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Large-scale preparation of purified monoclonal antibody from cell culture supernatant: A case study with a monoclonal antibody to ζ-globin chain

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ABSTRACT

Background: Nowadays, monoclonal antibodies have become important tools used in biomedical research, diagnosis and treatment of diseases. The mAbs are isolated from cell culture media, which usually contain different proteins in addition to antibodies. Obviously, antibody purification is becoming critical to guarantee its reliable application. Protein A and G resins are the most efficient and widely-used ligands in chromatographic methods. Nevertheless, some mAbs could not be purified by Protein A or G column.

Objectives: In order to explore the alternative method for purification of an IgG1 mAb, four different types of affinity chromatography were studied and compared in term of efficiency.

Materials and method: Chromatography using four commercial ligands, Protein A, Protein G, Protein L and engineered recombinant Protein A, was employed to purify the anti-zeta globin chain mAb clone PL3. The performance of the chromatography was compared in terms of yield, purity and biological activity of mAbs.

Results: The mAb PL3 could only be purified by using engineered recombinant Protein A column. The biological activity and purity of the purified mAbs were checked by ELISA and SDS-PAGE, respectively. It was found that the obtained purified mAbs were high purity and retained their biological activity.

Conclusion: In conclusion, engineered recombinant Protein A column is an alternative technique for large-scale purification of mAbs produced by unusual hybridoma clone that could not be purified by other columns.

Introduction

In 1975, Kohler and Milstein discovered hybridoma technology, which made possible the production of monoclonal antibodies (mAbs) possessing high affinity and specificity toward defined targets.[1-4] Nowadays, monoclonal antibodies have become obligatory tools used in biomedical analysis, purification, diagnosis and treatment of various diseases. For diagnosis, antibodies are the ideal biological recognition reagents; therefore, they are useful in a range of analytical platforms, e.g. immunohistochemistry, immunocytochemistry, enzyme linked immunosorbant assay (ELISA), flow cytometric analysis and immunosensors.[2, 5-7] The mAbs are usually isolated from cell culture media, which contain different proteins in addition to antibodies. Hence, the mAb purification becomes imperative for its reliable

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application and influences the reproducibility and stability of immunoassays and immunotherapy. Antibody purification can be achieved by several methods based on the specific physical and chemical properties of antibodies, such as size, solubility, charge, hydrophobicity and binding affinity.[5, 8-11] The techniques that are applied for antibody purification include precipitation, electrophoretic separation, filtration and liquid and affinity chromatography.[11] In general, affinity chromatography-based purification continues to be the most efficient and widely used.[5, 7-10, 12] However, the used ligands in chromatographic methods are species- and isotype-dependent and thus, adsorptions sometimes exhibit very low capacity.[5, 8-10, 12, 13]

From our previous study, mAb clone *PL3*, which is a mouse IgG1 anti-ζ-globin chain mAb, was generated by hybridoma technique.[14] The mAb clone *PL3* was used to develop the immunoassay for screening α-thalassemia 1 Southeast Asian (SEA)-type, which is the most common mutation of α-thalassemia 1 in the Thai population. Unfortunately, Protein G column, the common purification method for mouse mAb IgG isotypes, cannot be used to purify mAb clone *PL3*. Therefore, a suitable protein column ligand is needed to be investigated in order to produce a large amount of mAb clone *PL3*. We compared four commercial ligands (Protein A, Protein G, Protein L and modified Protein A) for the anti-ζ globin chain mAb clone *PL3* purification. The performance of the specific chromatography was compared in terms of yield, purity and biological activity of the mAb. Our study can be applied for purification of other unusual mAbs.

Materials and methods

Materials

Antibodies

Mouse anti-ζ globin chain mAb clone *PL3*[14]; mouse anti-γ4 globin chain mAb clone PB1[15]; mouse anti-γ4 globin chain mAb clone *Thal* N/B; mouse anti-Ag85B mAb clone *AM85B-8B*[16] were generated in our research center. The horseradish peroxidase (HRP)-conjugated rabbit anti-mouse immunoglobulin Abs were obtained from Dako (Glostrup, Denmark).

Affinity chromatographic resins

Four commercial affinity chromatographic resins were used in this study (Table 1). The first resin was Protein A-coated agarose (Thermo Fisher Scientific, Waltham, MA, USA). Protein A is a cell wall component produced by *Staphylococcus aureus*. This ligand has the ability to bind specifically to the Fc region of immunoglobulin molecules, especially IgG. The second resin was recombinant Protein G immobilized on agarose (GE Healthcare Bio-Sciences, Uppsala, Sweden). The recombinant Protein G contains two IgG binding regions. The albumin binding region of native Protein G has been genetically deleted to avoid undesirable cross-reactions with albumin. The third resin was recombinant Protein L immobilized on agarose (GE Healthcare Bio-Sciences). This resin has strong affinity to the variable region of the antibody's kappa light chain. The fourth resin was MabSelect SuRe (GE Healthcare Bio-Sciences), an engineered recombinant Protein A, which is composed of an alkali-stabilized Protein A derivative immobilized on agarose. This ligand has enhanced alkali stability and a high binding capacity for IgG.

Table 1 Chromatographic conditions used for each affinity chromatography.

Chromatography name	Binding buffer	Elution buffer	Regeneration buffer
Protein A	20 mM sodium phosphate bufferf, 150 mM NaCl, pH 7.5	0.1 M glycine-HCl, pH 2.7	15 mM NaOH and 5 N NaOH
HiTrap Protein G	20 mM sodium phosphate buffer, pH 7.0	0.1 M glycine-HCl, pH 2.7	15 mM NaOH and 5 N NaOH
HiTrap Protein L	20 mM sodium phosphate buffer, 150 mM NaCl, pH 7.0	0.1 M glycine-HCl, pH 2.7	15 mM NaOH and 5 N NaOH
Mabselect SuRe	20 mM sodium phosphate buffer, 150 mM NaCl, pH 7.0	0.1 M sodium citrate, pH 3.0-3.6	15 mM NaOH and 5 N NaOH

Hybridoma cell culture

Hybridomas producing anti-ζ globin chain mAb clone *PL3*, isotype IgG1,[14] were cultured in completed IMDM (cIMDM), which is 10% fetal bovine serum (FBS)-IMDM supplemented with Gentamycin (4 mg/mL) and Fungizone (5 mg/mL), at 37°C in 5% CO_2 humidified incubator. To eliminate IgG contamination from supplement serum before antibody purification process, the *PL3* hybridoma cells were adapted for culturing in Hybridoma-Serum Free Media (SFM) (Thermo Fisher Scientific) by gradually increasing the ratio of Hybridoma-SFM to cIMDM from 0:100 to 25:75, 50:50, 75:25 until 100% Hybridoma-SFM.

Preparation of hybridoma culture supernatant

The *PL3* hybridoma cells ($1x10^6$ cells/mL) were cultured in 100% Hybridoma-SFM at 37°C in 5% CO_2 humidified incubator for 5 days. The culture supernatant was collected, centrifuged and filtered through a 0.45 µm filter to remove cells and cell debris.

Purification of mAbs

All affinity chromatography was performed at ambient temperature using an ÄKTAprime plus equipped with a

fraction collector (GE Healthcare Bio-Sciences). One mL packed beads in 1 cm internal diameter columns, were carried out. The buffer system was suggested by the individual medium manufacturers and described in Table 1.

Before antibody purification, all materials were equilibrated at room temperature. Air bubbles were removed from all the buffers using a sonicator. Both buffers and hybridoma culture supernatant were filtrated through a 0.45 µm filter. The column was equilibrated with 10 column volumes of binding buffer at 1 mL/min of flow rate. Culture supernatant was loaded by pumping into the column, followed by at least 10 column volumes of binding buffer to wash unbound protein. After the absorbance reached a steady baseline, the column was eluted with elution buffer, maintaining a flow rate of 1 mL/min. The fractions were collected in tubes that contained neutralizing buffer (1 M Tris-HCl, pH 9.0) to neutralize pH. The concentration of protein obtained in each fraction was measured using a Nanodrop (Thermo Fisher Scientific). After the purification process, the column was washed by adding 10 column volumes of regeneration buffer followed by binding buffer and filled up with 20% ethanol for new round purification.

Indirect ELISA

Indirect ELISA was performed as described before.[14] Hb Bart's hydrops fetalis hemolysates containing Hb Bart's and ζ globin chain (kindly provide by Professor Dr. Suthat Fucharoen) or HbF were coated on the ELISA plate. *PL3* hybridoma culture supernatant was added to the antigen-coated plate. The antigen-antibody complexes were monitored by adding horseradish peroxidase-conjugated rabbit anti-mouse immunoglobulins antibody (Dako) for 1 hr at 37°C. After that, 3,3',5,5' tetramethylbenzidine (TMB) substrate (Zymed, South San Francisco, CA, USA) was added and incubated for 15 mins at room temperature in the dark. The reaction was stopped with 1 N HCl. The colorimetric signal was measured at OD 450 nm.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was performed to determine the purity of the purified mAbs. Five micrograms of proteins were loaded in each lane and separated on 10% polyacrylamide gel by electrophoresis. The SDS-PAGE was performed as described elsewhere. The protein bands were visualized by PageBlue Protein Staining (Thermo Fisher Scientific).

Results

Determination of monoclonal antibodies against ζ-globin chains

Firstly, we determined the activity of mAb *PL3* after thawing and propagating of the *PL3* hybrodoma cells in cIMDM by indirect ELISA. As shown in Figure 1, the hybridoma culture supernatant showed positive reactivity with Hb Bart's hydrop fetalis hemolysates and did not show cross-reaction with HbF. The positive control, anti-γ-globin chain mAb clone *Thal* N/B, showed positive reactivity with both Hb Bart's lysate and HbF. In contrast, the isotype-matched control mAb, *AM85B-8B*, showed negative reactivity with all tested antigens. These results indicated that the *PL3* hybridoma cells could still produce and secrete the mAbs specifically reacting to ζ-globin chains

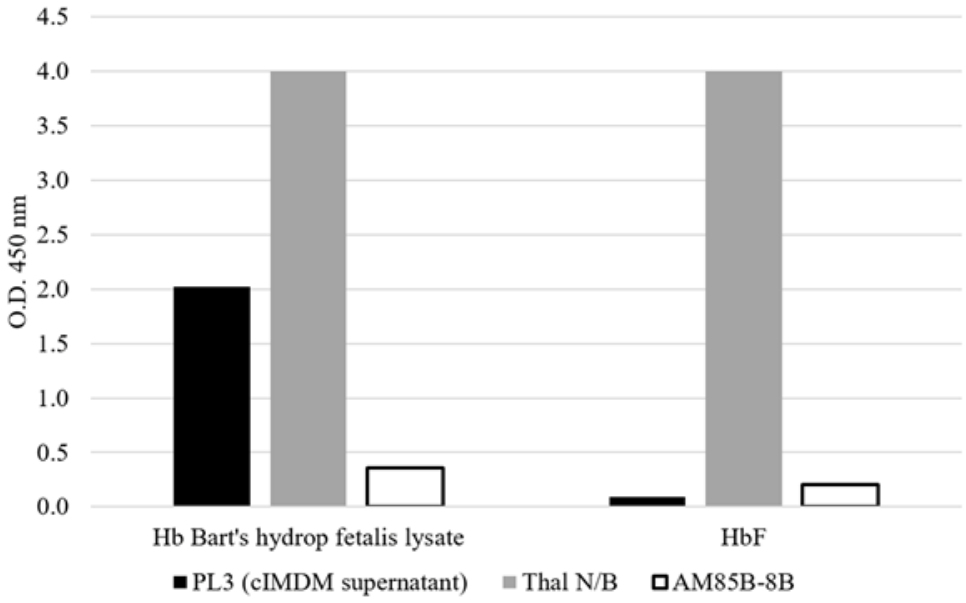

Figure 1. *Activity of mAb PL3 in hybridoma culture supernatant after culture in cIMDM. Indirect ELISA was performed using hemolysates of Hb Bart's hydrops fetalis (Bart's lysates) or purified HbF as antigen. Purified Thal N/B mAbs was used as positive control, whereas purified AM85B-8B mAb was used as isotope-matched control mAb. The antibody-antigen complexes were monitored by HRP-conjugated rabbit anti-mouse immunoglobulins.*

14 after long-term freezing.

For mAb purification, *PL3* hybridoma cells were adapted to culture in serum free media (SFM) to discard contaminated bovine immunoglobulins contained in cIMDM. We further checked the activity of mAbs produced by the *PL3* hybridoma cells cultured in SFM-IMDM. The *PL3* Hybridoma-SFM supernatant showed positivity with Hb Bart's hydrops fetalis hemolysates, as positive control mAb *Thal* N/B (Figure 2). No positive reactivity was detected in isotype-matched control mAb. This result suggested that mAb *PL3* was produced by hybridoma cells in serum free media condition. The culture of hybridoma in serum free media system could, therefore, be used to prepare culture supernatant for further experiments.

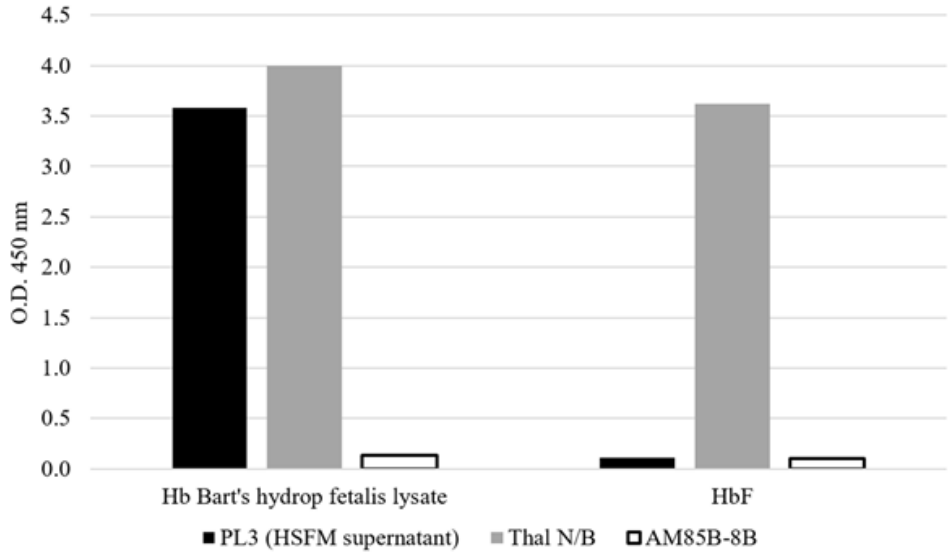

Figure 2. *Activity of mAb PL3 in hybridoma cell culture supernatant after culturing in hybridoma serum free media (HSFM) system. Bart's lysate or purified HbF was coated on plates. The culture supernatant and purified mAbs were added as indicated. HRP-conjugated rabbit anti-mouse immunoglobulins antibody was used to detect antigen-antibody complexes.*

Antibody purification

To purify mAb *PL3*, various types of affinity chromatography were employed. The yields of purified mAbs obtained from different affinity chromatographic columns were shown in Table 2. In fact, mAb *PL3* could not be purified using protein A, protein G and protein L columns. However, using the same columns, the irrelevant mAbs having the same isotype as mAb *PL3*, could be purified (Table 2). Surprisingly, mAb *PL3* could be purified only by the MabSelect SuRe column. These results suggested that different affinity chromatographic columns have a different ability in binding to immunoglobulins produced from various hybridoma clones. Using any affinity chromatographic column needs to be validated and optimized in order to purify different mAbs.

Table 2 Purification of mAb PL3 by using four different affinity chromatography.

Chromatography name	Protein binding capacity/mL	Purified PL3 mAb obtained (conc. per liter of culture supernatant)	Purified irrelevant mAb* obtained (conc. per liter of culture supernatant)
Protein A	12-19 mg human lgG	Not detected	0.773 mg (11 mg/L supernatant)
HiTrap Protein G	25 mg human lgG	Not detected	10 mg (100 mg/L supernatant)
HiTrap Protein L	25 mg human Fab	Not detected	5 mg (50 mg/L supernatant)
Mabselect SuRe	30 mg human lgG	2.064 mg (20 mg/L supernatant)	2.645 mg (37.8 mg/L supernatant)

*The irrelevant mAbs used in this study were IgG1 isotype. The anti-Ag85B mAb clone AM85B-9B were used for Protein A and anti-Hb Bart's mAb clone PB1 were used for Protein G, Protein L and MabSelect Sure.

Activity and purity of purified monoclonal antibodies *PL3*

The purified mAb *PL3* was further investigated for its activity and purity by indirect ELISA and SDS-PAGE, respectively. By indirect ELISA (Figure 3), the obtained mAb *PL3* purified using MabSelect SuRe column reacted specifically with Hb Bart's hydrop fetalis hemolysates and did not show cross-reaction with other hemoglobins. The positive control mAb *Thal* N/B showed positive reactivity with Hb Bart's hydrop fetalis hemolysate and HbF, whereas mAb *AM85B-8B* showed negative reactivity with all antigens.

The purity of the mAbs *PL3* eluted from the MabSelect SuRe column was examined by SDS-PAGE. The protein bands of purified mAb *PL3* under reducing condition appeared at approximately 55 and 25 kDa, corresponding to heavy chain and light chain, respectively (Figure 4). A major single band of approximately 180 kDa, corresponding to the intact IgG molecule, was observed in non-reducing condition (Figure 4). These results indicated that, using MabSelect SuRe column, purified mAb *PL3* could be obtained with high purity.

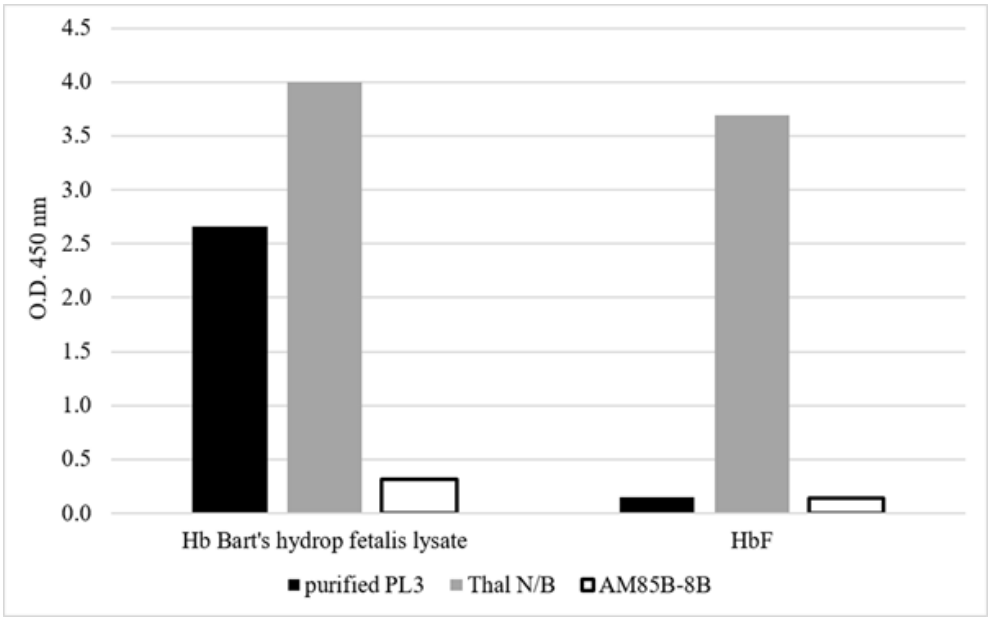

Figure 3. *Activity of purified PL3 mAb obtained by MabSelect SuRe column. Bart's lysate or purified HbF was used as the antigen of the poly-L-lysine pre-coated ELISA. Indicated purified mAbs were tested for their reactivity. The antigen-antibody complexes were detected using HRP-conjugated rabbit anti-mouse immunoglobulins antibody.*

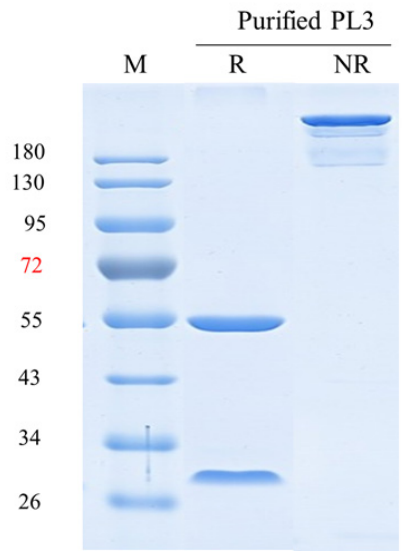

Figure 4. *SDS-PAGE analysis of the purified monoclonal antibody PL3. Five micrograms of proteins were loaded in each lane. The protein bands were visualized by PageBlue protein staining. Protein bands of purified mAb PL3 appeared at approximately 55 and 25 kDa in reducing condition. In non-reducing condition, the band showing molecular weight of PL3 appears at approximately 180 kDa.*

Discussion

In general, immunoglobulins or antibodies, are very important components of the vertebrate immune system.[17-21] Structurally, immunoglobulins are glycoproteins with a common Y-shaped building block comprised of two identical light chains (L) (~25 kDa) and two identical heavy chains (H) (~50 kDa), associated by disulphide covalent forces and by non-covalent interactions. Each chain is composed of constant (CL and CH) and variable (VL and VH) domains. These domains form into two antigen-binding fragments, Fab, and one constant region, Fc, involved in the effector function and biodistribution of the antibody. In addition to being the immune molecules in the body, antibodies can react to their specific antigen in vitro. This makes antibodies invaluable molecules for several applications. For the utilization of antibodies in several purposes, purified antibodies are required. Several methods for purification of antibodies have been developed; however, the most common and effective method is affinity purification. By affinity purification, antibodies of interest are captured in column by specific ligands and the unbound proteins are washed out. The retained antibodies in the column are eluted as purified antibodies.

Affinity purification of antibodies, actually, relies on the specific recognition between the antibody molecule and a complementary ligand. This can be achieved by two different approaches: 1) based on the specificity of antigen binding; and 2) targeting the constant part of the antibody.[5, 8, 9] Usually, the second option uses the biospecific ligand, a natural binding partner of antibody molecules, which is the most promising methodology for the large-scale production of purified mAbs.[5, 8-10] There are a number of naturally occurring immunoglobulin binding proteins that have been described.[10, 13] Protein A and Protein G for Fc portion of IgG[13, 22, 23] or Protein L for immunoglobulin light chains[10, 13] are the most common affinity ligands for the purification of antibodies. Protein A is a cell wall protein of Staphylococcus aureus, composed of five Ig-binding domains, which are independently capable of binding to the Fc part of IgG1, IgG2 and IgG4 with different affinities.[13, 22, 23] Protein A binds selectively to the Fc region of IgG (between the CH2 and CH3 region), but cannot form a complex with human IgG3 and binds to the Fab region of a subset of immunoglobulins with heavy chains belonging to the VH3 family.[13, 24-26] Protein G is a surface IgG-binding protein produced by Streptococci groups C and G and has a special affinity for the Fc region of IgG but also associates with the CH1 domain of the Fab portion through a β-zipper interaction.[13, 27] Protein L from Peptostreptococcus magnus recognizes κ1, κ3 and κ4, but not κ2 and λ light chains of antibodies and can be used to isolate antibody fragments that are not contained in the Fc domain.[10, 13, 28] The binding affinity of native biospecific ligands (Protein A, Protein G and Protein L) varies for the different IgG subclasses and species. Recently, recombinant and engineered forms of Protein A, G and L were developed in order to overcome the drawbacks associated with native proteins. The recombinant immunoglobulin ligands, currently, are procured by various commercial manufacturers and used as commercial products.[5, 8-10, 13]

Although various type of affinity column for purification of antibodies are available, in practice, some mAbs are difficult or cannot be purified. This becomes an obstacle for antibody utilization. In this study, we demonstrated an example for purification of an IgG1 mAb using four types of affinity chromatography. We revealed the use of four different biospecific ligands for purifying mouse IgG1, κ light chain mAb clone *PL3*. Protein L or Protein G beads were expected to be the best choice for mAb *PL3* purification, but it was not successful in this study. The reason might be that the mAb *PL3* consists of κ 2 light chain and imperfect CH1 domain resulting in mAb *PL3* unconfined. Surprisingly, the mAb *PL3* could only be purified by MabSelect SuRe (modified Protein A) column with high purity and it remained biologically activity. We hypothesized that mAb *PL3* contain complete CH2 and CH3 region, therefore it was captured by engineered recombinant Protein A-derived ligand of MabSelect SuRe. Moreover, Recombinant Protein A offers several potential advantages over the native Protein A. For recombinant Protein A construction, one of the Protein A domains has been engineered to favor a single-point oriented immobilization via thioether coupling, which results in enhanced binding capacity for IgG. Furthermore, amino acids that are particularly responsive to alkali on recombinant Protein A (Fab binding domain) were identified and replaced with more stable ones and the final construct was a tetramer of the engineered domain. Consequently, the Fab interaction is diminished, probably due to the fact that the engineered protein disturbed the interaction between the two molecules.[22, 29, 30] The different of IgG binding site and capacity between engineered recombinant Protein A and native Protein A revealed the different mAb *PL3* binding.

From our study, we demonstrated that each mAb has its own properties even though they have the same isotype. In order to obtain a large scale of purified mAbs, researchers need to optimize the purification process and also the affinity column used. In this case study, mAb *PL3s* are not possible to purify using normal Protein A or Protein G column. Nevertheless, the mAb PL3 can only be purified by an engineered recombinant Protein A column. Essentially, various factors and conditions such as the isotype of mAbs, concentration of mAb, the flow rate, are still needed to be optimized for each mAb.

Conclusion

Antibody purification is one of the key success points for the development of immunodiagnostics and immunotherapeutics. To this end, three bacterial immunoglobulin binding proteins (Protein A, G and L) have been extensively used to purify an anti-ζ globin chain mAb clone *PL3*. We demonstrated here the success of only using the new engineering recombinant Protein A-derived ligand, but not the others. Accordingly, this modified Protein A column is probably an alternative technique to purifying the unusual hybridoma clone that could not be purified by other columns for a large-scale purpose.

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Conflict of interest

The authors declared that they have no competing interests.

References

[1]. Apiratmateekul N, Phunpae P, Kasinrerk W. A modified hybridoma technique for production of monoclonal antibodies having desired isotypes. Cytotechnology. 2009;60(1-3):45.

[2]. Zhang C. Hybridoma technology for the generation of monoclonal antibodies. Methods in molecular biology. 2012;901:117-35.

[3]. Kohler G, Milstein C. Continuous cultures of fused cells secreting antibody of predefined specificity. 1975. Journal of immunology. 2005;174(5):2453-5.

[4]. Goding JW. Antibody production by hybridomas. Journal of immunological methods. 1980; 39(4): 285-308.

[5]. Ayyar BV, Arora S, Murphy C, O'Kennedy R. Affinity chromatography as a tool for antibody purification. Methods. 2012;56(2):116-29.

[6]. Lipman NS, Jackson LR, Trudel LJ, Weis-Garcia F. Monoclonal versus polyclonal antibodies: distinguishing characteristics, applications, and information resources. ILAR journal. 2005; 46(3): 258-68.

[7]. Huse K, Bohme HJ, Scholz GH. Purification of antibodies by affinity chromatography. Journal of biochemical and biophysical methods. 2002; 51(3): 217-31.

[8]. Salvalaglio M, Zamolo L, Busini V, Moscatelli D, Cavallotti C. Molecular modeling of protein A affinity chromatography. Journal of chromatography A. 2009; 1216(50): 8678-86.

[9]. Roque AC, Silva CS, Taipa MA. Affinity-based methodologies and ligands for antibody purification: advances and perspectives. Journal of chromatography A. 2007; 1160(1-2): 44-55.

[10]. Low D, O'Leary R, Pujar NS. Future of antibody purification. Journal of chromatography B, Analytical technologies in the biomedical and life sciences. 2007; 848(1): 48-63.

[11]. Gagnon P. Technology trends in antibody purification. Journal of chromatography A. 2012; 1221: 57-70.

[12]. Kabir S. Immunoglobulin purification by affinity chromatography using protein A mimetic ligands prepared by combinatorial chemical synthesis. Immunological investigations. 2002; 31(3-4): 263-78.

[13]. Choe W, Durgannavar TA, Chung SJ. Fc-Binding Ligands of Immunoglobulin G: An Overview of High Affinity Proteins and Peptides. Materials. 2016; 9(12).

[14]. Pata S, Khummuang S, Pornprasert S, Tatu T, Kasinrerk W. A simple and highly sensitive ELISA for screening of the alpha-thalassemia-1 Southeast Asian-type deletion. Journal of immunoassay & immunochemistry. 2014; 35(2): 194-206.

[15]. Tayapiwatana C, Kuntaruk S, Tatu T, Chiampanichayakul S, Munkongdee T, Winichagoon P, et al. Simple method for screening of alpha-thalassaemia 1 carriers. International journal of hematology. 2009; 89(5): 559-67.

[16]. Phunpae P, Chanwong S, Tayapiwatana C, Apiratmateekul N, Makeudom A, Kasinrerk W. Rapid diagnosis of tuberculosis by identification of Antigen 85 in mycobacterial culture system. Diagnostic microbiology and infectious disease. 2014; 78(3): 242-8.

[17]. Bonilla FA, Oettgen HC. Adaptive immunity. The Journal of allergy and clinical immunology. 2010; 125(2 Suppl 2): S33-40.

[18]. Chaplin DD. Overview of the immune response. The Journal of allergy and clinical immunology. 2010; 125(2 Suppl 2): S3-23.

[19]. Kennedy MA. A brief review of the basics of immunology: the innate and adaptive response. The Veterinary clinics of North America Small animal practice. 2010; 40(3): 369-79.

[20]. Abbas AK, Lichtman AH, S P. Cellular and Molecular Immunology. Philadelphia: Saunders; 2014.

[21]. Kindt TJ, Goldsby RA, Osborne BA., J. K. Kuby Immunology. 6 ed. New York: W. H. Freeman; 2007.

[22]. Hober S, Nord K, Linhult M. Protein A chromatography for antibody purification. Journal of chromatography B, Analytical technologies in the biomedical and life sciences. 2007; 848(1): 40-7.

[23]. Linhult M, Gulich S, Hober S. Affinity ligands for industrial protein purification. Protein and peptide letters. 2005; 12(4): 305-10.

[24]. Ey PL, Prowse SJ, Jenkin CR. Isolation of pure IgG1, IgG2a and IgG2b immunoglobulins from mouse serum using protein A-sepharose. Immunochemistry. 1978; 15(7): 429-36.

[25]. Moks T, Abrahmsen L, Nilsson B, Hellman U, Sjoquist J, Uhlen M. Staphylococcal protein A consists of five IgG-binding domains. European journal of biochemistry. 1986; 156(3): 637-43.

[26]. Graille M, Stura EA, Corper AL, Sutton BJ, Taussig MJ, Charbonnier JB, et al. Crystal structure of a Staphylococcus aureus protein A domain complexed with the Fab fragment of a human IgM antibody: structural basis for recognition of B-cell receptors and superantigen activity. Proceedings of the National Academy of Sciences of the United States of America. 2000; 97(10): 5399-404.

[27]. Akerstrom B, Bjorck L. A physicochemical study of protein G, a molecule with unique immunoglobulin G-binding properties. The Journal of biological chemistry. 1986; 261(22): 10240-7.

[28]. Rodrigo G, Gruvegard M, Van Alstine JM. Antibody Fragments and Their Purification by Protein L Affinity Chromatography. Antibodies. 2015; 4: 259-77.

[29]. Ishihara T, Nakajima N, Kadoya T. Evaluation of new affinity chromatography resins for polyclonal, oligoclonal and monoclonal antibody pharmaceuticals. Journal of chromatography B, Analytical technologies in the biomedical and life sciences. 2010; 878(23): 2141-4.

[30]. Hahn R, Bauerhansl P, Shimahara K, Wizniewski C, Tscheliessnig A, Jungbauer A. Comparison of protein A affinity sorbents II. Mass transfer properties. Journal of chromatography A. 2005; 1093(1-2): 98-110.