Journal of Applied Biological Sciences
E-ISSN: 2146-0108 13 (3): 177-181, 2019

Production of Polyclonal Rabbit IgG against Dog IgG: Orientation for Applications in Immunological Diagnosis of Dog Diseases

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Received: October 15, 2019
Accepted: December 5, 2019

Abstract
Dogs are one of the most popular pets in many parts of the world. The need to diagnose dog diseases using immunological assays is constantly increasing. This study aims to produce anti-dog IgG antibodies from rabbits for use in immunological diagnosis in dogs. To achieve this, the dog's refined IgG is used as an antigen to cause an immune response in rabbits. Specific rabbit IgG is then purified from serum by precipitation method with 45% saturated amonium sulfate salt, protein G affinity chromatography and dog IgG affinity chromatography. The purity of rabbit IgG reached 95.3% based on SDS-PAGE analysis. The specific activity of purified IgG is confirmed by dot blot assay. These results suggest that rabbit anti-dog IgG antibodies produced in this study can be applied to immunological diagnosis of dog diseases.

Keywords: Affinity chromatography, Dog IgG, Immunological diagnosis, Protein precipitation, Rabbit IgG.

INTRODUCTION

Immunoglobin G antibodies are biological molecules that plays an important role in the animal immune system. They are produced by B cells after animals are infected with antigens from microorganisms or other foreign protein molecules. In canine, there are four IgG subclases which engage distinct functions in the immune system via complement-dependent cytotoxicity or antibody-dependent cell-mediated cytotoxicity [1]. The production of antibodies against dog IgG (dIgG) is necessary for development of diagnostic tools to detect many immunological imprints of infectious diseases [2, 3]. Experimental animals for antibody production are diverse, including rabbits, goats, mice and other animals [4]. Among them, rabbits are considered a good choice, suitable for laboratory conditions because they are moderate in size, gentle and easy to take care of. Additionally, they have large amount of serum which is easily upscaled to produce antibodies for applications in many biological techniques.

There are many different approaches to purify IgG from rabbits (rIgG) including protein precipitating with saturated ammonium sulfate solution, ion exchange chromatography, affinity chromatography (protein A, protein G, targeted antigens, metals, lectin) [5-7]. In this study, rabbits were used to produce anti-dog IgG antibodies. The purification method of specific rIgG consists of protein precipitate using saturated ammonium sulfate, affinity chromatography with protein G and dIgG. The purified rIgG can be used for diagnosis by immunological techniques in dog diseases.

MATERIALS AND METHODS

Experimental animals

Two rabbits (2 months old, 2 kg weight) were purchased from the Pasteur Institute in Ho Chi Minh City and were raised in our animal house in Faculty of Biotechnology, Nguyen Tat Thanh University.

Rabbit immunization

The dIgG (>95% pure) was used to induce an immune response in rabbits by intradermal injection according to previous published work [5] but with modification. The regimen of immunization is shown in Table 1. Rabbit back skin was intradermal injected with dIgG prepared in complete Freund's adjuvant (CFA) for the primary injection and in incomplete Freund's adjuvant (IFA) for the boosters. The blood was taken from the ear vein, except for the last time the blood was drained from the heart.

Table 1. Schedule for rabbit immunization with dIgG.

Protocol day	Procedure	Description
Day 0	Control serum collection	Pre-immune bleed (5 ml per rabbit)
Day 1	Primary injection	Immunize with 0.25 mg dIgG in CFA
Day 14	1st booster	Bleed (5 ml per rabbit) Immunize with 0.1 mg dIgG in IFA
Day 28	2st booster	Immunize with 0.1 mg dIgG in IFA
Day 42	Serum collection	Bleed (25 ml per rabbit)
Day 56	3st booster	Immunize with 0.1 mg dIgG in IFA
Day 70	Serum collection	Bleed (25 ml per rabbit)
Day 84	4st booster	Immunize with 0.1 mg dIgG in IFA
Day 98	Serum collection	Bleed (50 ml per rabbit)

Ouchterlony assay

The assay was used to check for specific antibody production in the rabbits after each times of dIgG injection [8]. 1.5% agarose and 1.5 mm thickness gel was prepared. Six wells (2 mm diameter) were created on the gel in hexagonal form with a length of 1 cm each and another 1 well was created in the central position. The 6 peripheral wells were loaded with 10 µl of rabbit diluted (by a factor of 2) or undiluted serum. The central well was loaded with 10 µl

of dIgG antigen (concentration of 0.7 mg/ml). The gel was then incubated in a refrigerator at 2 – 8 °C for 36 hours. The forming complex of dIgG antigen and rIgG antibodes was detected by Coomassie Blue staining method.

Purification of total rIgG

Total rIgG was purified by using method showed previously [5] but with modification. Rabbit serum after the last booster was separated by centrifugation at 3000 x g for 15 minutes. To precipitate rIgG, 100% saturated ammonium sulfate solution was used to add to serum so that the final concentration of saturated ammonium sulfate was 45%. The precipitate mixture was kept for 4 hours at 2 – 8 °C. IgG precipitation was obtained by centrifugation at 6000 rpm for 15 minutes. The precipitate was washed twice with 45% saturated ammonium sulfate solution and centrifuged as above. The precipitate was collected and dialyzed to dissolve rIgG in sodium phosphate buffer. The dissolved rIgG was then purified by affinity chromatography with G protein column according to the manufacture (GE Healthcare). The rIgG product was quantitatively analyzed by spectral analysis at 280 nm wavelength and it's pure was assessed by electrophoresis under reduced condition (SDS-PAGE) with a 12.5% polyacrylamide gel. Proteins were detected by Coomassie Blue staining method.

Purification of dIgG specific rIgG

To purify specific rIgG against dIgG from total rIgG, SK2 affinity column was prepared in which dIgG was used as ligand. Thirty three mg of dIgG was used for conjugation with 12 ml of CNBr-activated gel according to the manufacturer's instructions (GE Healthcare). The effectiveness of the conjugate process was tested by quantification of protein in floating fluid after conjugation. The SK2 column was used in affinity chromatography which allows to bind specific rIgG which are then collected by washing buffer and quanlified by SDS-PAGE.

Dot blot assay

Specific rIgG activity was tested by dot blot assay shown previously but with modification [9]. One µl of dIgG antigen solution, containing 1 µg, 0.5 µg, 0.25 µg, 0.125 µg, 0.0625 µg, 0.03125 µg, 0.015625 µg, and 0 µg (negative control) of protein, was dripped onto nitrocellulose membrane and allowed to dry naturally. The membrane was then locked with 2.5% skim milk mixed in TBST buffer for 1 hour and washed 3 times with TBST buffer (every 10 minutes). The membrane was incubated with total rIgG (bound fraction in G protein column), specific rIgG (bound fraction in SK2 column) and non-specific rIgG (unbound fraction in SK2 column) at different concentrations including 500 ng/ml, 250 ng/ml, 125 ng/ml, 62.5 ng/ml, 31.25 ng/ml, 15.625 ng/ml, 7.8125 ng/ml, 3.90625 ng/ml and 0 ng/ml (negative control) for 1 hour. The membrane was then washed with a TBST buffer for 3 times (10 minutes each) and incubated with goat anti-rabbit IgG antibodies conjugated HRP at dilution of 1/8000 in 1 hour. The membrane was washed with TBST buffer for 3 times. To detect the antigen - antibodies complex, the membrane was incubated with enhanced chemiluminescence reagent and scanned with a C-digit blot scanner (LICOR Bioscience, Nebraska, USA). Scanned images were processed using Image Studio Lite software.

RESULTS AND DISCUSSION

Specific rIgG formation after immunization with dIgG

Ouchterlony assay was used to assess rIgG produced in rabbits after inducing immune response to dIgG antigens. Figure 1A shows that 14 days after the primary injection, there were occurrence of precipitating lines between the well 0 containing dIgG antigen with wells 2, 4 and 6 containing post-sensitive rabbit serum. In contrast, no observation of precipitation between well 0 and wells 1, 3 and 5 containing the pre-sensitive (control) rabbit serum. The results indicate the formation of specific antibodies in rabbits at the time of serum collection after hypersensitivity to dIgG.

In order to enhance the production of specific rIgG antibodies and enhance their affinity dIgG antigen, rabbits were boostered 4 times in accordance with the regimen outlined in Table 1. Antibody titer and affinity were assessed by Ouchterlony assay (Figure 1B). Rabbit sera after the 2nd, 3rd and 4th booster were continuously diluted by a factor of 2 and loaded accordingly to wells 1, 2, 3, 4, 5 and 6. Well 0 contained dIgG antigens. The results showed that after the 2nd booster, the precipitate appeared at dilution of $^1/_{16}$. From 3rd booster onwards, the precipitate was observed at dilution of $^1/_{32}$ indicating an increase in the production and affinity of specific rIgG antibodies.

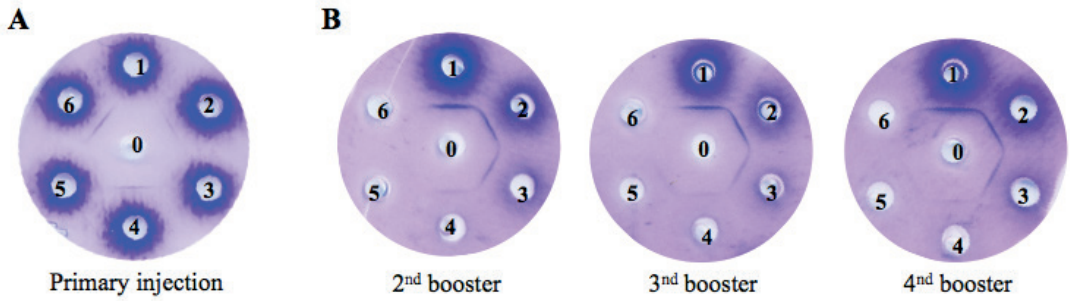

Figure 1. Examination of specific rIgG formation in rabbit sera using Ouchterlony assay. A: Serum collected 14 days after primary injection (Wells 2, 4 and 6) and untreated serum (Wells 1, 3 and 5). B: sera collected 14 days after 2nd, 3rd and 4th boosters were continuously diluted by a factor of 2 (½, $^1/_4$, $^1/_8$, $^1/_{16}$, $^1/_{32}$, $^1/_{64}$) and loaded into wells 1, 2 , 3, 4, 5 and 6. The well 0 is loaded with dIgG antigen.

Purification of total rIgG after immunization

Rabbit serum after the 4th booster was used to purify total rIgG. The purification was carried out in 2 steps including protein precipitation using 45% saturated ammonium sulfate (first step) and G protein affinity chromatography (second step). In the first step, after protein precipitation, SDS-PAGE analysis showed that there are 2 bands representing for heavy chain (~50 kDa) and light chain (~25 kDa) of rIgG (~150 kDa) found in the precipitate fraction but not in the supernatant (Figure 2) indicating that most rIgG has been precipitated and separated from serum. The rIgG precipitate was then dissolved and used in the second step in which only rIgG was principally trapped in G protein column resulting in formation of two peaks on chromatogram (Figure 3A). Analysis by SDS-PASE showed that as ecpected all rIgG was found in peak 2 but not peak 1 (Figure 3B). Estimation analysis based on the Quantity One software showed total rIgG reached 95.3% pure (data not shown). Our purification

results are equivalent to those showed in a previous study using ion exchange chromatography instead of G protein affinity chromatography [4].

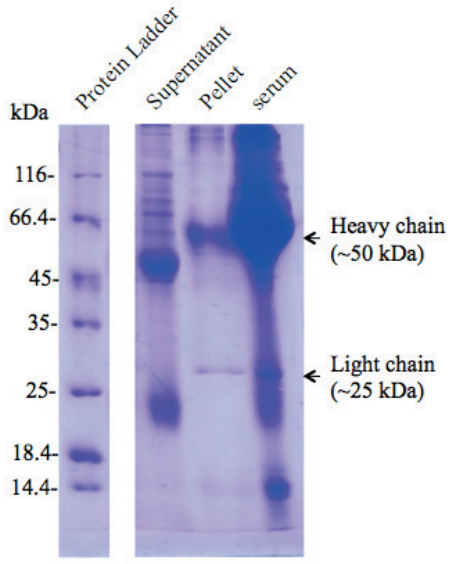

Figure 2. Analysis of precipitated rIgG by SDS-PAGE. rIgG was precipitated by 45% saturated ammonium sulfate.

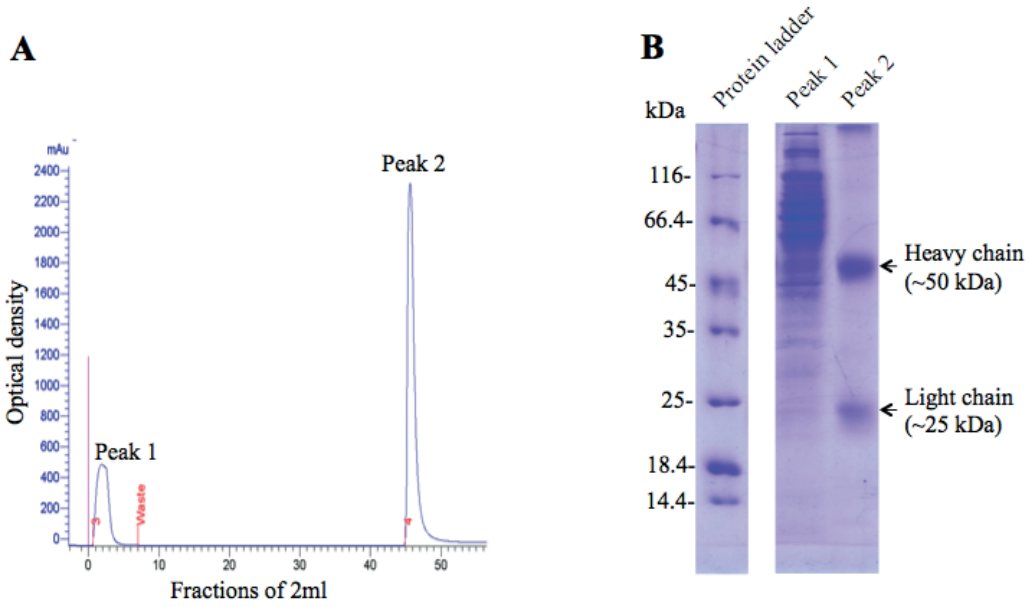

Figure 3. Analysis of total rIgG after G protein affinity chromatography by SDS-PAGE. A: Chromatogram; B: SDS-PAGE analysis.

Purification of specific rIgG against dIgG

The total rIgG (peak 2, Figure 3A) was applied to SK2 column containing dIgG ligand which traps only specific rIgG. On chromatogram showed in Figure 4, there are two peaks (designed as peak 2.1 and peak 2.2) of which the peak 2.1 contains non-specific rIgG (not captured in the column) but peak 2.2, in contrast, contains specific rIgGs (captured in column). Calculating the amount of rIgG recovered in each peaks showed that 38.5 mg of non-specific rIgG was found in peak 2.1 and 19 mg of specific rIgGs was found in peak 2.2 corresponding to 67% and 33%, respectively, of total recovered rIgG. The results demonstrate that the immunization procedure that we used in this study has produced a large amount of specific rIgG corresponding to ~ 1/3 of the total rIgG of rabbits.

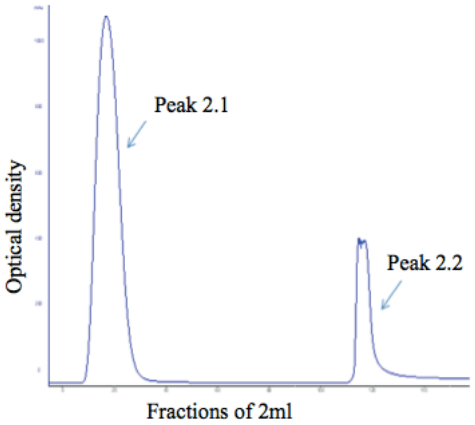

Figure 4. Chromatogram of specific rIgG purification using SK2 column.

Evaluation of purifed specific rIgG activity

Total rIgG (peak 2, Figure 3A), non-specific rIgG (peak 2.1, Figure 4) and specific rIgG (peak 2.2, Figure 4) were used to evaluate their activity in recognizing dIgG antigen using Dot blot assay (Figure 5). The results showed that total rIgG bound to dIgG mainly at high antibody concentrations ranging from 31.25 ng/ml to 500 ng/ml and high antigen amount ranging from 31.25 ng to 1000 ng. After specific and non-specific rIgG separation by affinity chromatography, the results showed that all specific rIgG was separated in peak 2.2 and that it bound to dIgG antigen at very low antibody concentration (3.90625 ng/ml) and very low antigen amount (31.25 ng). As expected, the non-specific rIgG could not bind to dIgG antigens at any situations.

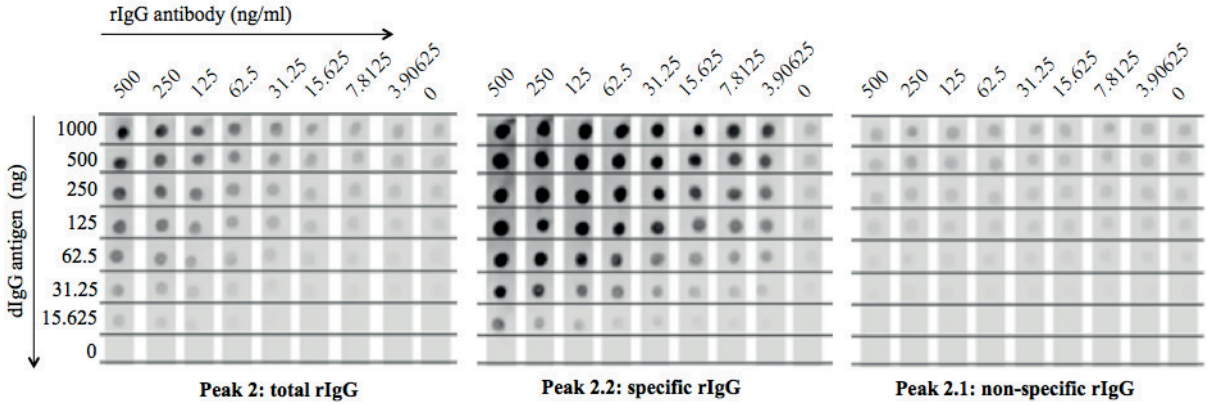

Figure 5. Acitvity of specific IgG after purification.

In a previous research, Sadeghi and colleagues carried out a similar procedure in which anti-dog IgG antibodies from rabbit were made [4]. However, they stopped at purifying total rIgG, meaning that in this rIgG mixture contains a significant portion of antibodies that are not used. Therefore, the use of this total rIgG for future applications can be difficult. In order to improve this and calculate the efficacy of antibody production, we created an affinity chromatography column in which the target dIgG antigen is used as a specific ligand for rIgG. Our results showed that approximately one-third of the specific rIgG was included in the total rIgG. The specific rIgG produced in this study achieves high purity, good activity, and can be applied to diagnostic tests in animals.

CONCLUSION

We present in this study a process of producing rIgG antibodies specific to dIgG antigen for applications in immunological diagnosis of dog diseases. The produced specific rIgG has 95.3% pure and remains active. This study can be extended to assess the cross-reactivity of the specific rIgG antibodies to the same types of antigens from humans and from other animals such as mice and goats. In addition, conjugation of HRP or fluorescence substances into this rIgG also helps to expand its scope of applications in immunological techniques (immunohistochemistry, ELISA, FACS). The process of antibody production in this study can also be extended and applied to other similar antigens.

ACKNOWLEGMENT

This work was funded by Nguyen Tat Thanh University's Scientific Research Fund (Project Nr. 2018.01.56/HD-KHCN).

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