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Development of Anti IgG Polyclonal Antibody Against Canine IgG and Their Roles in Diagnostic Purposes

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Abstract

Background: *IgG plays an important part in immunological studies and also in living bodies for providing secondary immune response which triggers as passive immunization against harmful pathogens in dogs.* ***Purpose:*** *The aim of this investigation is to separate, identify, and refine immunoglobulin G from canine serum.* ***Methods:*** *Ammonium sulphate precipitation was used to separate the dog's serum, which was then concentrated over night in a dialysis bag inside of a refrigerator. The protein sample's concentration was determined the following day using the Lowry method, and it was found to be 12.063 mg/ml. The investigation was then carried out using gel filtration column chromatography estimation, which produced a bell-shaped graph showing the absorbance vs the number of fractions. Next, SDS PAGE was used to determine the Rf values of the various bands. The marker graph was estimated using the molecular weight protein ladder log, and the unknown graphs were estimated using the antilog of the marker's molecular weight protein ladder marker. To conclude, a DID-TEST was conducted in which a single precipitin line of canine species' pure immunoglobulin G was seen against anti-serum produced in rabbits.* ***Results:*** *It was observed that there is a single line of precipitin of purified gigs of canine species were found against the anti-serum developed in the rabbit, and the immunoglobulin G was found to be immune reactive by DID-TEST and was confirmed fully by SDS PAGE.* ***Discussion:*** *The canine serum used in this investigation was separated using ammonium sulfate, purified using gel filtration column chromatography, and then subjected to immune-biochemical characterization. This was verified by SDS PAGE, which showed the presence of a single precipitin line of IgG by the DID test.*

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INTRODUCTION

Canine bodies produce secondary immune responses due to immunoglobulin G. The Y-shaped structure of antibodies was initially revealed to the world by Rodney Porter and Gerald M. Edelman. For this discovery, they were given the 1972 Nobel Prize in physiology or medicine. The subunits of immunoglobulins can be formed in two ways, according to Rodney Porter's 1966 description. The

first approach involves enzymatic hydrolysis, wherein papain or pepsin produces a combination site including a fragment (Fab) with a molecular weight of 45,000 and the synthesis of light chain and N terminals. In his Nobel speeches, he also gave an example of how precipitate dissociation and salt solution might produce antibodies. Immunoglobulin G (IgG), whose structure is made up of four polypeptide chains—two heavy (50 k Dalton) and two light (25 kDa) chains—was later identified as these Y-shaped entities. Disulphide bridges and non-covalent linkages bind these together. Heddle and Rowley used a single radial immune-diffusion fluid in 1975 to assess the amount of IgG in dog serum, colostrum, milk, parotid saliva, and small intestinal fluid. Using SDS PAGE, Abdel-Rehman et al. (2017) determined that the molecular weights of camel IgG of four bands were 63,52,40, and 33 kDa. Due of their apparent genetic resemblance to humans, dogs are utilized in scientific research. According to a study published in 2014 by Santos F.N. et al., the characterisation and synthesis of IgY antibodies against canine IgG are crucial for usage as a serological marker in infections and for developing diagnostic kits. Dogs provide good study subjects for medical research, particularly in the field of cardiovascular science because of their similar hearts to human hearts in size and connectedness. Dog experiments produced insulin, which is used to treat diabetic patients, blood transfusion procedures, and the electrical defibrillator, which is used to restart a heartbeat. Dogs with the same cancers as humans are used to assess the effectiveness of some new cancer medications since they receive the same advantages. Dogs are therefore employed in these experiments and studies, and it is important to provide them with the care and attention necessary to ensure that crude IgG is separated correctly for our immune-biochemistry research. Since IgG is the most often discovered antibody in blood and other bodily fluids, it is the primary focus of this study. This antibody is the only one that can cross a pregnant woman's placenta to shield the fetus from harm and is capable of defending an individual against a variety of illnesses by identifying which germs are present in the body. The goal of our current work is to separate, purify, and immunologically characterize the IgG that was recovered from dog serum using the aforementioned techniques.

MATERIALS AND METHODS

Isolation of IgG

The process of ammonium sulphate precipitation was used to remove the crude IgG from dog serum. In a centrifuge tube, the canine serum (6 ml) and conc. Ammonium sulphate (6 ml) solution were added and gently mixed. This tube was stored at 4°C in a refrigerator. The tube was removed from the refrigerator and centrifuged at 5000 rpm and 4°C after an hour. Subsequently, the supernatant was disposed of and the white pellet was extracted and diluted using 4 milliliters of PBS. Following dilution, the material was well combined and transferred into a dialysis bag, which was then stored at 4°C for the whole night. To get the supernatant, the sample was removed from the refrigerator the following day and centrifuged once more for 15 minutes at 5000 rpm.

This fresh sample which was obtained is known as Lowry's sample. After that in another test tube, 10 ml of 2% Na_2CO_3 and Na-K tartrate was taken and mixed properly. This test tube was wrapped carefully by aluminum foil to prevent it from degrading, and kept aside. The mixture of 2% Na_2CO_3 as sodium potassium tartrate was called as Lowry's reagent. Then 3 test tubes were labelled as BLANK, STANDARD and TEST, where they were labelled B, S, T respectively. In each of these 3 test tubes, 3 ml of Lowry's reagent was taken. After that, in blank, 100 microliter of distilled water was taken. 100 microliters of BSA (BOVINE SERUM ALBUMIN), which was created by adding 2 mg/ml to an ultrapure 0.9% sodium chloride solution, were taken as the standard sample and placed in the standard test tube. A 100 microliter sample of Lowry's was used for the test, and after thoroughly mixing each test tube, it was incubated for 15 minutes. A clean test tube was used to appropriately combine 600 microliters of distilled water and Folin's reagent while the other test tubes were being incubated. After 15 mins, the test tubes were taken out of the incubator and in all the 3 tubes (B, S, T), 300 microliters of Folin's reagent were given in each test tube. The color change was immediately noticed in all 3 test tubes, and then all 3 test tubes were again kept in the incubator for 30 minutes. The tubes were finally taken out and readings were taken in spectrophotometer of these 3 tubes at 750 nm.

Finally, the protein concentration was calculated to be 12.063 mg/ml. The rest of the protein serum acquired from the dog was used to get total protein concentration. From the remaining dog serum, in blank, 3 ml of Na_2CO_3 was taken only. In contrast, 30 microliter of sodium potassium tartrate was introduced to the test tube along with 3 milliliter of 2% Na_2CO_3. Thirty microliter of dog serum, three milliliters of 2% Na_2CO_3, and thirty microliters of sodium potassium tartrate were combined in the TEST sample. Following a thorough mixing process, each test tube was incubated for five minutes at 37°C. Following the incubation of each of these test tubes, a noticeable color shift was observed right away. The spectrophotometer was used to measure the concentration of proteins at 550 nm, and the result was 6.140 mg/ml of total protein.

Purification of IgG

36 portions of the crude IgG were extracted and refined using gel filtration column chromatography in preparation for additional research. Three milliliters of Lowry's sample were added to the column first, and the column was supposed to be closed at that point. PBS (Phosphate Buffer Saline) was used to wash the column. Thirty-six fractions were sorted, labeled, and stored beneath the column for the IgG separation. These 36 test tubes, which served as fractions, were filled, and readings at 750 nm were obtained for each test tube in a spectrophotometer. As a result, the readings produced a bell-shaped graph. Among these 36 test tubes, from the test tubes 17-26, the values were gradually increasing, and the final peak of values were received at test tubes 27-28-29. These 3 test tubes were kept overnight at 4°C and the samples were taken out and the others were discarded. Next, 500 ml of distilled water was used to dissolve 2 g of NaOH and 10 g of sodium carbonate, which were then well vortexed. In addition to the regular test tube, these three test tubes were taken. 15 milliliters of 2% Na_2CO_3 were placed in a new test tube, and 10 milliliters of the same solution were placed in a second new test tube. For the subsequent reaction, 300 microliter of sodium potassium tartrate were taken in the 15 ml tube and 200 microliter in the 10 ml tube. Lastly, there are five test tubes: three test tubes contain protein samples collected after gel, one fresh blank tube, one fresh standard tube, and one additional test tube. filtration chromatography. From the 10 ml and 15 ml tubes, 3 ml each was given in the blank, standard and the other 3 test tubes of protein IgG separated, then in Blank, distilled water was added (100 microliter). In standard, 100 microliters of Bovine serum albumin were added with predetermined concentration. And the other 3 test tubes T1, T2, T3 was taken together in a test tube rack. All these 5 test tubes were kept in the incubator for 15 mins at 37°C. After taking out the test tubes from the incubator, Folin reagent was added (made by adding Folin solution and distilled water 1:1) in a quantity of 300 microliter. This total material was mixed properly and color changed simultaneously. Then these tubes were incubated again for 30 mins. After the incubation period, these tubes were taken out and readings were taken at spectrophotometer at 750 nm. The yield of readings was as follows: 3.235 mg/ml, 4.65 mg/ml, and 4.72 mg/ml in the 3 test tubes of IgG tested.

Immuno-biochemical Characterization of IgG

After the gel filtration column chromatography was completed, SDS PAGE (Sodium Dodycyl Sulphate-Polyacrylamide Gel Electrophoresis) was also initiated. By taking the samples obtained after gel filtration chromatography and the crude sample made previously by Lowry method. After performing SDS PAGE, the bands were visible in the gel upon development. Next, a logarithmic excel graph of the marker's retardation factor value (Rf) was produced, and the anti-log of molecular weight vs. Rf of P1–P5 was shown in accordance with the Rf values thus far obtained. By plotting the logarithmic excel graphs of the markers and the P1–P5 data, additional research was carried out visually. Log (235) = 2.36, Log (173) = 2.23, Log (130) = 2.11, Log (93) = 1.96, Log (70) = 1.84, Log (53) = 1.72, Log (41) = 1.61, Log (30) = 1.47, Log (22) = 1.34, Log (18) = 1.25, Log (14) = 1.14, Log (9) = 0.95 are the log values that were computed. These values guided the creation of the marker graph.

The following are the log values of P1–P4, which were determined using antilog values:

Antilog (2.36) is 229.08, Antilog (2.23) is 169.82, Antilog (2.11) is 128.82, Antilog (1.96) is 91.2 Antilog (1.84) is 69.18, Antilog (1.72) is 52.48, Antilog (1.61) is 40.73, Antilog (1.47) is 29.51, Antilog (1.34) is 21.87, Antilog (1.25) is 17.78, Antilog (1.14) is 13.8, Antilog (0.95) is 8.91.

The graph was plotted on the basis of the given anti log values of P1-P5. After that, double immunodiffusion test was carried out with the sample.

Procedure: At first, a small beaker was taken and microwaved with distilled water for few minutes and then taken out. Then a conical flask was taken and 100 ml of PBS was poured and microwaved. After heating it enough, the conical flask was taken out and agar was mixed with it (1.5 gm). Then 2 slides were cleaned and washed thoroughly. The conical flask which is having agar and PBS mixed properly and dissolved were taken and again to heat and thoroughly dissolve the agar into it. With the help of a pipette, the mixture was poured on the 2 cleaned slides carefully. And left for solidification for approximately 2 hours. 4 wells were made in the slides after they were properly solidified, so 8 wells were made in total. In each well, in the slides, just 1 drop of agar and PBS mixture was given. After that, in the 1st slide, in the 1st well, 20 microlitre of canine IgG was given, and in 2nd well, 20 microliter of rabbit anti canine IgG was given. In the 3rd well, 20 microliter of rabbit anti canine IgG was added. In the 4th well, 20 microliters of canine IgG were added. These slides were incubated overnight. The slides were taken out the next day, from the incubator and bands were observed in the wells. The rabbit was given hyperimmune serum in response to the isolated crude IgG from the dog. When partially purified IgG was utilized for the test and it reacted with the hyperimmune serum, a precipitin line observation was recorded in the DID test.

The total protein concentration was calculated on basis of the formula given:

$$\text{Total protein concentration } \left(\frac{\text{mg}}{\text{ml}}\right) = \frac{\text{Absorbance of the Test}}{\text{Absorbance of Standard}} * \text{Concentration of standard X dilution factor}$$

RESULTS

Thus, the ongoing study was focused to the characterization of immunoglobulin G and its isolation from canine serum, by the ammonium sulphate precipitation method. This was followed by Lowry's test and the estimation of total protein concentration was done. In the next step, the purification of IgG was done by gel filtration column chromatography. And SDS PAGE was done on the same sample for immune-biochemical characterization of the antibody. In this case, the projected total protein concentration was 12.063 mg/ml, while the protein concentration was 4.65 mg/ml. The sequence of events for the purification of IgG was also approximated to be the collection of the sample from the gel chromatography column in 36 fractions of tubes, from which the spectrophotometric readings were obtained. And these readings, when graphically represented, formed a bell-shaped curve. Furthermore, the Rf values of the marker was calculated and the P1-P5 was measured with the help of a scale. The page's total length was 6.5 cm. 12 bands were observed in the first marker well. In the well P1, Rf values were calculated for the bands [1–7]. In the well P2, the Rf values of the bands [1-7] were calculated. In the well P3, the calculation of Rf values of the wells 1-7 was done accordingly. Finally, in the P4 well the calculation of Rf values was done accordingly too. The Rf values of the markers during the immune-biochemical assay of the immunoglobulin G was done and the 12 bands were calculated to be 0.061 cm, 0.12 cm, 0.076 cm, 0.18 cm, 0.24 cm. 0.33 cm, 0.47 cm, 0.56 cm, 0.73 cm, 0.78 cm, 0.90 cm, 0.95 cm, in case of the Pi, or the unknown one, the Rf values of the 6 consecutive bands were calculated to be 0.015 cm, 0.061 cm, 0.092 cm, 0.169 cm, 0.415 cm, 0.476 cm. In the case of P2, the 7 bands were found, whose Rf values were 0.015cm, 0.061 cm, 0.123 cm, 0.169 cm, 0.353 cm, 0.430 cm, 0.492 cm. in case of the P3, the Rf values were calculated of the 7 bands formed which were, 0.015 cm, 0.061 cm, 0.123 cm, 0.169 cm, 0.338 cm, 0.415 cm, 0.461 cm, in case of P4 , the Rf values of the 7 consecutive bands were calculated as follows:- 0.015 cm, 0.061 cm, 0.107 cm , 0.169 cm, 0.353 cm, 0.430 cm, 0.476 cm . Now, as a final step to this experiment, the logarithmic excel graph of marker was and P1-P5 were obtained, and plotted. .. The anti-log of the molecular weight vs. Rf value was computed and plotted for P1–P5, and the log of the molecular weight vs. Rf value was plotted for the marker. Log (235) is 2.36, Log (170) is 2.23, Log (130) is 2.11, Log (93) is 1.96, Log (70) is 1.84, Log (53) is 1.74, Log (41) is 1.61, Log (30) is 1.47, Log (22) is 1.34, Log (18) is 1.25, Log (14) is 1.14, and Log (9) is 0.95. These are the log values of the marker graph that were calculated. The

marker graph was obtained on the basis of these log values The Log values of P1-P4 (Unknown) were calculated to be Anti-Log (2.36) is 229.08, Anti-Log (2.23) is 169.82, Anti-Log (2.11) is 128.82, Anti-Log (1.96) is 91.2, Anti-Log (1.84) is 69.18, Anti-Log (1.72) is 52.48, Anti-Log (1.61) is 40.73, Anti-Log (1.47) is 29.51, Anti-Log (1.34) is 21.87, Anti-Log (1.25) is 17.78, Anti-Log (1.14) is 13.8, Anti-Log (0.95) is 8.91. On the basis of these Anti-Log values the graphs of P1-P4 were obtained.

DISCUSSION

Following treatment with a concentrated solution of ammonium sulphate and precipitation, the crude form of IgG was produced. It was then dialyzed against PBS, which is consistent with Duong-Ly K (2014) and Miller. Using the Lowry et al. 1951 method, the protein content in canine serum was measured; the estimated value was 4.65 mg/ml, and the total protein concentration was 12.063 mg/ml. Purified form of canine IgG was recovered by using the method of gel filtration column chromatography and the respective graphs were made out of the values received from the readings of the spectrophotometer. The result was almost similar to that of Still et al (1978). From the gel filtration column chromatography, the concentration of the protein sample which as needed for this experimental study was counted to be 0.982 mg/ml, 1.294 mg/ml, 1.198 mg/ml. These crude and pure form of samples were analyses through the process of SDS-PAGE by Reynolds et al (1970). The bands were obtained after the completion of this process and bands were shown in marker, and the columns: P1-P4. The Rf values of these bands of each well that were done was calculated to draw graphs as a result of the job done. After calculating the logarithms of the molecular weights of the protein ladder markers, the graphs were produced. These findings can line up with the research done by [19, 10, 6, 11, 21, 5], and [14]. Following that, a single precipitin line of purified canine IgG was seen against an antiserum developed in a rabbit (DID), which is comparable to the findings of Graham, [4, 13], who demonstrated the precipitin line in purified IgG desorbed from diphtheria bacteria using an in vitro technique to assess the toxin-producing capacity.

CONCLUSION

A novel avenue for the isolation, purification, and immunological biochemical analysis of immunoglobulin G from canine serum has been made possible by the completed study. In the realm of gel filtration column chromatography, the use of immobilized antigens for antibody isolation appeared to be the best approach. Furthermore, the foundation of these investigations was incredibly narrow biomolecule interactions. These produced antibodies can be utilized in recombinant experiments of protein purification in addition to existing techniques, or they can be used to purify the natural proteins. Protein separations from body fluids such as plasma, CSF, serum, urine, and others are typically performed for genomic research. These processes can also be utilized for unfolding novel antibodies from patients of auto immune disorders. Traditionally the IgG proteins were usually gram-positive bacteria which were utilized in antibody isolation processes. It is always expected that the improvements in these methods can be used to influence efficient antibody production in large scale. These steps will also help in increasing the no. of proteins derivatives of antibody available for the purpose of protein engineering.

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