ISOLATION, PURIFICATION AND IMMUNOBIOCHEMICAL CHARACTERIZATION OF IMMUNOGLOBULIN-G OF VANARAJA FOWL

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ABSTRACT

Immunoglobulin G (IgG) antibodies are primarily involved in secondary immune response in animals, and can be targeted for inducing passive immunization in chickens against pathogens. There is dearth of literature on the IgG status of chickens in India, particularly of *Vanaraja*, a new synthetic dual purpose (egg and meat) poultry breed, evolved in India. The purpose of the present study was isolation, purification, and immunobiochemical characterization of IgG, isolated from the serum of *Vanaraja* hens. The blood samples of *Vanaraja* hens maintained in the university farm were collected from heart, and clotted at 25°C for harvesting serum. Crude IgG was isolated from the serum of the birds by ammonium sulphate precipitation method, followed by purification by gel filtration chromatography on Sephacryl S-200. The antibody against crude *Vanaraja* IgG was raised in a clinically healthy New Zealand white rabbit. The protein concentration of purified IgG isolated from *Vanaraja* fowl was found to be 1.34 mg/ml. The molecular weight of purified IgG, analysed by one-dimensional sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was 181 kDa. There were two discernible polypeptide bands *viz*., IgG H of high molecular mass (64.95 kDa and 60.47 kDa) and IgG L of low molecular mass (28.39 kDa and 27 kDa). Purified IgG fractions showed immune reactivity against hyper immune sera raised in rabbit in double immunodiffusion (DID) test, and was duly confirmed by western blot.

KEY WORDS

DID, Immunoglobulin-G, SDS-PAGE, Serum, Western blot, Vanaraja fowl

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INTRODUCTION

Immunoglobulin G (IgG) antibodies are primarily involved in secondary immune response in animals. The existence of an immunoglobulin G (IgG) like molecule in chickens was reported by Leslie and Clem (1969). It is also known as IgY in birds, because of its phylogenetical distinction from mammalian antibodies, in spite of performing the same physiological function as IgG in mammals (Warr *et al*., 1995). These antibodies are not only part of the immune defense system of the chickens, but can also induce passive immunization in chickens against pathogens (Chalghoumi *et al*., 2009).

Vanaraja is a new synthetic dual purpose (egg and meat) Indian poultry breed, and has shown superior resistance and higher humoral immune response to SRBC and CBH in induced *Escherichia coli* infection than krishibro chicken broilers (Reddy *et al*., 2002) indicating genetic difference in immune response.

Dearth of available literature on the IgG status of Vanaraja impelled us to undertake the present study with the objectives of isolation, purification, and immuno-biochemical characterization of IgG from the serum of Vanaraja chicken.

MATERIALS AND METHODS

Isolation of IgG: The blood samples were collected from heart of Vanaraja fowls maintained in the university farm and clotted at 25°C in sterile viles for harvesting serum. The serum samples were further centrifuged at 5000 rpm for 5 minutes, to remove residual RBC, and were stored at -25ºC for analysis. Ammonium sulphate precipitation was carried out by addition of 6 ml saturated ammonium sulphate solution with equal volume of serum, and was kept in ice for 1 hour. The mixture was centrifuged at 10,000 rpm for 15 minutes and the precipitate was dissolved in phosphate buffered saline (PBS) at 7.2 pH of the original serum volume. The precipitation was repeated again by adding 3 ml of saturated ammonium sulphate solution to 6 ml of PBS dissolved precipitate. Thus, the final saturation came to 33.3 percent. It was kept in ice for 1 hour and centrifuged at 10,000 rpm for 15 minutes. The supernatant was discarded carefully, and the precipitate was dissolved in PBS and dialysis was carried out with several changes of the same buffer for 24 hours at 4°C, resulting in separation of crude IgG. The concentration of the crude IgG was determined as per the procedure adopted by Lowry *et al*. (1951).

Purification of IgG: Crude IgGs were purified by gel filtration chromatography on Sephacryl S-200, with a bed volume of 150 ml. Elution was carried out by a buffer containing PBS (pH 7.2), PMSF (0.03 mM) and 0.02% sodium azide at a flow rate of 24 ml per hour. Fractions of 4 ml each was collected in 40 different sterile test tubes. The distribution of protein was monitored by taking the absorbance of fractions at 280 nm in UV-VIS Spectrophotometer (Systronics-119). The behavior of antibody purification was studied by plotting the graph, taking fraction number along x-axis and absorbance along y-axis. The purified fractions (fraction numbers 20, 21, 22) of crude Vanaraja IgG was pooled (P_v), which contained a peak as well as upper ascending and descending parts of the peak. The protein concentration of the pooled fraction was determined as per Lowry *et al.* (1951).

Characterization of IgG: The crude and purified samples were analyzed by one-dimensional sodium dodecyl sulphate

polyacrylamide gel electrophoresis (SDS-PAGE) in vertical mPAGE (AE-6530) and vertical slab gel electrophoresis chamber (AE-6200) along with power supply and gel casting apparatus (ATTO Corporation, Japan) with power pack (Genei, Bangalore). The SDS-PAGE was performed under identical experimental conditions according to the method described by Laemmli (1970), with some modification under denaturing and reducing condition using 10% poly-acrylamide slab gel of 15 x 17 cm dimension. Each lane of gel was loaded with 40 µl of samples and sample buffer mixed in 1:1 ratio so that each lane contains 40 µg of protein. After separation, the gel was stained overnight in staining solution comprising of 0.25% Coomassie Brilliant Blue R-250 dye in methanol: acetic acid: water (50:10:40), followed by destaining it with destaining solution in methanol: acetic acid: water (30:10:60). Gels were finally preserved in a solution, containing 7% acetic acid.

Immunobiochemical assay: The antibody against crude Vanaraja IgG was raised in one clinically healthy New Zealand white rabbit weighing 1.5kg. The crude antigen was thoroughly mixed with equal volume of Freund's complete adjuvant (FCA) in 1:1 ratio, and 1 ml of this mixture was injected intramuscularly at 3 sites into the thigh muscle, and subcutaneously at 3 sites in the scapular region of the animal. Four booster doses of the same antigen, emulsified with Freund's incomplete adjuvant (FIA) in 1:1 ratio were given with increasing amount of 100 µg at 14 days interval following the first injection. Blood was collected from the rabbit, 7 days after the last booster dose, and the serum was stored at –20°C in aliquots for further use with sodium azide (0.02%) as preservative.

Double Immunodiffusion Test: Double Immunodiffusion test (DID) was performed according to Hudson and Hay (1989) with some modifications. Agarose solution (1.5%) was prepared with PBS (pH 7.4), and a small amount of sodium azide was added to it. After boiling, 5 ml of melted agarose was poured on two different clean and grease free glass slides to give a thickness of 1.5 mm. The slides were then kept at room temperature (25°C) for half an hour to solidify. Wells were then punched by gel puncher. The bottoms were sealed with melted agarose solution. The wells were then filled with 20µl of purified Vanaraja IgG, 20 µl of hyper-immune serum raised against crude Vanaraja IgG, and 20 µl of normal control serum in central and peripheral wells respectively. The slides were then placed in a humid chamber and incubated overnight at room temperature (25°C). The following day, the slides were washed in PBS (pH 7.4), dried by blotting paper, and stained with Coomassie staining solution. The stained slides were destained later with destaining solutions.

Western blot: The proteins were characterized by western blot technique according to Towbin *et al.* (1979) with modifications to the labeled antibody and its substrate. The proteins were separated by SDS-PAGE (Laemmli, 1970), and then the resultant proteins were electroblotted to nitrocellulose filter paper (Immobilon-NC) from gel according to the methods of Towbin *et al.* (1979) using a Milliblot semi-dry transfer system (Millipore Core, Bedford, MA, USA) at 50mA for twelve hours at 4°C. To minimize nonspecific protein binding, nitrocellulose sheets were incubated at 37°C for 2 hour in PBS containing 5% BSA and 0.05% Tween 20. All

additional washes and incubations were performed in PBS-Tween 20. After being washed, Nitrocellulose membrane was incubated with hyper immune serum raised against crude seminal plasma protein of 1:80 dilution in dilution buffer (1% BSA, 0.1%Tween 20, 0.5% control rabbit serum, and water 100 ml) for 2 hours. After washing with PBS-Tween 20, Nitrocellulose membrane incubated with goat anti-rabbit horse radish peroxidase conjugate of 1:1000 dilution in dilution buffer for 2 hours, followed by three times washing with PBS Tween 20. Then, the membrane was rinsed with substrate solution (10 ml Tris-HCl, 40µl H_2O_2 and 205mg Diaminobenzidine).The membrane was dipped into the distilled water to stop the reaction, and later, it was dried up and preserved.

RESULTS

The concentration of purified IgG, and their biochemical and immunological characteristics in Vanaraja fowl, are given below.

Concentration: The protein concentration of crude IgG in the serum of Vanaraja, obtained after 33.3% ammonium sulphate precipitation, and dialyzed against PBS was 8.04 mg/ml. The purified Vanaraja IgG, prepared by gel filtration chromatography on Sephacryl S-200 with the flow rate of 24 ml/hr, and plotted by taking the absorbance of eluted fractions, revealed a bell shaped curve (Figure-1), in which the protein was resolved into one major peak. The fractions showing the peak were pooled together into one part (P_v) comprising the fraction numbers 21, 22, and 23, that contained the peak as well as upper ascending and descending parts of the major peak. The protein concentration of the pooled fraction (P_v) was 1.34mg/ml, while the protein concentration of P_v, concentrated against sucrose using dialysis bag (cut off value 12,000) was 2.3mg/ml.

Biochemical characteristics: The purity of P_v fraction along with ammonium sulphate precipitated samples i.e. crude IgG, checked by SDS-PAGE analysis containing 10% acrylamide revealed polypeptide bands, when crude and purified IgGs were stained with Coomassie brilliant blue after gel electrophoresis. The major polypeptide bands of crude IgG were 114.13 kDa, 107.22 kDa, 64.95 kDa, 60.47 kDa, 28.39 kDa, and 27 kDa (Figure-2), while the major polypeptide bands of the purified fraction (P_v) were 64.95 kDa, 60.47 kDa, 28.39 kDa, and 27 kDa. (Figure-3).The molecular weight of purified IgG was 181 kDa.

Immunological characteristics: A single precipitin line was observed, when P_v reacted with corresponding hyperimmune sera raised in rabbit against crude IgG of Vanaraja in DID test. No precipitin line or band was formed against the normal control serum (Figure-4).

The purified Vanaraja IgG was found to be immunoreactive against the corresponding hyper immune serum raised in rabbit by western blot analysis. The P_v fraction revealed polypeptide bands of 64.95 kDa, 60.47 kDa, 28.39 kDa, and 27 kDa (Figure-5).

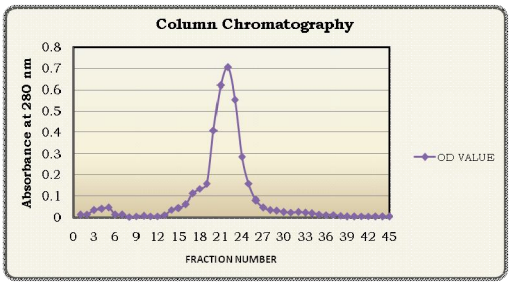

Figure-1. Partial purification of crude IgG from Vanaraja fowl serum by gel filtration chromatography on Sephacryl S 2000.

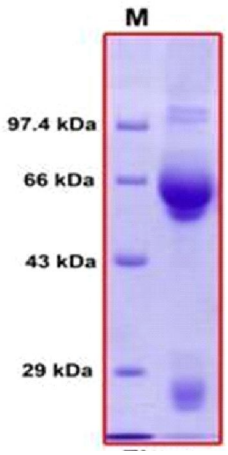

Figure-2. SDS-PAGE of crude IgG of Vanraja fowl

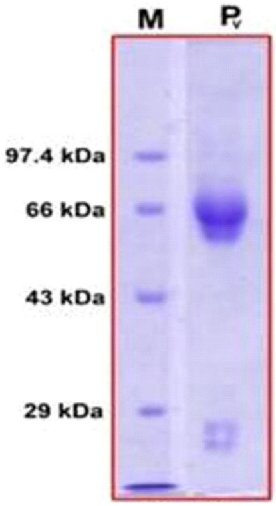

Figure-3. SDS-PAGE of purified IgG of Vanraja fowl

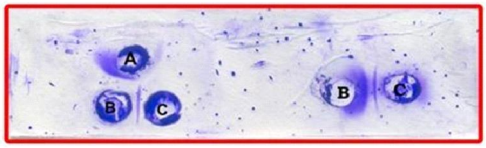

Figure-4. Single precipitin line of purified IgG of Vanaraja fowl observed against antisera developed in Rabbit (DID).

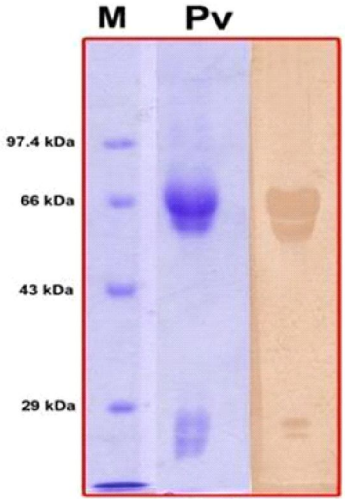

Figure-5. Western Blot Analysis of purified IgG of Vanaraja fowl

DISCUSSION

The present study reveled that the molecular weight of IgG isolated from the serum of Vanaraja fowl was 181 kDa, having two heavy chains of 64.95 kDa and 60.47 kDa, and two light chains of 28.39 kDa and 27 kDa. This study corresponds to Loeken and Roth (1983) who purified the IgG from White Leg Horn chicken serum adopting 10% SDS-PAGE after ion exchange chromatography and found polypeptide chains of 65 kDa as heavy chain and 30 kDa as light chain.

Purified IgG from Vanaraja fowl with hyper immune sera raised in rabbit against crude IgG in our study showed single precipitin

line in DID. This finding of the present study is in accordance with the study of Meyers and Dougherty (1971) that showed single precipitin band against corresponding hyper immune sera in chicken. Raj *et al*. (2004) observed that IgY isolated from egg yolk gave a single precipitin line with rabbit anti-chicken serum and a single band of about 180 kDa, which under reducing conditions produced two bands of 60 kDa and 25 kDa as found by western blot analysis with no other contaminating band, indicating similar banding pattern of IgG isolated from both serum and egg of chickens. Our study confirmed the report of Oliviera *et al.* (2010), who recognised the IgG H chain of 65 kDa and IgL chain of 25 kDa of the IgG isolated from serum as well as egg yolk in Japanese quail.

CONCLUSION

Serum IgG from Vanaraja fowl could be isolated and purified by gel filtration chromatography. The molecular weight of purified IgG was 181 kDa, which was comparatively higher than the other fowl species. Western blot analysis of purified IgG revealed immunodominant polypeptide band and showed specific immuno reactivity.

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