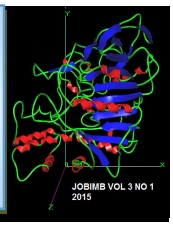
HIBISCUS PUBLISHER

JOURNAL OF BIOCHEMISTRY, MICROBIOLOGY AND BIOTECHNOLOGY

Website: http://journal.hibiscuspublisher.com/index.php/JOBIMB/index



Optimization of Monoclonal Antibodies Purification Expressed in H-192 cells using Preparative Native-Polyacrylamide Gel Electrophoresis

Santhana Krishnan[1], Zularisam Abdul Wahid[2], Chua Yeo Gek Kee[2], Chew Few Nee[2] and Mimi Sakinah Abdul Munaim[1,2*]

[1]Faculty of Engineering Technology, Universiti Malaysia Pahang, 26300 Kuantan, Pahang, Malaysia.
[2]Faculty of Chemical and Natural Resources Engineering, Universiti Malaysia Pahang, 26300 Kuantan, Pahang, Malaysia.

*Corresponding author:
Prof. Dr. Mimi Sakinah Abdul Munaim,
Faculty of Engineering Technology,
Universiti Malaysia Pahang, 26300 Kuantan, Pahang, Malaysia,
E-mail: mimi@ump.edu.my
Tel.: +609 549 2825
Fax: +609 549 2889

HISTORY

Received: 9th October 2015
Received in revised form: 11th of October 2015
Accepted: 12th of October 2015

KEYWORDS
monoclonal antibodies (mAbs)
preparative n-PAGE
Hybridoma murine cell lines
Congenital Adrenal Hyperplasia
17-Alpha-Hydroxyprogesterone

ABSTRACT

Monoclonal antibodies (mAbs) are unique and specific drug molecules targeting the treatment of various diseases such as arthritis, immune disorders, infectious diseases, and cancer etc. Different methods such as antibody coupled affinity chromatography, hydrophobic interaction chromatography, etc., can be applied to purify mAbs from various sources. This article provides a simple, cost effective, preparative native-polyacrylamide gel electrophoresis (n-PAGE) technique to purify mAbs expressed in H-192 cells (Hybridoma murine cell lines) against an antigen i.e. 17-alpha-hydroxyprogesterone (17-OHP), which further can have diagnostic application to detect *Congenital Adrenal Hyperplasia* (*CAH*). Furthermore, different parameters such as concentration and volume of the feedstock (medium containing antibodies), pore size of gel, height of resolving gel etc. were optimized to obtain the maximum purity and yield of mAbs.

INTRODUCTION

Monoclonal antibodies (mAbs) are pivotal therapeutic agents in the pharmaceutical industry, having applications in diagnostics and therapeutic areas because of their high specificity [1,2].The mAbs can be purified using diverse methods such as antibody coupled affinity chromatography, hydrophobic interaction chromatography, expanded bed adsorption, magnetic microspheres etc. [3–5]. However, all of these methods are expensive and multistep methods can be replaced with an efficient, less time consuming and cost effective method i.e. preparative native polyacrylamide gel electrophoresis (n-PAGE) for the purification of mAbs. n-PAGE has been employed for purifying many complex proteins and other biomolecules from various sources [6]. The technique works on the principle of size exclusion and charge based separation of a molecule [7].

The mAbs against 17-alpha-hydroxyprogesterone (17 OHP) have already been purified by chromatography techniques [8,9]. Here we report for the first time to our knowledge, the purification of mAbs against 17-OHP expressed in H-192 cells using an n-PAGE technique. High levels of 17-OHP have been detected in the serum of patients suffering from Congenital Adrenal Hyperplasia (*CAH*) [10]. *CAH* occurs due to the deficiency of 21-hydroxylase enzyme which results in the decreased production of glucocorticoid, mineralocorticoid and increased secretion of adrenocorticotropic hormone (ACTH) [11]. Some conventional diagnosing methods (e.g., use of polyclonal antibody) are used to detect the 17-OHP, which is insensitive and non-specific. Alternatively, mAbs are specific and have high affinity towards the antigens (in this study, 17-OHP) and can be used for effective treatment of *CAH*.

The goal of this study is to purify mAbs for 17-OHP antigen using a single step n-PAGE technique. Moreover, different parameters such as, concentration and volume of the feedstock (medium containing antibodies), pore size of gel,

height of resolving gel and voltage for electrophoresis were optimized to obtain the maximum purity and yield of mAbs.

MATERIALS AND METHODS

Materials

Dulbecco's Modified Eagles Medium (Sigma chemicals, St Louis, MO), Foetal Bovine Serum (FBS) and antibiotic–antimycotic, Sodium dodecyl sulphate Gel electrophoresis products (Gibco-Invitrogen Corporation), Bovine serum albumin (Invitrogen Corporation), Dialysis membrane–10 K MWCO–(Thermo scientific), Nitrocellulose membrane (Pall Corporation), Semidry transfer unit (Bio-Rad), Laboratory bottles (Scott), Protein assay kits (Bio-Rad) were obtained from specified sources. All antibodies and HRP substrate were obtained from Sigma corp. Other Lab reagents were purchased from Fisher scientific, UK.

Cell culture and mAbs isolation

H-192 cells were obtained from the animal cell culture laboratory, University of Malaya, Malaysia. The cells were maintained in Dulbecco's Modified Eagles Medium (DMEM) supplemented with 2% (v/v) Foetal Bovine Serum (FBS) (Gibco, Invitrogen Corp), 1% (v/v) antibiotic – antimycotic and 4mM glutamine. Further, H-192 cells were incubated at 37°C, with 86% humidity and 5% CO_2 in an incubator. About 50 ml of cell culture broth (containing H-192 cells, mAbs, host cell proteins etc.) were centrifuged at 10000 rpm for 15 minutes to separate mAbs from the cells.

The supernatant containing the mAbs was precipitated using 50 ml ammonium sulphate (100%), further centrifuged at 10000 rpm for 10 minutes. The pellet was dissolved in 5 ml phosphate buffer (pH-6.5). Further, 1 ml of protein sample mixed with 1 ml of sample loading dye (pH-8.3) was loaded on an n-PAGE column.

Preparative n-PAGE and optimization

The schematic picture of Electro-elution n-PAGE setup is shown in **Fig.** 1. In this study, preparative scale native gel systems were used for the purification of mAbs against 17-hydroxyprogesterone. The gel column (1.7 cm inner diameter of height 12 cm) was loaded with two layers of gel mixture (resolving gel and 4% (w/v) acrylamide stacking gel).

The gel was run under a constant current of 30 mA in a buffer containing 0.025 M Tris and 0.192 M glycine as the electrode buffer. An electrode made of platinum wires were fixed in both reservoirs and connected to the power supply. The lower end of the gel column was attached to dialysis tube to collect the mAbs elute, prior to it was boiled in 10mM sodium bicarbonate for 1 h and again boiled for another 1 h in 10mM EDTA, then washed with distilled water thoroughly and then moistened with electrode buffer. Further, the concentration of mAbs in elute was determined using ELISA and total protein concentration was analysed using Lowry method. Moreover, preparative n-PAGE was optimized by assessing the effects of different operating parameters such as, volume and concentration of feedstock i.e. 250–2000µl and 5–25% respectively, by varying the concentration of acrylamide to optimize the pore size of the resolving gel i.e. 4–16% (w/v), the height of the resolving gel i.e. 0.5–3.5 cm and Voltage (25-175v) for effective separation.

Western blotting analysis

The mAbs extract was size-fractionated by 12% gel electrophoresis and transferred to nitrocellulose membranes using a semi-dry transfer. Blots were incubated with 17 OHP hormone (1:2500) used as primary antibody and HRP conjugated anti-mouse IgG (1:5000) used as a secondary antibody. The mAbs band were visualised by the addition of phosphate substrates, ABTS (0.18 mg/mL).

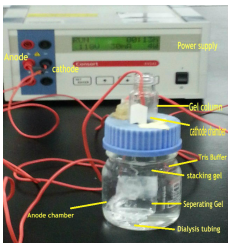


Fig. 1. Electro-elution setup (n-PAGE system).

ELISA

ELISA was performed to determine the concentration, yield and purity of mAbs in the harvested broth. 17-hydroxyprogesterone (17-OHP), 1 µg per well was coated on 96 well microtitre plate as capture antigen to detect the specificity of mAbs.

RESULTS AND DISCUSSION

Cell density and mAbs expression

During cell growth, the maximum viable cell density and specific growth rate achieved were 1.79 -0.20 $\times 10^6$ cells mL^{-1} and 0.044 - 0.003 h^{-1} respectively. The maximum mAb titre was found to be 12.32 - 4.79 µg mL^{-1} with the average specific production rate of 0.369 -0.128 pg $cell^{-1}$ h^{-1}.The values were similar to that of results obtained by Chua et al. [12].

Conditions optimization of preparative n-PAGE

The effect of volume of feedstock

On the n-PAGE when using a 1.7 cm internal diameter gel column, the mAbs purity and yield was significantly influenced by the volume of the feedstock. Both the parameters i.e. purity and yield of mAbs was unaffected by the volume of feedstock up to 250-1000 µl and the purity and yield of mAbs obtained with mentioned volume of feedstock was about 0.94 and 91% respectively (**Fig.** 2A). The subsequent increase in the feedstock volume of up to 2000 µl decreased the purity to 60% as well as the yield to 0.78. The volume of the feedstock is directly proportional to biomass concentration. Hence, the higher feedstock volume (2000µl) caused the blockage on the surface of the gel and prevented the target mAbs from entering into small pores of the gel network. As a result, it caused gel shrinkage, which resulted in the bands distortion for mAbs [13] and thus affected the purity and yield of mAbs. Therefore, a smaller volume of the feedstock is preferred to obtain the highly purified mAbs using this method.

The effect of concentration of feedstock

The purity and yield of mAbs increased initially with the concentration of the feedstock from 5 to 15% (w/v). But, when the feedstock concentration was further increased to 20% (w/v), a slight decline in the mAbs purity was observed. Moreover, in contrast to purity, the mAbs yield was increased as the feedstock concentration increased from 10 to 20% (w/v). However, the decrease in the yield was noticed when the feedstock concentration was increased further to 25%. Hence, the optimal concentration for effective results was found to be 15% (w/v), with high purity values of (0.96) and yield (91%) were achieved (**Fig.** 2B). The decrease in both the parameters is

because very high mAbs loads in concentrated feedstock modified the pH, voltage gradient and ionic strength, within the sample stacking zone. It resulted in the interaction between the sample components and consequently denaturation reactions which further waned the yield of mAbs [14].

In addition, the loading capacity of the system was relative to the concentration of protein components present in the sample. Higher load concentration increased the broadening of the mAbs band due to a rise in the diffusion coefficient. Our observations were nearly similar to the results reported by Suck et al [15].

The effect of pore size of resolving gel

The movement of any molecule highly pivots on matrix arrangements in the polyacrylamide gel. Therefore, optimization is indispensable for the pore size of the resolving gels to provide optimal resolution of the protein bands. As shown in **Fig.** 2C, the purity and yield of mAbs were unaffected from 4 to 10% (w/v) acrylamide concentration, obtained 80% and 0.7 respectively. A 12% (w/v) resolving gel has shown the optimum mAbs purification, where the purity and yield achieved were 0.92 and 90% respectively.

A decrease in the yield was observed with further increase in the gel concentration. During electrophoresis, heat energy is generated from electrical energy, which in turn affected both the density and viscosity of the electrophoretic medium. Therefore, in maximal porosity band spreading was observed due to increasing in diffusion with little loss in resolution of bands in the gel [16]. Moreover, produced heat also caused mAbs denaturation [17], enzymatic digestion [18] and aggregation that led to the protein clogging within matrices of the gel [19], consequently low yield and less pure mAbs.

The effect of height of resolving gel

Using a 2 cm gel height, the mAbs purification achieved better results of 0.93 purity and 90% yield (**Fig.** 2D). A gel height of 2 cm was adequate to separate mAbs from other contaminants such as the host cell protein. However, the purity was unaffected even with a gel height increased to greater than 2 cm. On the other hand, when the height of gel was increased to above 3 cm the yield was decreased about 25%. The reason could be an increased concentration and height of the gel lengthens the migration of mAb through the gel matrix. So the electrophoresis required longer time for the complete elution of proteins from the gel, causing a pH shift in the electrode buffer that possibly could damage the electrophoretic performance [20].

Current is directly proportional to its voltage applied, so the variation in the electrophoretic voltage from 25 to 150 V changes the operating current and also the temperature. In this experiment, the outer surface of the gel column was cooled in the buffer. The increase in voltage might have formed a radial temperature gradient across the path of the migrating bands. Generally, the heat is increased with time at a high electrophoretic voltage (150 V). So the mAbs band moving from the top to the bottom of the gel column exhibited a curved out distortion because the molecules moved faster in warmer regions [6]. The generated heat, distorted the mAbs band and increased diffusion rate, which interfered with the electrophoretic resolution or caused protein aggregation and denaturation, resulting in poor mAbs recovery [19]. In spite of that, the electrophoretic time is inversely proportional to the voltage applied and thus higher voltage increased mAbs migration velocity in the gel column. If heat dissipation from the gel is suitable, a higher voltage is suitable for faster electrophoretic elution [6,21,22]

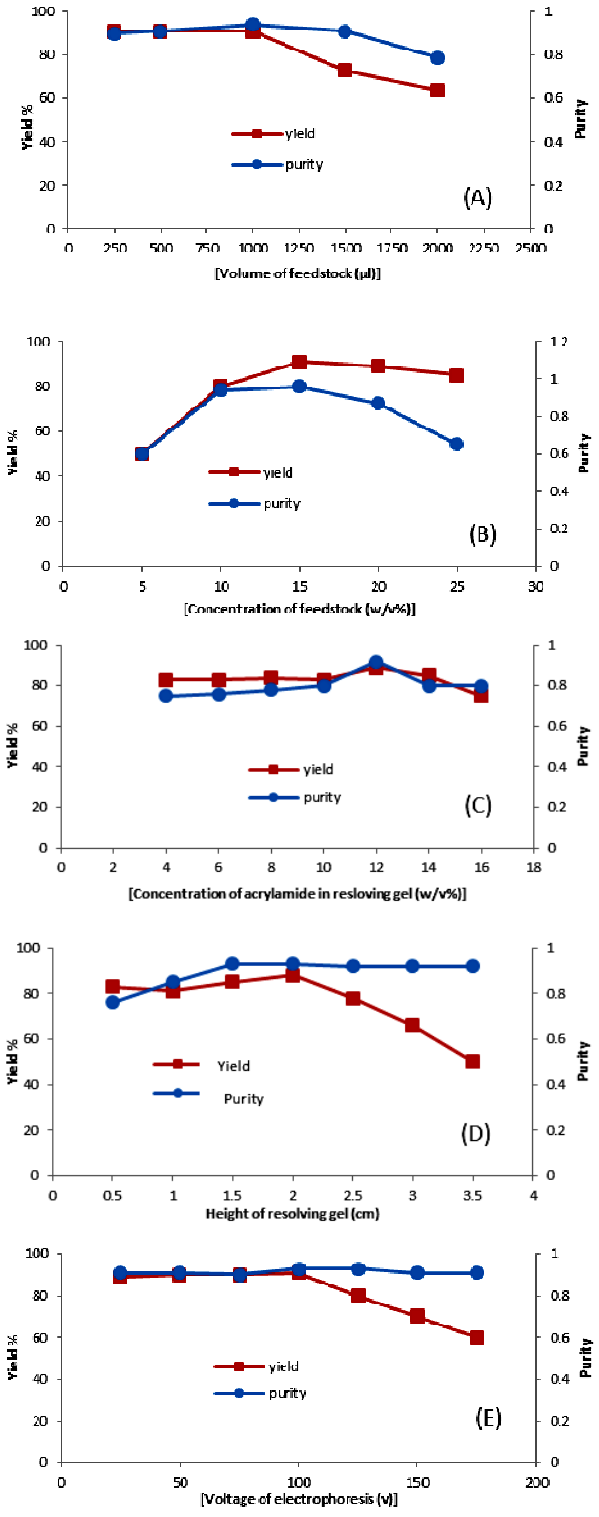


Fig. 2. Optimization patterns of mAbs in n-PAGE- (A) Volume of feedstock vs. purity and yield, (B) Concentration of feedstock vs. purity and yield, (C) Pore size vs. purity and yield, (D). Height of resolving gel vs. purity and yield, (E) Applied voltage vs. purity and yield.

SDS-PAGE analysis

The purified antibody samples were analyzed by 12% SDS-PAGE for qualitative analysis. The low molecular weight proteins like albumin passed initially through the end of the gel in 0-30 minutes leaving behind the mAbs upwards (lane 2). After 30 minutes operation of electrophoresis until 90 minutes, purified form of mAbs was obtained, shown in **Fig.** 3A (lane 3 and 4), while it is clear that initial feedstock sample contains many impurities (lane 1). A 150kDa of mAbs was reduced by SDS into two proteins of molecular weight 50 kDa and 25 kDa respectively, as it was observed in the gel (lane 3 and 4). The electro-elution profile data's were paralleled with SDS-PAGE gel results. Negatively charged mAbs was electro-eluted out from the gel column and found in large quantity between 31–90 min operation periods, which formed a Gaussian peak (see **Fig.** 3B). Other contaminant proteins were slowly eluted out from the gel column after 91-120 minutes operation periods (**Fig.** 3A, lane 5).

The results showed no evidence of antibody molecule degradation during the purification. All impurities eluted within the first 0-30 and last 91-120 minutes gel operation, leaving the antibody fraction free of any contaminants with a purity of ≥92%. The purified mAb fractions were of high purity and that very little contaminants proteins were detected. The electropherogram showed that IgG (150 kDa) always appeared as two major bands at 50 kDa and 25 kDa. These results demonstrate that the purification methods in this study significantly improved the purity of the mAbs. In our experiments, we were able to achieve excellent purification and recovery without pre-treating

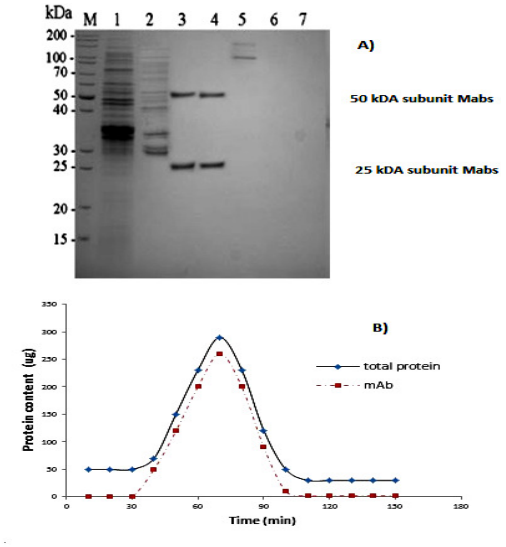


Fig. 3. Purity profile of mAbs. (A) SDS Page analyses of the pooled mAbs fraction collected between 0-120 min. M: molecular mass markers in kDa (sigma), lane 1: initial feedstock, lane 2: samples collected between 0-30 min, lane 3: samples collected between 31-60 min (H-chain and l-chain), lane 4: samples collected between 61-90min (H-chain and l-chain), lane 5: samples collected between 91-120 min (showing some impurities), lanes 6 and 7 were blanks. (B) Electro-elution profile of mAbs.

Western blotting analysis

Besides, the eluates from the native gel column were also analysed using native PAGE (slab gel) and western blotting was carried out to confirm the presence of mAbs specific against 17-OHP (**Fig.** 4A & B). The results showed the expression of mAbs on nitrocellulose membrane specific towards 17 OHP antigens while residual non-specific antibodies restricted from transfer through the membrane.

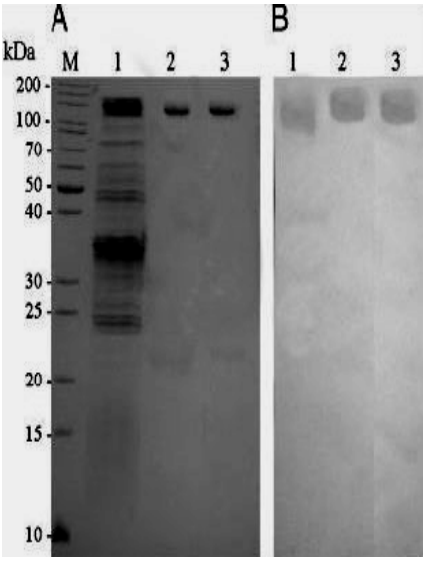


Fig. 4. (A) Native-PAGE (slab gel) and (B) Western blotting analyses of the pooled mAbs fraction collected between 31-90 min from 50 mL of 15%w/v feedstock using a preparative PAGE. Lane M: molecular mass markers in kDa (Fermentas), lane 1: initial feedstock, lane 2: purified mAbs (run 1); lane 3: purified mAbs (run 2). The same amount of total protein (0.1 mg) was applied in each lane.

ELISA analysis

The activity and quantitation of mAbs were confirmed through the 17 OHP specific ELISA. 17-OHP antigens were coated in ELISA plates to capture mAbs in samples and standards. The colour change from colourless to greenish indicates the presence of specific mAbs for the antigen 17-OHP in the samples (**Fig.** 5A). Row B; well number 6, 7, 8 were 31-60 minute's samples. Row C; well number 6, 7, 8 were 61-90 minute's sample. Both observed greenish colour change indicating the mAbs presence in the well. Row A and D (wells 6, 7, 8) shows an absence of mAbs. A standard curve is drawn (**Fig.** 5B) relating absorbance to the concentration of mouse IgG specific for 17-OHP (150 kDa) in the standard dilutions. Table 1 shows the concentrations of preparative n-PAGE purified mAbs present in a 96 well plate at different time intervals. Purified mouse IgG against 17-OHP was used in the experiments to derive a standard curve. The concentrations of mAbs in 96 well plates (100 µl/ well) were 12.3 µg and11.9 µg for 31-60 and 61-90 min respectively. Moreover, ELISA results suggest no significant loss in antibody during recovery and free any of the major contaminants. It was confirmed from the assay that mAbs molecules were still active and specific against 17-OHP.

Table 1- Quantitation of OHP specific MAbs in 96 well plates using purified mouse IgG as standards.

Time intervals (min)	96 well plate	Sample volume/well (ml)	mAbs Conc. (µg)
0-30	A-6,7,8	0.1	0
31-60	B-6,7,8	0.1	12.337
61-90	C-6,7,8	0.1	11.971
91-120	D-6,7,8	0.1	0.001

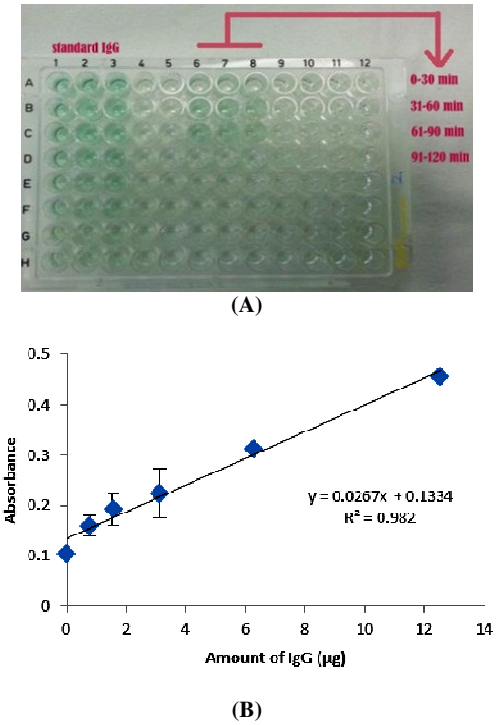


Fig. 5. (A) 17 OHP specific ELISA to detect 17 OHP specific mAbs purified using preparative PAGE. Triplicate samples-[(Well 1-3(A-H) is Standard IgG (150 kDA) Sigma Aldrich. Row A; well 6, 7, 8 – 0-30 minute's samples. Row B; well 6, 7, 8 – 31-60 minute's samples. Row C; well 6, 7, 8- 61-90 minute's sample. Row D; well 6, 7, 8- 91-120 minute's sample. Remaining wells were blanks. (B) Standard curve of mouse IgG.

CONCLUSIONS

In conclusion, we developed an efficient n-PAGE method to provide an efficient platform to purify and optimize the mAbs from cell culture feedstock in one-step. The technique reduced time, equipment cost, conventional multiple steps and protein degradations. The optimum conditions for this process were found to be 1000µl of 15% (w/v) feedstock with 12% (w/v) resolving gel of height 2 cm, achieved 93% purity and 91% yield of mAbs. Moreover, the results suggested that preparative n-PAGE has a number of advantages over conventional affinity chromatography for the purification of antibodies. The mAbs against 17-OHP, purified from this method can be further utilized in a diagnostic kit for *CAH*.

ACKNOWLEDGEMENT

The authors are grateful to the Universiti Malaysia Pahang for its financial support (Grant No. RDU131413) to carry out this research.

REFERENCES

[1] Karg SR, Kallio PT. The production of biopharmaceuticals in plant systems. Biotechnol Adv. 2009;27(6):879–94.

[2] Marissen WE. Production of Human Monoclonal Antibodies using Mammalian Expression Systems. In: Current Laboratory Techniques in Rabies Diagnosis, Research and Prevention. 2014. p. 277–90.

[3] Naik AD, Menegatti S, Gurgel PV, Carbonell RG. Performance of hexamer peptide ligands for affinity purification of immunoglobulin G from commercial cell culture media. J Chromatogr A. 2011;1218(13):1691–700.

[4] Selimoglu SM, Ayyildiz-Tamis D, Gurhan TD, Elibol M. Purification of alginate and feasible production of monoclonal antibodies by the alginate-immobilized hybridoma cells. J Biosci Bioeng. 2012;113(2):233–8.

[5] Kishishita S, Katayama S, Kodaira K, Takagi Y, Matsuda H, Okamoto H, et al. Optimization of chemically defined feed media for monoclonal antibody production in Chinese hamster ovary cells. J Biosci Bioeng. 2015;120(1):78–84.

[6] Chew FN, Tan WS, Ling TC, Tey BT. Optimization of a native gel electrophoretic process for the purification of intracellular green fluorescent protein from intact *Escherichia coli* cells. Process Biochem. 2011;46(1):399–403.

[7] Branco AT, Ferreira B d. S, de Souza Filho GA. A paper-based electroelution system for protein recovery from stained sodium dodecyl sulfate-polyacrylamide gels. Anal Biochem. 2008;381(2):267–9.

[8] Jun-ichi S, Tadao T, Shun-Ichi I, Masako M, Akio T, Hiroshi H, et al. Production and characterization of monoclonal antibodies to 17α-hydroxyprogesterone. J Steroid Biochem. 1987;28(4):405–10.

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

[10] Merke DP, Bornstein SR. Congenital adrenal hyperplasia. Lancet. 2005;365(9477):2125–36.

[11] Witchel SF, Azziz R. Congenital Adrenal Hyperplasia. J Pediatr Adolesc Gynecol. 2011;24(3):116–26.

[12] Chua GK, Abdul-Rahman B, Chisti Y. Production and scale-up of a monoclonal antibody against 17-hydroxyprogesterone. Biotechnol Prog. 2013;29(1):154–64.

[13] Galland-Irmouli AV, Pons L, Luçon M, Villaume C, Mrabet NT, Guéant JL, et al. One-step purification of R-phycoerythrin from the red macroalga *Palmaria palmata* using preparative polyacrylamide gel electrophoresis. J Chromatogr B Biomed Sci App. 2000;739(1):117–23.

[14] Seelert H, Krause F. Preparative isolation of protein complexes and other bioparticles by elution from polyacrylamide gels. Electrophoresis. 2008;29(12):2617–36.

[15] Suck R, Petersen A, Weber B, Becker W-M, Fiebig H, Cromwell O. Analytical and preparative native polyacrylamide gel electrophoresis: Investigation of the recombinant and natural major grass pollen allergen Phl p 2. Electrophoresis. 2004;25(1):14–9.

[16] Shoji M, Kato M, Hashizume S. Electrophoretic recovery of proteins from polyacrylamide gel. J Chromatogr A. 1995;698(1-2):145–62.

[17] Jain S, Teotia S, Gupta MN. Purification of green fluorescent protein overexpressed by a mutant recombinant *Escherichia coli*. Protein Expr Purif. 2004;36(1):76–81.

[18] Saeed SA, Boyde TR. Preparative gel electrophoresis at high sample load. The effect of some experimental variable on separation performance. Prep Biochem. 1980;10(4):445–62.

[19] Lim S, Manusu HP, Gooley AA, Williams KL, Rylatt DB. Purification of monoclonal antibodies from ascitic fluid using preparative electrophoresis. J Chromatogr A. 1998;827(2):329–35.

[20] Dunn SD. Effects of the modification of transfer buffer composition and the renaturation of proteins in gels on the recognition of proteins on western blots by monoclonal antibodies. Anal Biochem. 1986;157(1):144–53.

[21] Thomas TM, Shave EE, Bate IM, Gee SC, Franklin S, Rylatt DB. Preparative electrophoresis: A general method for the purification of polyclonal antibodies. J Chromatogr A. 2002;944(1-2):161–8.

[22] Kueakhai P, Changklungmoa N, Chaithirayanon K, Songkoomkrong S, Riengrojpitak S, Sobhon P. Production and characterization of a monoclonal antibody against recombinant saposin-like protein 2 of Fasciola gigantica. Acta Trop. 2013;125(2):157–62.