Advanced Pharmaceutical Bulletin

Adv Pharm Bull, 2015, 5(1), 109-113
doi: 10.5681/apb.2015.015
http://apb.tbzmed.ac.ir

Research Article

Production and Purification of a Polyclonal Antibody Against Purified Mouse IgG2b in Rabbits Towards Designing Mouse Monoclonal Isotyping Kits

Sadeq Eivazi[1], Jafar Majidi[1,2]*, Leili Aghebati Maleki[1,2], Jalal Abdolalizadeh[3,1], Mehdi Yousefi[1,2], Majid Ahmadi[1], Somayeh Dadashi[1], Zahra Moradi[1], Elmira Zolali[4]

[1] *Immunology Research Center, Tabriz University of Medical Sciences, Tabriz, Iran.*
[2] *Department of Immunology, Faculty of Medicine, Tabriz University of Medical Sciences, Tabriz, Iran.*
[3] *Drug Applied Research Center, Tabriz University of Medical Sciences, Tabriz, Iran.*
[4] *Faculty of Pharmacy, Tabriz University of Medical Sciences, Tabriz, Iran.*

Article info

Article History:
Received: 16 July 2014
Revised: 9 September 2014
Accepted: 11 September 2014
ePublished: 5 March 2015

Keywords:
· Mouse IgG2b
· Affinity chromatography
· Ion-exchange chromatography
· Horse radish peroxidase conjugation

Abstract

Purpose: Mouse IgG subclasses containing IgG1, IgG2a, IgG2b and IgG3 have been defined and described both physiochemically and immunologically.

Methods: Sepharose beads conjugated with protein A affinity chromatography was used for purification of mouse IgG2b. Sodium citrate buffer (0.1 M, pH: 3.5) was used for separation of mouse IgG2b. Verification of the purified fractions was monitored by SDS-PAGE (polyacrylamide gel electrophoresis) in reducing condition. Immunized rabbit serum was collected and precipitated at the final concentration of 50% ammonium sulfate. After dialysis against tris-phosphate buffer (pH: 8.1) ion exchange chromatography column was used for purification of rabbit anti-mouse IgG2b. The periodate method was performed for conjugation with some variations. After conjugation, direct ELISA was used to determine the titer of HRP conjugated rabbit IgG against mouse IgG2b.

Results: The titer of rabbit anti-mouse IgG2b that determined by ELISA was 32000. The purity of rabbit anti-mouse IgG2b was about 95%. The optimum dilution of prepared HRP conjugated IgG was 1:10000. This study showed that ion-exchange chromatography and affinity chromatography could be appropriate techniques for purification of mouse IgG and IgG subclasses respectively.

Conclusion: This study showed that affinity chromatography could be an appropriate method for purification of IgG2b antibodies.

Introduction

Antibodies (or immunoglobulins) are circulating proteins that are produced in response to exposure to foreign antigens. Mice immunoglobulins contain IgG, IgA, IgM and IgE. Four major subclasses of mouse IgG containing IgG1, IgG2a, IgG2b and IgG3 have been defined and described, both physiochemically and immunologically.[1-3] Isolation methods of immunoglobulins in mice have been described by Kalpaktsoglou et al.[4] Many years ago, protein A was shown to interact with mouse antibodies such as IgG2a, IgG2b and IgG3, but not with IgG1, IgM or IgA.[3,5] Protein A from Staphylococcus aureus has bacterial Fc (or fragment crystalline) receptors which bind to IgG. Protein A, with two binding sites, interacts with the Fc fragment of antibodies.[6,7] The isolation and purification of proteins that have close functional and structural relationships requires many specific procedures. The two most common techniques that are applied in order to purify antibodies are affinity chromatography and ion-exchange chromatography. The selection of an appropriate technique for the isolation and purification of immunoglobulins depends upon the purity and yield of the immunoglobulins.[8] Immunoglobulins are essential tools used by researchers in their experiments, and their usage has led to many medical developments. The manufacture and use of antibodies as identification and purification ligands has helped to develop bioresearch and diagnostic techniques. Mammalian sources of immunoglobulin are efficient and notable resources used extensively in analytic and therapeutic applications.[9,10] The purification of these antibodies is beneficial for many types of detection methods. For example, polyclonal antibodies are used for the assessment, detection, and purification of specific proteins. These antibodies can be used in biochemical and biological research, usually as ligands for the preparation of immunoaffinity columns. They are also used for coating or labeling reagents for the determination of molecules in immunoassay tests, such as enzyme-linked immunosorbent assay (ELISA), Western blot tests, immunoradiometric assay (IRMA), radioimmunoassays (RIA), and other applications.[11-13] Purity of the antibodies

***Corresponding author:** Jafar Majidi, Tel: +98 (41) 33364665, Fax: +98 (41) 33364665, Email: Majidij@Tbzmed.ac.ir

is essential because other contents in the source material may interfere with the detection process.[8] Horseradish peroxidase (HRP) is used often as an enzyme label in medical diagnostic and investigation.[14] However, enzyme-labeled antibodies are used in the ELISA method, immunoblotting and histochemical staining. Enzyme-labeled antibodies offer several advantages over fluorescent and radio-labeled substances. Furthermore, they can provide an instant visual result and good sensitivity. The consistency of enzyme immunoassay reagents is excellent, and they do not have the safety problems related to radio isotopic labels. Enzyme assays can also be as sensitive as radioimmunoassays. In addition, several enzyme detection methods are observable or use a standard spectrophotometer, reducing the need for expensive and sophisticated equipment. In this present study, our aim was the purification of mouse IgG2b, and the production, purification and HRP conjugation of polyclonal IgG against mouse IgG2b in rabbits, towards designing mouse monoclonal isotyping kits.

Materials and Methods

Purification of mouse IgG2b

For production of polyclonal antibodies against mouse IgG2b, fifty mice were bled and the collected serum was pooled. First, they were clarified by centrifuge (1000 g, 15 min) and then diluted 1:1 with a phosphate buffer saline solution (PBS, pH: 7.2).[15] After dilution, equal volumes of saturated ammonium sulfate and the diluted serum were mixed by gentle stirring and the gradual addition of the saturated ammonium sulfate solution. After centrifugation (1000 g for 20 min.), the precipitate was washed twice with a 50% saturated ammonium sulfate solution. The final precipitate was dissolved in PBS, and then overnight dialysis was performed against the PBS. After dialysis was performed against PBS for purification use, Sepharose beads conjugated with Protein A, and the column affinity chromatography equilibrated with 5-10 column volumes of the same buffer. In this study, for the purification of IgG2b, in the first stage, the isolation of IgG1 and then IgG2a was performed by a specific buffer in a defined pH. The initial immunoglobulin fraction was loaded onto the column, which was equilibrated at a flow rate of 60 cm/h with the selected buffer. After elution of the unbound material and separation of IgG1 and IgG2a, the isolation of IgG2b (the eluent) was changed to a 0.1 M sodium citrate buffer (pH: 3.5) in order to purify the IgG2b subclass. We confirmed the purified fractions by performing a SDS-PAGE test.

Confirmation of the IgG2b purity by SDS-PAGE

The purity of the eluted fractions from the affinity column was checked by the SDS-PAGE test in a reducing condition according to the standard Laemmli protocol.[16] The final concentration of the polyacrylamide solution was 13%. Samples were boiled with 2% SDS for 10 min, and were loaded onto an electrophoresis gel. After they separated, we tested for detection of the protein bands by staining them with Coomassie Brilliant Blue G 250.[17]

Immunization of rabbits with mouse IgG2b

300 µg/300 µl of the purified IgG2b was mixed with equal volumes of Complete Freund's adjuvant (Sigma) and was then injected intra-muscularly (IM) into a 6-month–old New Zealand white rabbit. The rabbit was fed a regular commercial diet. The second and third injections were performed on days 21 and 35 with Freund's incomplete adjuvant (Sigma), and finally an injection was done on day 45 with Freund's incomplete adjuvant, or without any adjuvant. After the last immunization, blood samples were collected from the rabbit and its antibody titer was checked by ELISA tests. This study was approved by the Regional Medical Sciences Research Ethics Committee of Tabriz University of Medical Sciences.

Purification of rabbit anti-mouse IgG2b

Immunized rabbit serum was collected and precipitated using a 50% ammonium sulfate. After dialysis against a tris-phosphate buffer (pH: 8.1), the protein concentration was determined by UV spectrophotometer (280 nm) and loaded onto an ion-exchange chromatography column packed with diethylaminoethyl (DEAE)-Sepharose fast flow (Pharmacia), which was equilibrated with tris-phosphate buffer (pH: 8.1). The column elution was performed in two steps, the first eluting with tris-phosphate buffer, and second eluting with tris-phosphate buffer containing 100 mM of NaCl. The collected samples for protein analysis were assayed by using a UV spectrophotometer (set on a 280 nm absorbance). The washed proteins were collected in 3 mL fractions and analyzed by the SDS-PAGE test previously described.

Conjugation of rabbit IgG with peroxidase (HRP)

The periodate method was performed for conjugation with some variations.[18] First, 2 mg of peroxidase (Sigma) was dissolved in 0.5 mL of distilled water in a dark glass bottle. Then 100 µl sodium periodate (Merck) was added to the solution, and the container was kept at room temperature on a stirrer for 20 min. The blend was dialyzed against a sodium acetate buffer (0.1 mM, pH: 4.4) at 4°C overnight followed by the addition of 10 µl of carbonate-bicarbonate buffer (0.2 M, pH: 9.5). Four mg of the purified rabbit anti-mouse IgG2b in 1 mL of carbonate-bicarbonate buffer (10 mM, pH: 9.5) was added to the active enzyme, and the bottle was put on the stirrer. Then 100 µl of fresh sodium borohydrate solution (Merck) was added to the solution and was kept at 4°C for 1.5 hours on the stirrer. The product was then dialyzed overnight against PBS at 4°C with the addition of BioStab antibody stabilizer (Sigma Alderich).

Enzyme linked immunosorbent assay (ELISA)

A direct ELISA was used to determine the titer of the HRP conjugated rabbit anti-mouse IgG2b. For this test, 100 µl of purified mouse IgG2b, which was diluted 1:100 in PBS (10 µg), was added to each well of a 96-well micro titer plate and incubated at 4°C for 24 hours. The wells were washed with a PBS-Tween (0.05% Tween 20) three times and blocked with 200 µl blocking

solution (PBS–0.5% Tween 20). After the washing step, 100 µl of 1:500, 1:1000, 1:2000, 1:5000, 1:10000 and 1:20000 dilutions of prepared HRP conjugated anti-mouse IgG2b were added to each well. The reaction was developed using 100 µl of 3, 3', 5, 5'-tetramethylbenzidine (TMB) as a substrate and the absorbance was determined at 450 nm after stopping the reaction using a 5% sulfuric acid solution (Sigma).

Results

Purification of mouse IgG2b

After initial purification of mouse IgG2b, the purity of the eluted fraction was analyzed by SDS-PAGE, proceeding in descending order. The purity of the fraction was up to 90%. This indicated the electrophoretic pattern of purified mouse IgG2b (Figure 1).

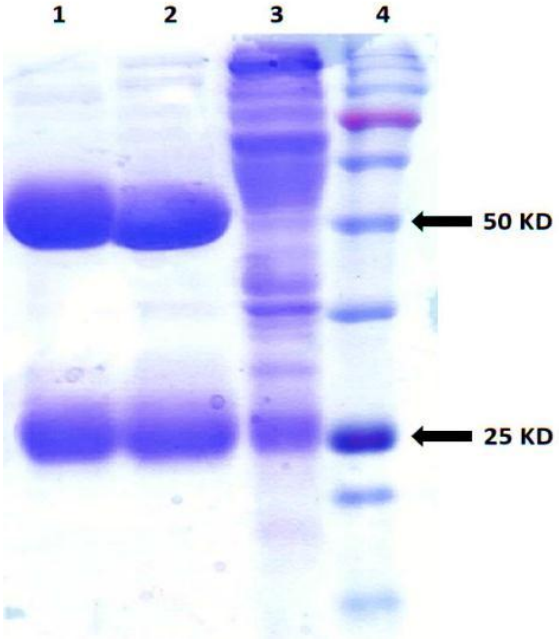

Figure 1. SDS-PAGE of mouse IgG2b subclass, purified by protein A affinity chromatography in reduced conditions and stained with Coomassie Brilliant Blue G-250. Purified mouse IgG2b (Lanes 1 and 2), unbounded material (Lane 3) and molecular weight markers (Lane 4).

Production of rabbit anti-mouse IgG2b

In order to check the production of antibodies in the rabbit and evaluate the effectiveness of immunization, ELISA tests were performed. The titer of anti-mouse IgG2b immunoglobulins determined by ELISA was 32000.

Purification of rabbit anti-mouse IgG2b immunoglobulins

The purification of a polyclonal antibody from an immunized rabbit by DEAE ion-exchange chromatography resulted in a highly pure fraction. The protein content of this fraction after elution from IEC was 11 mg, which was about one third of the primary protein content (Figure 2).

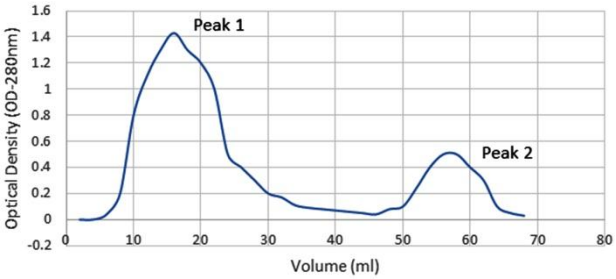

Figure 2. Chromatographic pattern of purified rabbit anti-mouse IgG2b by ion-exchange column with Tris-phosphate buffer (pH: 8.1) (peak 1) and 100 mM NaCl elution (peak 2). Sample, Rabbit IgG; Matrix, DEAE Sepharose; working buffer, first step is Tris-phosphate buffer and second step is Tris-phosphate buffer +100 mM NaCl.

SDS-PAGE analysis

The results of the SDS-PAGE for determining the purity of rabbit anti-mouse IgG2b (which were purified by ion-exchange chromatography) have been shown on Figure 3. A distinct band with a molecular weight of about 50 kDa indicates that there are heavy chains of rabbit IgG, and bands between molecular weights of 20-30 kDa indicate that there are light chains of rabbit IgG. The purity of the rabbit anti-mouse IgG2b was about 95%. The SDS-PAGE analysis showed that purification of IgG by ion-exchange chromatography resulted in a highly pure and acceptable product.

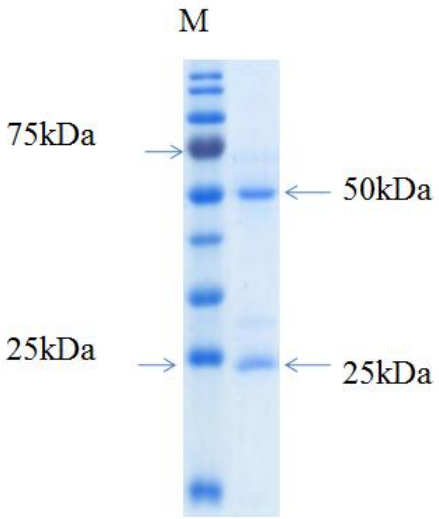

Figure 3. SDS- PAGE of purified rabbit anti-mouse IgG2b in reduced form. Two bands of 25 and 50 kDa, corresponding to the heavy and light chains were detected.

Enzyme linked immunosorbent assay

A direct ELISA test was used to determine the optimum titer of HRP-conjugated rabbit anti-mouse IgG2b. The optimum dilution of prepared HRP to conjugated IgG was found to be 1:10000.

Discussion

There is a long history of research regarding methods of isolation and purification of antibodies in the effort to reach the purest method. These achievements in the mass production of antibodies will allow widespread usage of

antibodies as diagnostic tools and as pharmaceutics in order to better humans' health. After the first antibody-based therapy was introduced, many researchers attempted to use immunoglobulins for the diagnosis and treatment of numerous diseases.[19-21] In this study, mouse IgG2b was purified and used as an immunogen for the immunization of a rabbit. Purification of the mouse IgG2b was performed by affinity chromatography. In the affinity chromatography, protein A was used for the isolation of mouse IgG2b. Studies have widely shown the occurrence of immunoglobulin binding in many mammalian species to Staphylococcal protein A, particularly with regard to the subclasses of IgG.[5,22] The ability of protein A to bind to Fc fragments of immunoglobulins make them good ligands for the analysis of antibodies.[23] Protein-A chromatography is the purification technique which is often selected to purify antibodies on a large scale. The binding of mouse IgG to protein A-Sepharose is pH-dependent and is most useful for the purification of mouse IgG subclasses. After the isolation and purification of IgG2b, the purity of IgG2b was confirmed by SDS-PAGE. Then, the rabbit was immunized with IgG2b. After several immunizations, the blood of the rabbit was collected, and ion-exchange chromatography was used to purify the polyclonal antibody. To estimate the polyclonal antibody titer, an ELISA test was the most suitable method. Defining a titer of 32000 in an ELISA test shows the high quality of the product. Therefore, this antibody is highly economical, and in regards to the 30 mL volume of serum taken from the rabbit, a noticeable amount of anti-mouse IgG2b could be obtained, which would meet many educational and research requirements in the country's programs. When it comes to electron microscopy, a polyclonal antibody is preferable over a monoclonal antibody where detection of an antigen with different epitopes is the target of study, since the polyclonal antibody can bond to more connective sites, resulting in better sensitivity. The purification of immunoglobulins has numerous applications; especially for polyclonal antibody production.[24] The purification of rabbit anti-mouse IgG2b involves several stages, including salt precipitation, dialysis, and anion exchange. We used ion-exchange chromatography for the isolation and purification of the rabbit anti-mouse IgG2b antibody. The isolation of proteins from ion-exchange chromatography are related to factors such as buffer type and pH, flow rate of the mobile phase, length of gradient, characteristics of the proteins, charged ligand bound as stationary phase and ionic strength. The best conditions for antibody purification must include changing some or all of these factors. By changing the mobile phase so that more counter ions are present, the proteins elute in order of increasing interactions with the stationary phase.[25] This method was well established in our laboratory for the purification of the IgG antibody.[26] After purification, we achieved a protein with a purity of about 95%. The results of the SDS-PAGE showed that proteins with a molecular weight of about 50 kDa were rabbit IgG heavy chains, and bands between molecular weights of 20-30 kDa were rabbit IgG light chains. In a direct ELISA test against mouse IgG2b (10 µg/mL), the optimum dilution of prepared HRP conjugated IgG was 1:10000. This antibody purification is beneficial for many types of detection methods.

Conclusion

In conclusion, purified immunoglobulin and its conjugation with HRP can be used for research and diagnosis using mouse monoclonal isotyping kits. Polyclonal antibodies can be used for the assessment, detection, and purification of specific proteins.

Acknowledgments

We would like to thank the Immunology Research Center (IRC) and Drug Applied Research Center, Tabriz University of Medical Sciences for their kind assistance. This work was supported by a grant from the Immunology Research Center (IRC). The manuscript was written based on a dataset of a master thesis registered in Tabriz University of Medical Sciences.

Ethical Issues

Not applicable.

Conflict of Interest

The authors report no conflicts of interest in this work.

References

1. Fahey JL, Wunderlich J, Mishell R. The Immunoglobulins of Mice. I. Four Major Classes of Immunoglobulins: 7s Gamma-2-, 7s Gamma-1-, Gamma-1a (Beta-2a)-, and 18s Gamma-1m-Globulins. *J Exp Med* 1964;120:223-42.
2. Grey HM, Hirst JW, Cohn M. A new mouse immunoglobulin: IgG3. *J Exp Med* 1971;133(2):289-304.
3. Prouvost-Danon A, Binaghi R, Rochas S, Boussac-Aron Y. Immunochemical identification of mouse IgE. *Immunology* 1972;23(4):481-91.
4. Kalpaktsoglou PK, Hong R, Good RA. The five classes of immunoglobulins in normal C3H and BALB/c mice. *Immunology* 1973;24(2):303-14.
5. Kronvall G, Grey HM, Williams RC, Jr. Protein A reactivity with mouse immunoglobulins. Structural relationship between some mouse and human immunoglobulins. *J Immunol* 1970;105(5):1116-23.
6. Forsgren A, Sjoquist J. "Protein A" from *S. Aureus*: I. pseudo-immune reaction with human immunoglobulin. *J Immunol* 1966;97:822-7.
7. Goudswaard J, Van Der Donk JA, Noordzij A, Van Dam RH, Vaerman JP. Protein A reactivity of various mammalian immunoglobulins. *Scand J Immunol* 1978;8(1):21-8.
8. Huse K, Bohme HJ, Scholz GH. Purification of antibodies by affinity chromatography. *J Biochem Biophys Methods* 2002;51(3):217-31.

9. Gallacher G. Polyclonal catalytic antibodies. *Biochem Soc Trans* 1993;21(4):1087-90.
10. Gathumbi JK, Usleber E, Martlbauer E. Production of ultrasensitive antibodies against aflatoxin B1. *Lett Appl Microbiol* 2001;32(5):349-51.
11. Shin K, Hayasawa H, Lonnerdal B. Purification and quantification of lactoperoxidase in human milk with use of immunoadsorbents with antibodies against recombinant human lactoperoxidase. *Am J Clin Nutr* 2001;73(5):984-9.
12. Calabozo B, Duffort O, Carpizo JA, Barber D, Polo F. Monoclonal antibodies against the major allergen of Plantago lanceolata pollen, Pla l 1: affinity chromatography purification of the allergen and development of an ELISA method for Pla l 1 measurement. *Allergy* 2001;56(5):429-35.
13. Cheung HY, Chan KM, Ng TB, Cheng CH. Production of a polyclonal antibody against recombinant goldfish prolactin and demonstration of its usefulness in a non-competitive antigen-capture ELISA. *Comp Biochem Physiol B Biochem Mol Biol* 2002;131(1):37-46.
14. Handley RS, Akhavan-Tafti H, Schaap AP. Chemiluminescent Detection in High Volume Ligand-Binder Assays. *J Clin Lig* Assay 1998;20:302-12.
15. Stec J, Bica L, Kuzmak J. Isolation and purification of polyclonal IgG antibodies from bovine serum by high performance liquid chromatography. *B Vet I Pulawy* 2004;48:321-7.
16. Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 1970;227(5259):680-5.
17. Blakesley RW, Boezi JA. A new staining technique for proteins in polyacrylamide gels using coomassie brilliant blue G250. *Anal Biochem* 1977;82(2):580-2.
18. Nakane PK, Kawaoi A. Peroxidase-labeled antibody. A new method of conjugation. *J Histochem Cytochem* 1974;22(12):1084-91.
19. Robinson MK, Weiner LM, Adams GP. Improving Monoclonal Antibodies for Cancer Therapy. *Drug Dev Res* 2004;61(3):172-87.
20. Schrama D, Reisfeld RA, Becker JC. Antibody targeted drugs as cancer therapeutics. *Nat Rev Drug Discov* 2006;5(2):147-59.
21. Enever C, Batuwangala T, Plummer C, Sepp A. Next generation immunotherapeutics--honing the magic bullet. *Curr Opin Biotechnol* 2009;20(4):405-11.
22. Kronvall G, Williams RC, Jr. Differences in anti-protein A activity among IgG subgroups. *J Immunol* 1969;103(4):828-33.
23. Hage DS. Affinity chromatography: a review of clinical applications. *Clin Chem* 1999;45(5):593-615.
24. Verdoliva A, Basile G, Fassina G. Affinity purification of immunoglobulinss from chicken egg yolk using a new synthetic ligand. *J Chromatogr B* 2000;749(2):233-42.
25. Tishchenko GA, Bleha M, Skvor J, Bostik T. Effect of salt concentration gradient on separation of different types of specific immunoglobulins by ion-exchange chromatography on DEAE cellulose. *J Chromatogr B Biomed Sci Appl* 1998;706(1):157-66.
26. Aghebati Maleki L, Majidi J, Baradaran B, Abdolalizadeh J, Kazemi T, Aghebati Maleki A, et al. Large Scale Generation and Characterization of Anti-Human CD34 Monoclonal Antibody in Ascetic Fluid of Balb/c Mice. *Adv Pharm Bull* 2013;3(1):211-6.