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Polyclonal Antibody Production against Mouse Purified IgG2a towards Use in Basic Research

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Abstract

Background: The ability of polyclonal antibodies to react with many epitopes of an antigen makes them valuable reagents in research and diagnosis. The aim of this study was purification of mouse IgG2a and production of polyclonal antibody against purified mouse IgG2a subclass.

Materials and Methods: Mouse IgG2a was purified by ProA affinity. Verification method of the purified antibody was SDS-PAGE and ELISA by a mouse isotyping Kit. Rabbit was immunized with purified IgG2a. The production of antibody in rabbit was investigated by direct ELISA method. Rabbit serum was collected and precipitated at the final concentration of 50% ammonium sulfate. Polyclonal antibody was purified by ion-exchange chromatography and labeled with HRP. The titre and cross reactivity of product was detected by direct ELISA method.

Results: The results of SDS-PAGE in reduced and non-reduced conditions showed bands with 50-KDa, 25-30 KDa MW and a distinct band with 150 KDa MW. Isotype determination showed the presence of mouse IgG2a in related fraction. The titer of Anti-mouse polyclonal antibody was 200000. The optimum titer of prepared HRP conjugated IgG was 4000. Conjugated rabbit IgG has more cross reactivity with mouse IgG2b.

Conclusion: Taking together, affinity chromatography and ion-exchange chromatography are appropriate techniques for purification of mouse IgG subclasses and rabbit IgG, respectively.

Keywords: Affinity chromatography; Purification, Polyclonal antibody; IgG2a; ProA; ProG

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Introduction

There is a need for using purified antibodies for several reasons. Aside from therapeutic applications, antibodies are important tools in medical researches. Polyclonal Antibodies (pAbs) owing to greater avidity to a polyvalent antigen has wide applications in areas where such multiple interactions are required as in the case of heamagglutination, or whole-bacterial agglutination and complement mediated lysis. PAbs are used as ligands for the preparation of immunoaffinity columns and as coating or labeling reagents for the qualitative and quantitative determination of molecules in a variety of assays, such as enzyme linked immunosorbent assay (ELISA), double diffusion, radial immuno-diffusion, western blot and radioimmunoassay. The spatial expression of an antigen relative to an individual cell, or in the context of whole tissue, can be analyzed with antibodies using immunofluorescence and immunohistochemistry, respectively. The advantages of such polyclonal antibody generation are distinct

typically in vivo situations. Despite their widespread use, pAb purification presents various problems which are due to the complex composition of sera, and to the heterogeneity of pAbs in terms of charge and size. Countless restrictions such as: problems with batch-to-batch variations upon re-immunization with the same antigen, cross-reactivity, and safety issues limit their use in therapy (1-6).

Antibodies are often used in a labeled form. The Ig purification is the first step for obtaining labeled or conjugated antibodies (4, 7).

Protein G (ProG) and Protein A (ProA) affinity chromatography are the fastest methods for Ab purification. ProG, a bacterial cell wall protein with affinity for Immunoglobulin G (IgG), has been isolated from group C and G streptococcal strain (8-10). Staphylococcal ProA is one of the first discovered immunoglobulin binding molecules and has been extensively studied during the past decades (11). It removes > 99.5% of product impurities in a single step with high throughput (12). In our laboratory Agebati *et al* and Eyvazi *et al* have obtained protein with suitable purity after purification by ProA (13-15).

The aim of this work was purification of mouse IgG2a and production of polyclonal antibody against purified mouse IgG2a subclass. Purified mouse IgG2a is widely used as immunogen to obtain monoclonal and PAb. Purified rabbit polyclonal IgG could be used in designing mouse monoclonal isotyping kits.

Materials and Methods

Mouse Serum preparation

50 Balb/c mice were exsanguinated under anesthesia by ketamin. Sera were collected and diluted in PBS, pH 7.4 (1:1), and then its immunoglobulins were precipitated by equal volume of saturated ammonium sulphate, followed by overnight dialysis against PBS pH 7.4. In all stages, protein content of the fluids were assayed by a UV spectrophotometer (pharmacia biotech Uppsala, Sweden) at 280 nm.

Purification of mouse IgG by affinity chromatography

Affinity chromatography was performed on a ProG coupled column (Pharmacia, Uppsala, Sweden) for purification of mouse IgG. The column was equilibrated by 5 column volumes of 0.1 M sodium acetate buffer, pH 5 and IgG eluted by 0.1M glycine at pH 2.7. The flow-rate used at this stage was 0.5 ml/min and in all stages the volume (V) of each fraction was 2ml. The collected fractions were immediately treated by 1 M Tris-HCl buffer at pH 9.0, then were combined and precipitated by equal volume of saturated Ammonium sulfate. After dialysis, for purifying mouse IgG2a, this precipitate (25mg/3ml) was applied on a ProA affinity chromatography column (Pharmacia, Uppsala, Sweden). The column was washed by several volumes of PBS, pH 7.4. IgG1 and IgG2a elution was performed by 0.1M Sodium phosphate buffer, PH 6 and 0.1M Sodium citrate buffer, pH 4.5, respectively. The elution rate was 0.5ml/min. Finally the column was washed by PBS buffer containing 0.1% sodium azide (NaN3) at pH 7.4.

SDS-PAGE analysis

In all stages the chosen method for verification of the purified fractions was non-reduced and reduced Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE). The process was carried out according to laemmli method using 12% polyacrylamide with SDS (Merck, Darmstadt, Germany) (16). Protein bands were visualized by Coomassie blue (G250) staining technique.

Isotype determination

The subclass of the purified IgG was determined by ELISA using a mouse monoclonal sub isotyping kit (Thermo, Rockford, USA) containing rabbit anti-mouse IgG1, IgG2a, IgG2b, IgG3, IgM, and IgA following the procedure provided by the manufacturer.

Rabbit immunization and serum preparation

Six-month–old New Zealand white rabbit was chosen for this study. Guidelines on the production of pAbs have been published by various organizations, for example the Scientists Centre for Animal Welfare (17).

The first inoculation was done by 300µg/300µl of mouse IgG2a and equal volume of freund's complete adjuant (Sigma, Deisenhofen, Germany). The second, third and fourth inoculations using freund's incomplete adjuant (Sigma, Deisenhofen, Germany) were inoculated intramuscularly on days 21, 35 and 45. Final immunization was done without any adjuant on day 60. One week after final booster, blood was taken from rabbit's ear and production of antibody was investigated by ELISA test. The huge blood samples were taken from the rabbit's heart with moral considerations. Rabbit serum was collected and precipitated at the final concentration of 50% saturated ammonium sulfate. After dialysis, the protein concentration was determined by UV spectrophotometer (pharmacia, Uppsala, Sweden) at 280 nm (14, 18).

Purification of rabbit polyclonal antibody

For the purification of rabbit polyclonal IgG, ion-exchange chromatography was used on a DEAE-

sepharose column (pharmacia, Uppsala, Sweden). The column was equilibrated by 40 mM Tris-phosphate buffer at pH 8.1 until the pH of the external buffer was the same as the pH of the internal buffer. Elution was performed in two steps, first by tris-phosphate buffer at pH 7.4, then by tris-phosphate buffer containing 100 mM NaCl. Finally the column was washed by PBS containing NaN3 (0.1%). The flow-rate at all stages was 1 ml/min and each collected fraction volume was 2 ml. The collected fractions were assayed and purity was analyzed according to laemmli SDS-PAGE method (13, 16, 18).

Conjugation of rabbit IgG with HRP

The conjugation was performed by the Nakane and Kawaoi' s periodate method (19). Two mg Horseradish Peroxidase (HRP) (Sigma, Deisenhofen, Germany) was dissolved in 0.5cc distilled water in a dark glass. Then 100 µl of sodium periodate 0.1M (Merck, Germany) was added to the solution. Then the mixture was dialyzed against sodium acetate buffer (0.1mM, pH 4.4) followed by adding10 µl of 0.2 M carbonate-bicarbonate buffer and pH 9.5. Eight mg of purified rabbit IgG in 1 mL of sodium-carbonate buffer (10 mM, pH 9.5) was added to the active enzyme. Then 100µl fresh sodium borohydrate solution (Merck, Darmstadt, Germany) was added to the solution. The product was dialyzed overnight against PBS at 4 °C (13, 14).

Determination of titer and cross reactivity of conjugated IgG

Determination of conjugated IgG titer was done by direct ELISA. Mouse IgG2a (10µg/100µl) was added to each well of a micro plate and incubated at 37 °C for 45 min. The wells were washed by PBS-Tween (0.05% Tween 20) and blocked with 250 µl of blocking solution PBS-Tween (0.5% Tween 20). Then 100µl of 1:400, 1:800, 1:1600, 1:3200, 1:6400, and 1:12800 dilutions of prepared HRP conjugated anti-mouse IgG2a were added to each well. The reaction was developed using 100 µl of 3, 3', 5, 5'-tetramethyl benzidine (TMB) as substrate and the absorbance was determined at 450 nm through stopping the reaction by 5% sulfuric acid (Merck, Darmstadt, Germany) (14) .

Direct ELISA method was also used for cross reactivity determination with this differentiation that the coated Antigens were mouse IgG1, IgG2b, IgG3, IgM, and IgA. These antigens were coated on micro plate wells at 1/2000 and 1/4000 dilutions.

Ethics Statement

All procedures were performed according to the guidelines proposed by Animal Laboratory and approved by the Regional Medical Sciences Research Ethics Committee of Tabriz University of Medical Sciences

Results

Mouse Serum and affinity chromatography:

Protein content of the mice sera (V: 5cc) were 105 mg that reduced to 90 mg after precipitation by ammonium sulphate and dialysis. Purification by ProG column affinity chromatography yielded about 25 mg of IgG antibody which was about one third of the primary protein content. Optical Density (OD) of collected fractions is shown in Table 1.

Table 1. Optical Density of collected fractions in washing and elution stages of protein G affinity chromatography for purification of mouse IgG

Fraction	5	6	7	8	9	10	11
OD (Washing)	2.5	2.8	3	2.5	2.3	2	1.8
OD (Elution)	0.5	1.3	2	2.5	1.9	1.2	0.9

Protein content of the loaded fluid on ProA column was 25 mg and purification by this column yielded about 8 mg of mouse IgG2a subclass. The step wise IgG subclasses elution from the ProA column is shown in Figure 1. For the purification of IgG2a, in the first step, isolation of IgG1 was performed by phosphate buffer at pH 6 and then IgG2a eluted by Sodium citrate buffer at pH 4.5. Collected fractions were immediately treated by neutralizing Tris-HCl.

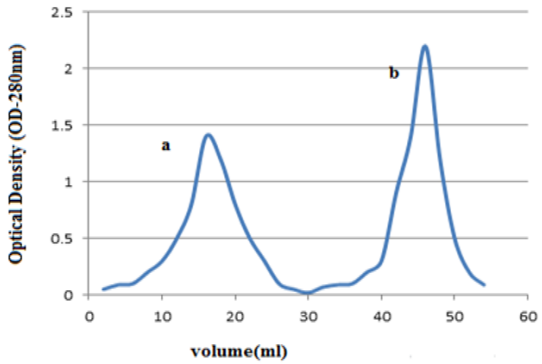

Figure 1. Chromatographic pattern of mouse IgG2a purification by ProA affinity chromatography a: IgG1 elution by phosphate buffer at pH 6 b: IgG2a elution by citrate buffer at pH 4.5.

SDS-PAGE analysis and isotype determination of purified mouse IgG2a

The results of reduced SDS-PAGE for determining the purity of mouse IgG2a showed a distinct band in 50-KDa molecular weight (MW) position corresponds to IgG heavy chains and the bands between 25-30 KDa MW positions correspond to IgG light chains (Figure 2A). In non-reduced SDS-PA

only one band with 150 KD MW was appeared that demonstrated purified antibody (Figure 2B).
The results of isotype determination by ELISA with mouse monoclonal isotyping kit showed that purified IgG2a has high absorbance (OD: 1.4) with pre-coated anti IgG2a and Anti kappa light chain (OD: 1.6) antibody in isotyping kit, but about pre-coted rabbit anti-mouse IgG1, IgG2b, IgG3, IgM, and IgA absorbance was < 0.2. These results demonstrated the presence of mouse IgG2a in purified fraction.

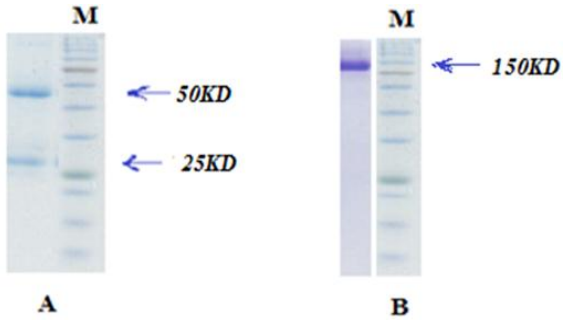

Figure 2. SDS-PAGE analysis of purified mouse IgG2a by ProA affinity chromatography. A: in reduced form, two bands were seen in 50 & 25 KDa MW positions B: in non-reduced condition only one band was seen in about 150 KDa MW position.

Purification of rabbit polyclonal antibody
The serum of the immune rabbit at 1/200000 dilution, indicated the highest absorbance in reaction with mouse IgG2a using ELISA method.
Protein content of the rabbit serum (V: 4cc) was 50mg. After precipitation by ammonium sulfate, 30 mg protein was loaded on the Ion exchange chromatography column. In the first step polyclonal IgG was eluted by Tris phosphate buffer, pH 8.1 (peak a- Figure 3). IgM, IgA and remnant of IgG were eluted by Tris phosphate buffer containing 100mM NaCl (peakb-Figure 3). Purification by Ion exchange chromatography yielded about 10mg IgG which was exactly one third of the loaded protein content.

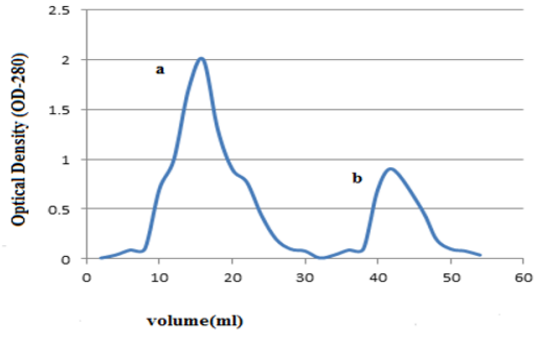

Figure 3. Purification of rabbit polyclonal IgG by ion-exchange chromatography. Peak a: elution of IgG by Tris-phosphate buffer, pH 7.4. Peak b: elution of other proteins by tris-phosphate buffer containing 100 mM NaCl.

SDS-PAGE analysis of rabbit polyclonal IgG
The results of reduced SDS-PAGE for determining thepurity of rabbit polyclonal IgG showed distinct band in 50-KDa and the bands between 25-30 KDa MW positions (Figure 4).

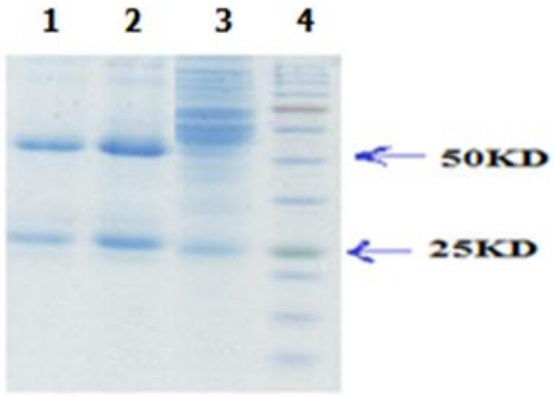

Figure 4. Reduced SDS-PAGE analysis of rabbit polyclonal IgG purified by ion exchange chromatography. Lanes1, 2: eluted IgG by Tris-phosphate buffer, pH 8.1. Lane 3: eluted proteins by 100 mM NaCl. Lane4: molecular weight marker.

Determination of titer and cross reactivity of conjugated IgG
The optimum titer of HRP conjugated IgG in direct ELISA was 4000. Conjugated anti mouse IgG2a polyclonal Ab at 1/2000 and 1/4000 dilutions didnot show any cross reactivity with mouse IgM & IgA (Table2).

Table 2. OD of cross reactivity determination of rabbit polyclonal Anti mouse IgG2a with other mouse IgG subclasses in 1/2000 and 1/4000 dilutions. IgG2a and PBS were coated as positive and negative control, respectively.

Mouse Ab	IgG2a	IgG1	IgG2b	IgG3	IgA	IgM	PBS
1/2000	2.06	0.5	0.9	0.5	0.09	0.08	0
1/4000	1.3	0.3	0.6	0.03	0	0	0

Discussion

Our antibody sources were mice and rabbit sera. Mammalian sera are a remarkable source of immunoglobulins. Mouse serum normally contains appreciable quantities of IgG1, IgG2a, IgG2b, and lesser amounts of IgG3. As a source of the IgG2a, Balb/c mice sera were used in our study. IgG2a subclass is a major component of mouse serum immunoglobulins. Observations suggested that IgG2a could predominate in antiviral immune responses (20-22).
In this study IgG2a was purified by affinity chromatography which appears to be a simple, one-step and time-efficient approach for capturing

proteins with high purity (19). A low pH condition is often required for elution of antibodies from ProA column, such a harsh condition, leads to degradations and conformational changes of the antibodies (1) - (2, 23). To prevent this problem, our collected fractions were immediately treated with neutralizing Tris-HCl.
Mouse IgG2a subclass with purity higher than 95% is a suitable and economic product.
Rabbit was used as host animal for polyclonal antibody production, because it has a convenient size, is easy to handle and bleed and produce adequate volumes of high-titer, high-affinity antiserum. Obtained polyclonal antibody titer (titer: 200000) could be beneficial for many types of detection methods and shows the high quality of the injected mouse IgG2a (14, 24).
Due to the high purity of injected mouse IgG2a, rabbit immunization was very effective using this product and produced polyclonal Ab had high titer and less cross reactivity with other mouse IgG subclasses except IgG2b. Anti-mouse IgG2a pAb has highest cross reactivity with mouse IgG2b. In one study, when the y2a chain nucleotide sequence was compared with the y2b chain nucleotide sequence, the percent homology of corresponding segments was 82% for the 5' flanking sequence, 87% for C H 1, 84% for intervening sequences (IVS1), 96% for the hinge, 95% for IVS 2, 94.6% for CH2, 86% for IVS 3, 74% for CH3, 89% for the3' un translated region, and 92% for the 3' flanking region. Obtained high cross reactivity of IgG2a and IgG2b can be resulted from a such high homology (25).
Rabbit polyclonal IgG was purified by Ion exchange chromatography. This method is used commonly in protein purification due to its high binding capacity and cost benefits. Rattana Wongchuphan *et al* successfully purified IgG with Ion exchange chromatography (26-28).This technique was well established in our laboratory for purification of IgG, also, Majidi *et al*, Abdolalizadeh *et al* and Eyvazi *et al* obtained polyclonal IgG with approximate purity of 95% (14, 18, 29).
The chosen method for purity verification of purified Abs in all stages was SDS-PAGE. In reduced SDS-PAGE results (Figure 2 and 4) the diffused bands between molecular weights of 25-30 KDa could be related to different levels of deglycosilation of protein during manipulation process (30).
Rabbit polyclonal IgG was conjugated with HRP. HRP is one of the most widely used enzyme labels in medical diagnostics and research applications (19). Using enzymes as labels offer several advantages over fluorescent and radio-labeled substances. Enzyme immunoassays reagents are more stable and do not have safety problems and are at least as sensitive as radioimmunoassay (30). HRP conjugated polyclonal antibody against mouse IgG2a could be used in isotype determining kits and would meet many educational and research requirements. In regards to production of antibodies it can last for a lifetime, a noticeable amount of anti-mouse IgG2a could be obtained, which would meet many educational and research requirements (14, 31). In other words, this product is an economical and suitable product towards self-sufficiency of the country.

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Author contributions

ZM contributed to doing laboratory methods, researching data, discussing the content and writing the Draft. JM and LA contributed to making study design, Help to laboratory methods, discussing the content, editing the manuscript.TK Help in design of the study. JA, SE, SD, MA and NM Help to laboratory methods and sampling.

Conflict of Interest
The authors report no conflicts of interest in this work.

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