

EVALUATING PRE-TREATMENT METHODS IN THE ISOLATION AND PURIFICATION OF MONOCLONAL IgG ANTIBODY

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ABSTRACT

In this study, the effects of different sample preparation techniques on the separation of monoclonal antibody IgG1 were investigated experimentally. Monoclonal IgG1 was obtained from hybridoma cell line TB/C3 transfected with bcl-2 carrier plasmid, which was grown in serum-free medium. Three different pre-treatment techniques prior to Protein G affinity chromatography have been used in order to concentrate and partial purify the monoclonal antibody. The pre-treatments researched in this paper are precipitation of the antibody by ammonium sulfate, dilution of the antibody in the binding buffer of affinity chromatography and ultrafiltration through an Amicon Ultra-15 filter with molecular weight cut-off at 100 kDa. Purification through direct application of the antibody onto the Protein G affinity column without pre-treatments was used as a control method. The results indicate that the ultrafiltration through an Amicon filter was an effective method for both concentration and partial purification of the antibodies in laboratory scale.

Key Words: Pre-treatment- IgG- Protein G- affinity chromatography

1.0 INTRODUCTION

The processing of complex biological solutions is a major bottleneck in the biotechnology industry and there is a demand for the cost-effective technologies for the purification of naturally occurring or recombinant proteins. This is particularly true for monoclonal antibodies, where there has been an on-going search for simple generic methods of purification [1]. Monoclonal antibodies have had an increasing number of research, diagnostics, probes for fine structural analysis, histological examinations, immunoaffinity chromatography and immunotherapy [2]. Some of these applications require monoclonal antibodies in ample quantities and in a highly purified form.

However, the diversity of monoclonal antibody structures that can be engineered and the variety of hybridoma cell lines that can be utilized for their production, complicate the efficient isolation of these antibodies, such as the approach of new purification technique [3, 4]. A growing list of purification procedures has emerged and considerable attention has been paid to alternative sample preparation methods [5]. Basically, the purification procedures for most monoclonal antibodies involve sample preparation steps such as precipitation, and in subsequent purification steps using chromatographic techniques. Purification of monoclonal and even polyclonal antibodies from different range of biological material, for instance, monoclonal antibodies from

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mouse ascites and monoclonal antibodies from cell culture supernatant, require different combination of techniques [6]. Many schemes of purification procedures have been developed and carried out by different researchers to purify their monoclonal antibodies to homogeneity. Therefore, the downstream processing of the biotherapeutics has challenged both research and industry, and inspired the combination and uses of different types of interactions and separation techniques [7].

Generally, the crude starting material containing many impurities, possibly particulate matter, need to reduce its volume and concentrated prior directed to chromatographic methods. The classical methods for the sample preparation and purification of IgG antibodies were frequently long and tedious. Ammonium sulphate is the most commonly used in salt precipitation because it is highly soluble in water, stabilizes most proteins in solution and helps to reduce lipid content of sample, yet the product yields are low. For example, the purification of antibodies by conventional precipitation with ammonium sulphate followed by dialysis which has been used to purify polyclonal and monoclonal antibodies requires several days to complete [8]. Precipitation with polyethylene glycol 6000 (PEG 6000) and finally reprecipitated using an ammonium sulphate procedure were also used to purify monoclonal antibodies from the culture supernatant [9].

The method described in this paper uses three different fundamental methods to concentrate and prepare the monoclonal antibody secreted from a hybridoma cell line prior to the main chromatography. The samples were pre-treated differently using ammonium sulphate precipitation followed by dialysis, dilution with phosphate buffer and ultrafiltration to allow a comparison between the different techniques.

2.0 EXPERIMENTAL

2.1 Samples

The murine hybridoma cell line TB/C3 was provided by R. Jefferis from University of Birmingham, UK. TB/C3 cell line is based on NS1-derived myeloma cell, which produces immunoglobulin G subclass 1 (IgG1) monoclonal antibody specific to the hapten C γ 2 domain at the Fc region of human IgG. This cell line was transfected with bcl-2 carrier plasmid, and was termed TB/C3.bcl-2.

Cells thawed from a working cell bank were firstly grown in Roswell Park Memorial Institute (RPMI 1640, Gibco Invitrogen, USA) medium supplemented with 10% Foetal Bovine Serum (FBS, Gibco, Invitrogen, USA) and later adapted by a stepwise procedure to serum-free medium (Gibco, UK). No antibiotics were used in any of the medium. All cell stocks were harvested with at least 90% viability and were in log phase growth in order to collect crude monoclonal IgG1 antibody for further purification steps.

All samples were filtered through 0.45 μ m cellulose acetate filters (Vivascience AG, Germany) prior to experimental procedures. All buffers used in this study were also filtered through 0.45 μ m cellulose nitrate filters (Whatman International Ltd., England) to remove small particles.

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2.2 Purification of monoclonal IgG

Affinity chromatography was performed at room temperature on a Hi-Trap Protein G (5 mL) column from Amersham Bioscience GE Healthcare, England. Equilibration buffer was 0.02 M sodium phosphate, pH 7.2 and eluting buffer was 0.1 M glycine-HCl, pH 2.5. The flow rate used; at all stages was 2.5 mL/min. The collected fractions of 1 mL volume were immediately treated with neutralizing 1 M Tris-HCl, pH 9.0. The chromatographic profiles were monitored at 280 nm using Lambda 25 UV/VIS spectrophotometer from Perkin Elmer Instruments, UK.

To explore the effects of different pre-treatment techniques on the purification yield, each sample was subjected to the three following pre-treatments prior to application into the Hi-Trap Protein G affinity column.

Crude monoclonal IgG1 sample was diluted 1:1 with 0.02 M sodium phosphate buffer (equilibration buffer) at pH 7.2 before applying into the Hi-Trap Protein G affinity column.

The crude sample was concentrated by ultrafiltration using an Amicon Ultra-15 filter centrifugal filter device (Millipore Corporation, Ireland) with molecular weight cut-off of 100 kDa. The Amicon filter was centrifuged at 5000 rpm, 4°C for 30 minutes using a Mikro 22 R refrigerated fixed-rotor centrifuge (Hettich Zentrifugen, Germany). The concentrated supernatant (200 µL) was then applied to the Hi-Trap Protein G affinity column.

The precipitation was performed at 4°C. One part of 1 M Tris-HCl, pH 8.0 was added to ten parts of sample volume to maintain pH. Equal volumes of crude sample and saturated ammonium sulphate solution (50% saturation) were mixed by slow addition of the ammonium sulphate solution with gentle stirring. On the next day this solution mixture was centrifuged at 5000 rpm, 4°C for 30 minutes and the supernatant removed. The pellet was washed twice by resuspension in an equal volume of ammonium sulphate solution of the same concentration. The precipitate was dissolved in 0.02 M sodium phosphate, pH 7.2 and desalted using PD-10 Desalting column packed with Sephadex-G25 (Amersham Biosciences GE Healthcare, England) and eluted using the same buffer. The eluted fractions were collected and determined at 280 nm. The fractions that showed IgG concentration were pooled and applied to the affinity column.

2.3 SDS-PAGE electrophoresis

The purity of the various IgG preparations was checked by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) carried out on a BIO-RAD electrophoresis system (Richmond, CA, USA) with Mini-Protean II electrophoresis cell (gel size: 6 cm × 8 cm × 0.75 mm), according to Laemmli (1970) method. The sample protein was mixed with equal volume of 5x sample buffer (20µL) containing 5 mM 2-mercaptoethanol, boiled for 5 minutes and centrifuged prior loaded onto gel. The total polyacrylamide concentration in the separating gel was 12%. The electrophoresis was carried out at a constant voltage of 100V for 90 minutes at room temperature. Silver staining was used to visualize the protein bands. As a standard, the Broad Range Protein Molecular Weight Marker from Promega was used.

2.4 Enzyme-linked immunosorbent assay

IgG concentration was quantified using a sandwich ELISA, a Mouse IgG1 ELISA Quantitation Kit from Bethyl Laboratories Inc., USA. The wells of an ELISA microplate (TPP) were coated with 100 μ L of anti-mouse IgG1 developed in goat (1 mg/mL) in coating buffer (0.05 M carbonate-bicarbonate buffer, pH 9.6) overnight at 0-4°C. After three washing steps with TBST (50 mM Tris, 0.14 M NaCl, 0.05% (v/v) Tween 20, pH 8.0), the plate was blocked (200 μ L/well) with postcoat solution (50 mM Tris, 0.14 M NaCl, 1% BSA (v/v), pH 8.0) and incubated for one hour at room temperature. Standards and samples were prepared by diluting mouse reference serum calibrator with conjugate diluent (50 mM Tris, 0.14 M NaCl, 1% (v/v) BSA, 0.05% (v/v) Tween 20, pH 8.0). Standard dilutions from 250 to 3.9 ng/mL of mouse IgG1 were used.

The plate was washed three times with TBST and 100 μ L of diluted standards and samples were transferred to assigned wells. After one hour of incubation (room temperature), the standards and samples were removed and each well was washed five times. Goat anti-mouse IgG1 conjugated to horseradish peroxidase diluted 1:20 000 in conjugate diluent [50 mM TBS pH 8.0 + 0.14 M NaCl + 1% (v/v) BSA + 0.05% (v/v) Tween 20] (100 μ L) was transferred to each well and the plate incubated for one hour at room temperature. After incubation, the plates were carefully washed five times with TBST. Substrate solution [3, 3', 5, 5'-tetramethylbenzidine (TMB) liquid substrate system] was freshly prepared and 100 μ L added to the wells. The plates were incubated at room temperature for 5-30 minutes. After the incubation period, 100 μ L of 2 M H₂SO₄ were added to each well and the absorbance read at 450 nm.

3.0 RESULTS AND DISCUSSION

In the first set of experiment, monoclonal antibody IgG1 crude sample supernatant was directly subjected to Hi-Trap Protein G affinity chromatography. This experiment was carried out as a control method to allow comparison for the different pre-treatments conducted. The main peak of IgG1 was separated by affinity chromatography on Protein G column with a 0.1 M glycine-HCl eluting buffer at pH2.5, but the remaining ballast proteins were removed with sodium phosphate starting buffer as shown in Figure 1. From the figure shown, the IgG1 peak was eluted in fractions 57, 58 and 59. The IgG fractions were collected and analyzed by reducing SDS-PAGE for purity assessment. Figure 6 has shown that IgG has appeared as two major bands on 50 kDa and 25 kDa due to broken -S-S- bonds with 2-mercaptoethanol, and that no any other protein were found in the IgG fraction. The ballast proteins present in the crude sample consisted mainly of transferrin and insulin, which were supplemented in the serum-free medium during the cell growth. From the result shown in SDS-PAGE, the contaminating proteins were successfully separated from the IgG1 antibody.

The first pre-treatment carried out was a simple dilution of crude monoclonal IgG1 sample with 0.02 M sodium phosphate buffer, pH 7.2 before loading into the Hi-Trap Protein G affinity column. There was obtained a well separated peak from the ballast proteins, which was later identified as IgG1 by SDS-PAGE (Figure not shown). From the chromatogram shown on Figure 2, it can be seen that the square-shaped peak

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area of the unbound material during washing step was approximately two times the size of it in the control chromatogram (Figure 1). This happens due to the addition of sodium phosphate buffer, where it adds to the time of washing. The chromatogram also shows that a higher IgG1 peak was detected at 280 nm. This indicates that the IgG1 concentration eluted from the affinity column after a dilution with sodium phosphate buffer is higher compared to the control. This result was further proven by the IgG immunological activity detection using ELISA (Table 1).

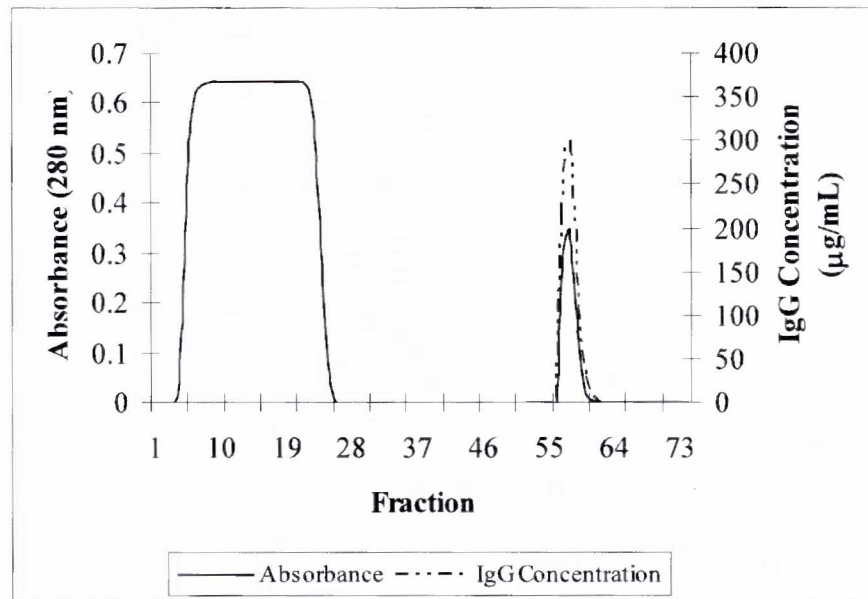


Figure 1 Isolation of monoclonal IgG1 on Hi-Trap Protein G column (Control).

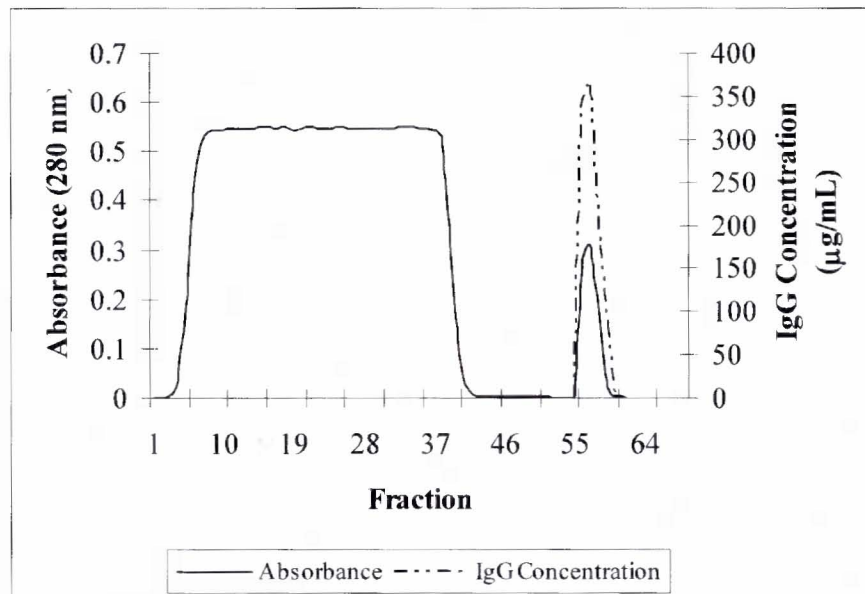


Figure 2 Chromatographic pattern for monoclonal IgG1 antibody obtained on Hi-Trap Protein G column with a sample dilution of 1:1 with 0.02 M sodium phosphate buffer before loading.

In the third set of experiment, the crude monoclonal IgG1 sample was subjected to ammonium sulphate precipitation before affinity chromatography. The ammonium sulphate precipitate was then desalted by performing buffer exchange on Sephadex-G25. The collected fractions of 1-mL allowed for complete separation of a protein peak from salt of a previous buffer (ammonium sulphate), as shown on Figure 4. The precipitate containing IgG1 in ammonium sulphate solution was removed and changed with 0.02 M sodium phosphate buffer, pH 7.2 as the starting buffer for the Hi-Trap Protein G affinity column. The fractions that showed IgG1 peak after ELISA analysis were pooled and applied to affinity column with the same loading and elution conditions. The chromatogram on Figure 5 shows a different profile from both of the previous affinity chromatography, but a well separation of IgG1 from the contaminating proteins.

Ultrafiltration was carried out as the last method in this comparison of different pre-treatments. From Figure 5, it can be seen that the chromatographic profile of the separation of retentate (ultrafiltration) after affinity chromatography is different from the other separation profiles. The washing profile gave a large, sharp but asymmetrical peak, unlike the rectangular ones given by the previous chromatograms. This peak did not include any IgG1 when examined by SDS-PAGE. The second peak was separated very well from the first one and was later identified as IgG1 when analyzed by SDS-PAGE.

The recovery of antibody of the various pre-treatment methods was analyzed by ELISA and summarized in Table 1.

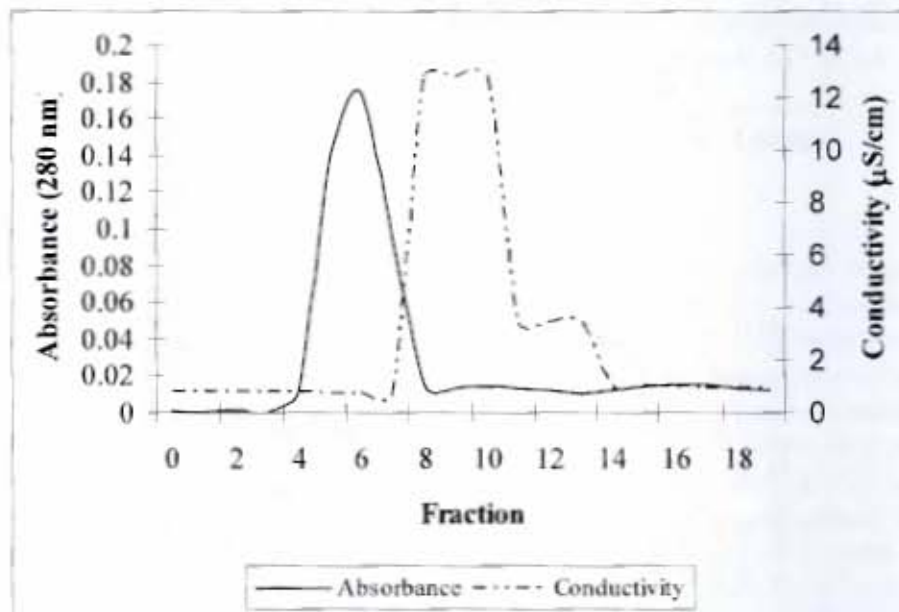


Figure 3 Buffer exchange on Sephadex G-25. The sample load was after ammonium sulphate precipitation. Isocratic elution with 0.02 M sodium phosphate buffer, pH 7.2.

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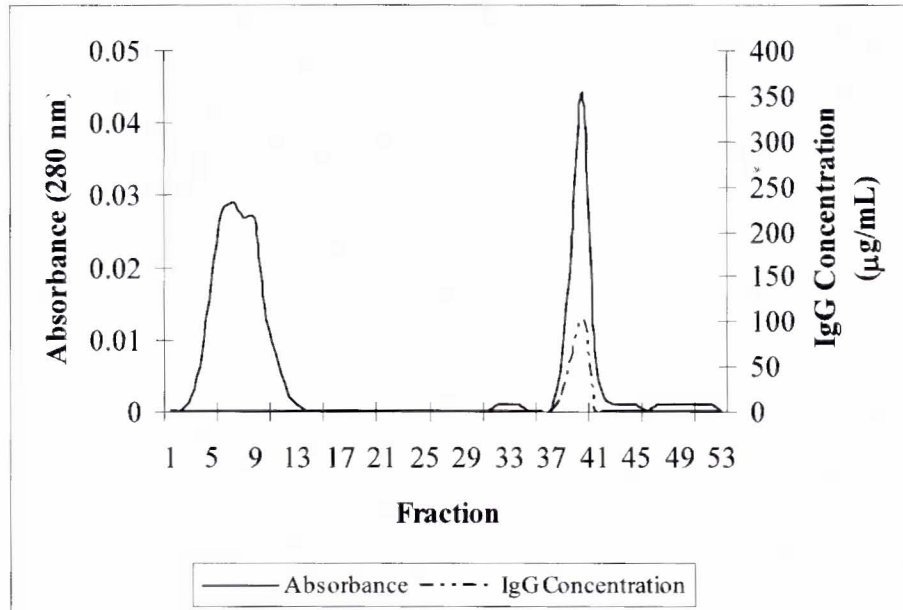


Figure 4 Chromatographic separation of sample after ammonium sulphate precipitation and buffer exchange on Hi-Trap Protein G affinity column.

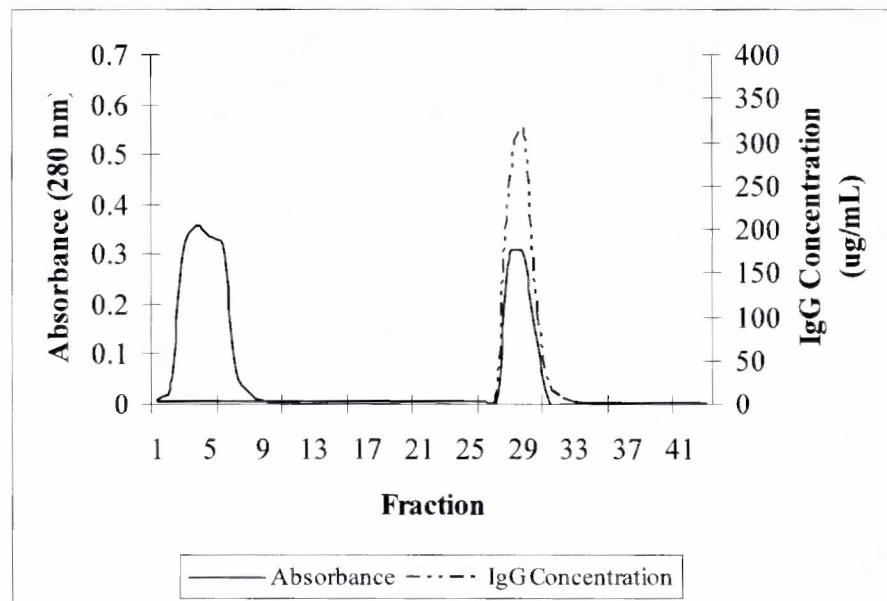


Figure 5 Purification of concentrated monoclonal IgG1 (ultrafiltrate) on Hi-Trap Protein G affinity column.

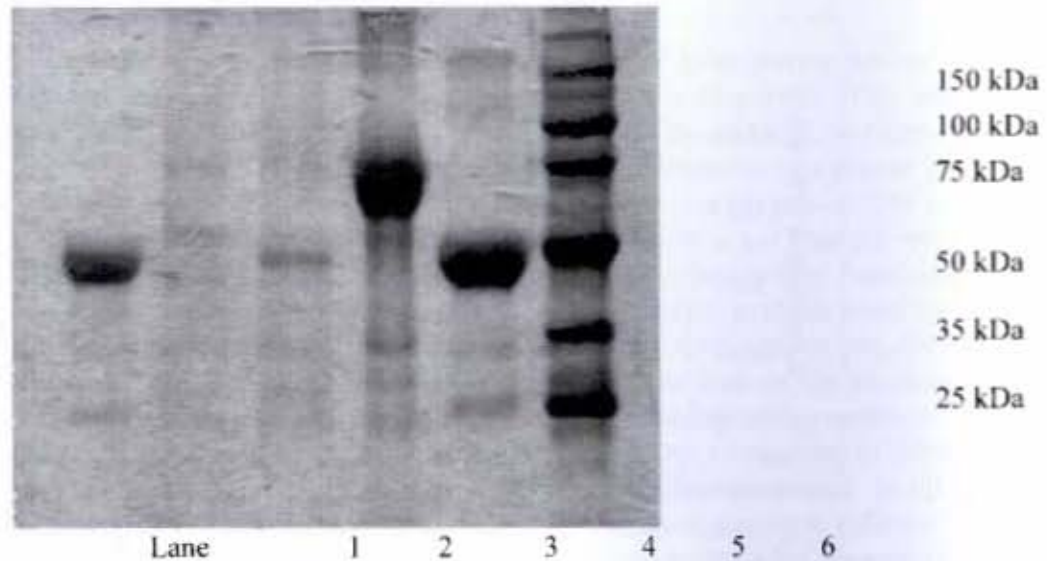


Figure 6 SDS-PAGE analysis of pooled IgG1 peak from Hi-Trap Protein G affinity chromatography reduced with 2-mercaptoethanol. Lane 1: eluted IgG1(1:1); lane 2: flowthrough (1:1); lane 3: standard IgG1 (1:1); lane 4: transferrin (1:1); lane 5: sample loading (1:4); lane 6: molecular weight marker.

Table 1 Summary of the recovery of antibody activity in fractions obtained from affinity chromatography after different pre-treatment methods

Protein Recovery	Pre-treatment							
	Control		Dilution with PBS		NH ₂ SO ₄ precipitation		Ultrafiltration	
	(ug)	(%)	(ug)	(%)	(ug)	(%)	(ug)	(%)
Crude sample	1156	100	1339	100	1345	100	1114	100
After pre-treatment	1156	100	1339	100	600	44.6	926	83.0
After affinity chromatography	621	53.8	874	65.2	367	27.3	669	60.1

In this study, we have obtained purified monoclonal IgG1 antibody from supernatant of hybridoma cell line TB/C3.bcl-2 using different pre-treatment methods which were followed by a main purification of affinity chromatography. Affinity chromatography on Protein G was chosen as the main purification step because the protein G was covalently bound to Sepharose, and is able to trap all mouse IgG subclass, especially mouse IgG1 which either do not bind or bind weakly to protein A.

Affinity chromatography with Hi-Trap Protein G Sepharose at pH 2.5 was shown to be a powerful method for the isolation and purification of monoclonal IgG antibody from cell used, and the recombinant form of protein G has its albumin-binding region genetically deleted. This column is also used for the isolation and purification of immunoglobulins G from lipids [10].

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According to the results shown in the previous section, the dilution of crude monoclonal IgG has proven to increase the antibody recovery from the affinity chromatography. The cell supernatant harvested has a pH of less than 6 due to the metabolism of cell line and the secretion of cell wastes. Therefore, the dilution with sodium phosphate, pH 7.2 increased the sample's pH to neutral. This coincides with the binding pH of the protein G to the IgG with isoelectric points between pH 7.0 to 8.5. The neutral pH of starting buffer has almost purified the IgG antibody to homogeneity, with a recovery of about 80%.

By ammonium sulphate precipitation, the recovery of IgG protein was found to be 55%, as shown in Table 1. The precipitation step acts to concentrate the sample into a pellet and stabilizes the sample protein. However, some antibodies may be damaged by ammonium sulphate and the yield is low. For routine, reproducible purification, precipitation with ammonium sulphate is best avoided. In this study, desalting with gel filtration on Sephadex G-25 was carried out in favour of the conventional dialysis because dialysis is generally a very slow technique and requires large volumes of buffer. From the results shown (Table 1), the desalting step gave a high recovery at 80%. The ammonium sulphate was removed during desalting and the fraction of IgG was loaded onto affinity column. The recovery after affinity chromatography was found to be 61%. However, the total recovery of this set of experiment was found to be only 27%.

Attempt was also made to purify the monoclonal antibody IgG1 by ultrafiltration followed by affinity chromatography. The Amicon Ultra-15 filter has a molecular weight cut-off of 100K and theoretically will only retain proteins of molecular weight in excess of 100 kDa. However, it can be seen on the chromatogram after affinity chromatography (Figure 5) that during washing, a peak was resulted and this peak was later identified as transferrin on SDS-PAGE by comparing with a standard. Therefore, this means that some transferrin was retained by Amicon Ultra-15 filter although it has a molecular weight of significantly less than 100 kDa. The supernatant was concentrated 69 fold by ultrafiltration from 15-mL sample to only 200 μ L. The recovery was also high at 83% as analyzed by ELISA. After affinity chromatography, about 60% of monoclonal antibody IgG1 was recovered.

4.0 CONCLUSION

In conclusion, all the pre-treatment methods in this study are suitable as sample preparation and concentration of the cell supernatant from TB/C3. *bcl-2* before loading onto affinity column. The diluted sample supernatant which has been altered its pH before loading onto affinity column has also shown a significant improvement in the IgG recovery. This method was a favourite among researchers when ammonium sulphate precipitation must be avoided because high concentrations of ammonium sulphate may cause contamination of the precipitate with unwanted proteins. Our results also show that ultrafiltration is an extremely effective method for concentration of the sample supernatant at 4°C due to its ease of preparation and time-saving. However, the higher cost of this ultrafilter may become a constraint in the separation process.

REFERENCES

- [1] Lim, S., H.P. Manus, A.A. Gooley, K.L. Williams, D.B. Rylatt. 1998. Purification of monoclonal antibodies from ascetic fluid using preparative electrophoresis. *J. Chromatogr. A.* 827: 329-335.
- [2] Clausen, A. M., A. Subramaniam, P.W. Carr. 1999. Purification of monoclonal antibodies from cell culture supernatants using a modified zirconia based cation-exchange support. *J. Chromatogr. A.* 831: 63-72.
- [3] Serpa, G., E.F.P. Augusto, W.M.S.C. Tamashiro, M.B. Riberio, E.A. Miranda, S.M.A. Bueno. 2004. Evaluation of immobilized metal membrane affinity chromatography for purification of an immunoglobulin G1 monoclonal antibody. *J. Chromatogr. B.* 816: 259-268.
- [4] Yan, Z., J. Huang. 1999. Chromatographic behaviour of mouse serum immunoglobulin G in protein G perfusion affinity chromatography. *J. Chromatogr. B.* 738: 149-154.
- [5] Stocks, S. J. 1989. Production and isolation of large quantities of monoclonal antibody using serum-free medium and fast protein liquid chromatography. *J. Hybridoma.* 8: 241-247.
- [6] Stec, J., L. Bicka, J. Kuźmak. 2004. Isolation and purification of polyclonal IgG antibodies from bovine serum by high performance liquid chromatography. *Bull Vet Inst Pulawy.* 48: 321-327.
- [7] Roque, A. C. A., M.A. Taipa, C.R. Lowe, C. R. 2004. An artificial protein L for the purification of immunoglobulins and Fab fragments by affinity chromatography. *J. Chromatogr. A.* 1064: 157-167.
- [8] Perosa, F., R. Carbone, S. Ferrone, F. Dammacco. 1990. Purification of human immunoglobulins by sequential precipitation with caprylic acid and ammonium sulphate. *J. Immunol. Methods.* 128: 9-16.
- [9] Brooks, D. A., T.M. Bradford, J.J. Hopwood. 1992. An improved method for the purification of IgG monoclonal antibodies from culture supernatants. *J. Immunol. Methods.* 155: 129-132.
- [10] Kitsouli, E., E.M. Lekka, G. Nakos, C. Cassagne, L. Maneta-Peyret. 2002. Lipids are co-eluted with immunoglobulins G during purification by recombinant streptococcal protein G affinity chromatography. *J. Immunol. Methods.* 271: 107-111.