION PROFILE OF CANINE SERUM IgG ON PROTEIN A-SEPHAROSE



monospecific antisera are indispensable reagents for some immportant serological procedures as indirect imunofluorescence technique and for primary binding techniques such as Enzyme Linked Immuosorbent Assay (ELISA).

Immuonglobulin G is the main immunoglobulin of canine serum and early colostrum (Reynolds and Johnson, 1970; Heddle and Rowley 1975). Besides its protective function, IgG plays an important role in conferring specificity to serological tests used in the diagnosis of infectious diseases. Different method have been used to isolate IgG. Some workers produced IgG by gel filtration chromatography on sephadex G 200 or sepharose 6-B (Heddle and Rowley 1975).

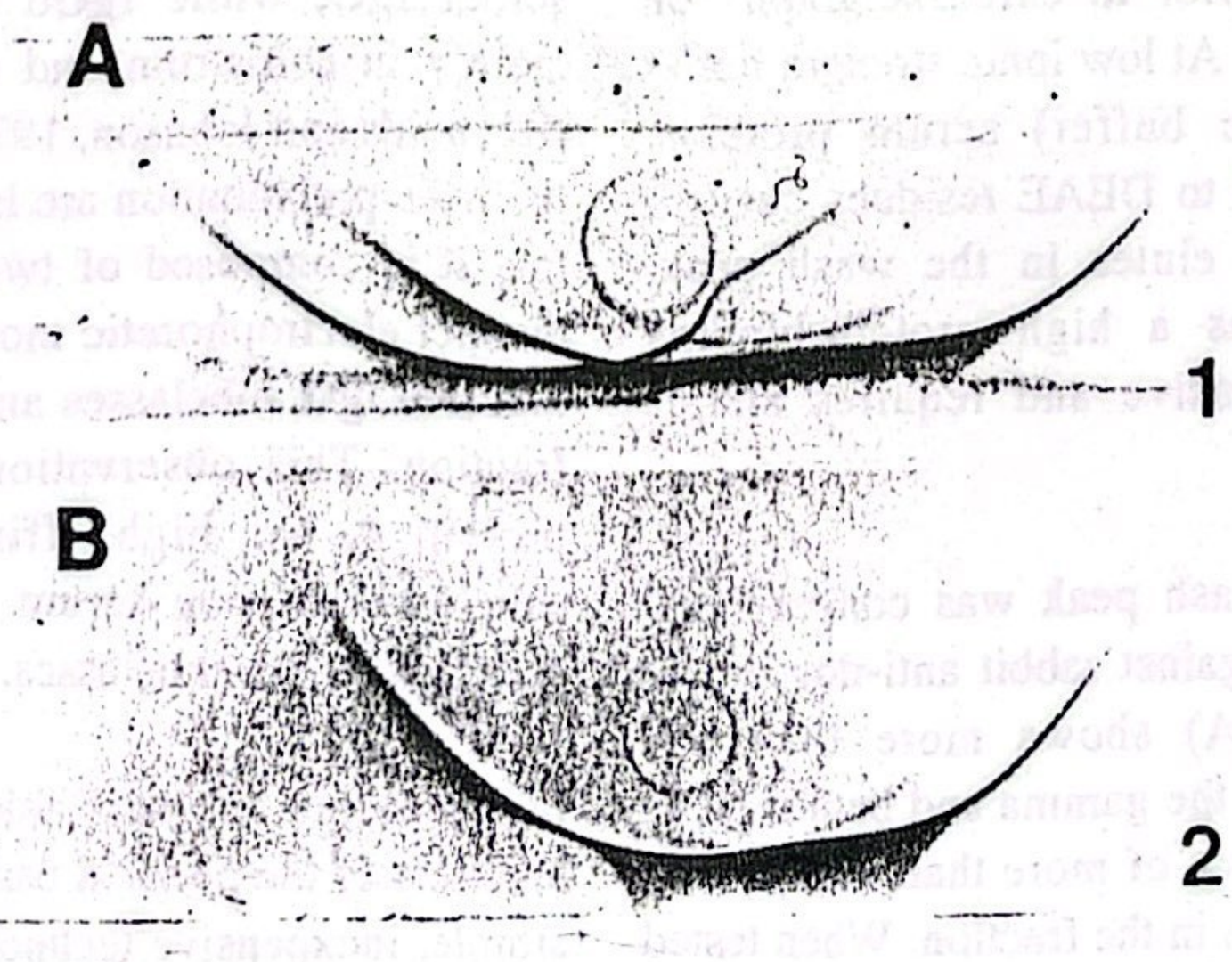


Figure 2: Cellulose wash peak fraction, A.; protein A sepharose peak, B. 1. Anti dog-whole serum 2. Anti dog- IgG.

However, this method is time-consuming, laborious and contamination of IgG with IgA is likely (Fey et al., (1976). Anion-exchange chromatography is a simpler method for IgG isoaltion (Butler and Maxwell, 1972). However, IgG fraction is usually over diluted and requires several steps of concentration. Also, both gel filtration and anion-exchange chromatography require expenisve equipments for chromatography that may not be available in most of our laboratories.

An important property of protein A is its ability to bind canine serum IgG as well as serum IgM (Goudswaard et al., 1978; Warr and Hart, 1979). This property constituted a problem for the simple one-step isoaltion of IgG directly from serum as both immunoglobulins will bind to the affinity column. So, It was necessary to eliminate IgM from serum prior to affinity purification. Some workers eluted both immunoglobulins from protein A column then isolated IgG from the eluate through gel filtration on sephadex G200 (Warr and Hart, 1979). This step is complicated and requires equipments that are not available in our laboratory. This step was replaced by a single passage of serum on anion-exchanger (DEAE-cellulose) prior to chromatography on protein A-sepharose. At low ionic strength buffer (0.01 M phosphate buffer) serum proteins, including IgM, bind to DEAE residues but IgG does not bind and elutes in the wash peak. DEAE-cellulose has a high protein-binding capacity, less expensive and requires simple technology.

Cellulose-column wash peak was concentrated and tested by IEF against rabbit anti-dog whole serum. Figure 2 (A) shows more than one precipitation lines in the gamma and beta regions indicating the presence of more than one protein and traces of albumen in the fraction. When tested against anti-dog IgG arcs in the gamma region proved to be IgG subclasses. Testing of the same fraction against anti-dog IgA proved that the b region arc to be IgA. The fraction was devoid any IgM activity when tested against anti-d IgM (data not shown). This finding clearly prov that passage of serum over DEAE-cellulo eliminated most of serum proteins and all serum IgM from the sample.

The anion-exchanger wash peak w chromatographed over the protein A affini column. Figure 1 shows the typical elution profi of IgG on protein A column eluted with 0.01 glycine-HCL at pH 2.8. The frac'ion tub representing the peak (fractions 4-8) were poole and concentrated then tested by IEF agains anti-dog whole serum and anti-dog IgG. Th fraction developed the same pattern c precipitation against both anti-whole serum an anti-IgG (Fig. 2 B). In both patterns n contaminating precipitation lines were evide against other serum proteins indicating that th fraction is a pure IgG preparation. Also, th obtained arcs showed the same anodi electrophoretic mobility characteristic for IgG.

Canine IgG is differentiated into four subclasses IgGab, IgGd and IgGc. IgGab is the dominan serum IgG, while IgGd and IgGc are foun mainly in colostrum and other secretory fluid (Reynolds and Johnson, 1970). By fine analysis o the wide precipitation are in Fig. 2 (b) ; it is clea that it is composed of two overlapping arcs o distinct electrophoretic mobility, which indicate that two IgG subclasses are found in the isolate fraction. This observation clearly proves tha protein A has high affinity for two distinc subclasses of dog serum IgG, which are mos probably IgGab subclasses.

In conclusion, we were able to isolate appreciable amounts of the purified canine serum IgG using a simple, inexpensive technoloyg. Chromatography on protein A sepharose proved to be an uncomplicated procedure that does not require

sophisticated equipments and can be performed under our laboratory condition. Regenerated columns can be used repeatedly with excellent resolution. Using simple technology we were able to prepare monospecific antiserum that will enable us to quantify dog serum IgG levels.

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