Pemurnian Antibodi Monoklonal terhadap Virus Hepatitis B yang diisolasi dari Asites Mencit Balb/c Menggunakan "Homemade" Protein A Spin Mini Kolom

Purification of Monoclonal Antibody against Hepatitis B Virus isolated from Balb/c Ascites Using "Homemade Protein A Spin Mini-column"

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KATA KUNCI *Protein A; purifikasi; antibody monoclonal; hepatitis B; mini kolom*
KEYWORDS *Protein A; purification; monoclonal antibody; hepatitis B; mini-column*

ABSTRAK *Penelitian ini bertujuan mengembangkan pemurnian antibodi monoklonal menggunakan eks-mini kolom yang isinya diganti dengan partikel gelas silika yang terkonjugasi dengan Protein A. Matriks Protein A yang digunakan adalah Prosep vA, sedangkan antibodi target adalah antibodi monoklonal (MAb) terhadap antigen permukaan virus hepatitis B yang diisolasi dari asites mencit Balb/c. Sebelum pemurnian, sampel asites dibagi menjadi 3 yang masing-masing diinkubasikan dengan Protein A dalam 3 perlakuan. Perlakuan A dan B adalah inkubasi 30 dan 60 menit pada suhu kamar, sedangkan perlakuan C adalah inkubasi semalam pada suhu 4°C. Hasil pemurnian menunjukkan bahwa aktivitas spesifik dan tingkat kemurnian MAb secara berurutan adalah 358.45 U/mg, 237.83 U/mg untuk perlakuan C dan B, dan 229.67 U/mg untuk perlakuan A. Hasil penelitian ini menunjukkan bahwa 'homemade' mini kolom yang dikembangkan dalam penelitian ini dapat digunakan untuk memurnikan MAb untuk kebutuhan riset skala laboratorium.*

ABSTRACT *This study was aimed to develop a reliable antibody purification technique using the ex-mini-columns in which its content was replaced with silica glass Protein A conjugated. Protein A matrix used was Prosep vA, whereas the target antibody was a monoclonal antibody (MAb) against hepatitis B virus surface antigen from the ascites of Balb/c mice. Prior to purification of the ascites, samples were incubated with Protein A in three different treatments. Treatment A and B were 30 and 60 minutes incubation time at room temperature, and treatment C with overnight incubation at 4°C. The results showed that the specific activity and the purity level of the MAb obtained from the studies were 358.45 U/mg, 237.83 U/mg and 229.67 U/mg for treatment C, B and A, respectively. These results indicated that the 'homemade' mini-column developed in this study can be used to purify MAb to the needs of the laboratory-scale research.*

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Antibody(ies) with sufficient purity is necessary and needed in immunological research activities. For this purpose a variety of procedures or methods to isolate and purify antibody in order to get not only optimum activity but also efficient and economical values has been done. Purification of antibody using Protein A attached to a variety of matrices such as silica glass is one of the methods of separation and purification commonly used. This is due to the nature of the Protein A (42 kDa), a protein of the bacterium *Staphylococcus aureus,* which can bind to the Fc arms of immunoglobulin, especially IgG (Graille *et al*., 2000; Liu *et al*., 2010).

Protein A column of silica glass is commonly used for antibody purification in small or even large scale and available on the market. In certain circumstances, particularly in several laboratories such as those in Indonesia, a bench top small-scale purification method with optimal results is needed. Therefore research on the development of small-scale antibody purification method using homemade Protein A column was conducted.

In this study monoclonal antibody against the surface antigen of Hepatitis B virus (HBsAg) from mouse ascites was used. Hepatitis is still considered as a serious health problem in the world with more than 350 million people infected with hepatitis B virus (HBV), some of them will have cirrhosis and liver cancer (Mulyanto *et al*., 2011). The prevalence of HBV infection is generally high in Asia and Africa, with most infections develop at birth or during childhood (Lee, 1997). In Indonesia, the prevalence of HBV infection is different in each region with a relatively high population of HBV carriers, about 4.0 to 20.3% (Khan *et al*., 2004).

Until now there has been no effective drug available to treat HBV infected persons. WHO recommends early immunization as the most efficient means to prevent transmission of HBV (McMahon, 2005). In addition, screening for the blood donor on a regular basis is important as well. To support all of these, reliable and accountable diagnostic kits are necessary to be available. Consequently, research to ensure continuous availability of antibodies, including antibody purification process is needed.

This paper reports the success of developing a "homemade Protein A mini-column" to isolate and purify monoclonal antibody against HBV antigen from ascites of Balb/c mice.

MATERIALS AND METHODS

Materials

Antibodies used in this study were monoclonal antibody (MAb) specific for hepatitis B virus surface antigen (HBsAg), derived from ascites fluid of Balb/c mice. This MAb (Lot. KO436) is produced in the Laboratory of Hepatitis Nusa Tenggara Barat. Mini-column used was a 2 ml plastic mini-column regenerated from Invitrogen (Esser *et al*., 2005). The used silica in the tube was removed and replaced with 250 µl fresh glass silica Protein A (Prosep vA, Millipore).

Preparation of Buffers

Buffer used consisted of buffer A (washing buffer) 500 mM NaCl containing 100 mM Na_2HPO_4-$12H_2O$ pH 8.5, buffer B (sample dilution) 1 M NaCl containing 200 mM Na_2HPO_4-$12H_2O$ pH 8.5, and buffer C (elution buffer) 150 mM NaCl containing 100 mM Citric acid, pH 3, and neutralization solution 2 M Tris. Chemicals were purchased from Merck, Germany.

Purification process was initiated by diluting the sample (4 times dilution) of ascites fluid containing monoclonal antibody using buffer B. Subsequently the diluted samples were divided into 3 treatment groups (A, B, and C). One ml of diluted

sample from each group was mixed with 250 µl of Protein A conjugated glass silica in a 1.5 ml micro tube. Micro tubes were assigned for group A, B and C, representing incubation time for the mix between Protein A conjugated silica and ascites fluid for 30 and 60 minutes at room temperature and overnight at 4°C, respectively.

Following the incubation, the silica and samples of representative group was transferred and loaded into the mini-column placed into a collection tube, and then centrifuged using a micro centrifuge (TOMY, MC-140, Japan) for 1 minute, at room temperature with maximum speed. The flow-through (FT-1) was collected and the collection tubes were assigned in accordance to the treatment group and purification step (Table 1, 2 and 3). Then, the column was washed twice, firstly with 500 µl and then 3 ml of buffer A. The flow-through was collected and assigned as FT-2 and FT-3 respectively. After washing with buffer C, the target antibody was removed with elution buffer and the flow-through was collected (assigned as Elutant) and neutralized with 2 M Tris. All of the procedures were repeated 3 times for all treatment groups. The whole flow-through collected was kept at 4°C until assayed for their antibody activities, protein concentration and purity.

Assay for Antibody Activity

The antibody activities were assessed by passive hem-agglutination (PHA) method using the Kit "Entebe" PHA-Cell (Laboratorium Hepatika, NTB, Indonesia) according to the manufacturer's instructions, without any modification.

Protein Determination

Protein (antibody) determinations were carried out using Bradford microassay method in accordance with the manufacturer's instructions. Bovine serum albumin (BSA) was used as standard. A total of 100 µl standard or sample was mixed with 1 ml of Bradford reagent then the absorbance changes were read using UV-Vis Spectrophotometer (Shimadzu, Japan) at a wavelength of 595 nm. Antibody concentration was calculated by interpolation of standard curves.

Data Analysis

The data obtained were tabulated and analyzed according to Depamede and Rosyidi (2009) and calculation for the recovery, activity and purity of the antibody throughout the purification steps were based on Xiao-lan *et al*., (2005).

RESULTS

The aim of this study was to develop methods of isolation and purification of the antibody in a small scale using a mini-column of "homemade protein A glass silica". The results of the purification process are presented in Tables 1, 2 and 3 with the representation of each treatment of 30 and 60 minutes incubation time at room temperature and overnight at 4°C respectively.

Table 1. Antibody recovery and activity during purification process using homemade Protein A mini-column with 30 minutes incubation at room temperature

Purification Step	Tot.Vol. (ml)	Prot.Cons. (mg/ml)	Total Prot. (mg)	Total Activity (Unit)	Specific Activity (U/mg)	Purification (fold)	% Recovery
Ascites	1.00	2.04	2.04	15.50	7.61	1.00	100.00
FT-1	0.80	1.39	1.11	9.00	8.10	1.06	58.06
FT-2	0.50	0.47	0.24	11.00	46.45	6.10	70.97
FT-3	3.00	0.06	0.18	11.00	60.80	7.98	70.97
Eluting	0.25	0.19	0.05	11.17	229.67	30.16	72.04

FT: Flow through

Table 2. Antibody recovery and activity during purification process using homemade Protein A mini-column with 60 minutes incubation at room temperature

Purification Step	Tot.Vol. (ml)	Prot.Cons. (mg/ml)	Total Prot. (mg)	Total Activity (Unit)	Specific Activity (U/mg)	Purification (fold)	% Recovery
Ascites	1.00	2.04	2.04	15.50	7.61	1.00	100.00
FT-1	0.80	1.48	1.19	11.00	9.27	1.22	70.97
FT-2	0.50	0.39	0.20	11.00	55.94	7.35	70.97
FT-3	3.00	0.10	0.20	11.00	55.61	7.31	70.97
Eluting	0.25	0.24	0.06	14.50	237.83	31.86	94.62

FT: Flow through

Table 3. Antibody recovery and activity during purification process using homemade Protein A mini-column with overnight incubation at 4°C

Purification Step	Tot.Vol. (ml)	Prot.Cons. (mg/ml)	Total Prot. (mg)	Total Activity (Unit)	Specific Activity (U/mg)	Purification (fold)	% Recovery
Ascites	1.00	2.04	2.04	15.50	7.61	1.00	100.00
FT-1	0.80	1.29	1.04	10.00	9.65	1.27	64.52
FT-2	0.50	0.30	0.15	10.00	67.29	8.84	64.52
FT-3	3.00	0.02	0.05	10.00	204.67	26.88	64.52
Eluting	0.25	0.15	0.04	13.50	358.45	47.08	87.10

FT: Flow through

DISCUSSION

Currently a lot of kits for isolating antibodies are available in the market (Berruex *et al*., 2000) but in some circumstances when we want to do the isolation and purification of antibody in small-scale, some technical constraints are often encountered. On the other hand, antibody with sufficient purity for bench-top experiments is truly needed.

In general the "homemade Protein A mini-column" was successfully used to isolate the antibody derived from ascites in a small scale volume. As presented in Tables 1, 2 and 3, if we look closer at the recovery, which was the percentage of total activity of purified MAb against the total activity of crude MAb, the highest yield was obtained when the antibody exposed or incubated for 60 minutes with Protein A glass silica at room temperature prior to further purification process. Subsequent level of recovery was derived from the overnight incubation time at 4^{o}C and for 30 minutes at room temperature, respectively.

Considering the antibody specific activity, overnight incubation time at 4^{o}C was superior (358.45 U/mg) compared to the other treatments (i.e. 237.83 and 229.67 U/mg for 60 and 30 minutes at room temperature respectively). The specific activity was also corresponding to antibody purity i.e. 47.08, 31.86 and 30.16 folds. Although a recovery percentage of 94.62% by incubation time of 60 minutes was obtained, it did not remove many proteins (Mondal and Gupta, 2006) that might interfere further usage of the antibody compared to the overnight incubation times with more pure yield. Ideally, we expected to get high recovery with high specific activity and purest antibody as well. However, this is actually quite a compromise. If the processing time limit is not an issue and the degree of purity is preferred, then the overnight incubation protocol is the right choice.

What can be obtained from the results of this study is that we can take advantage of the used mini-column and replace its content with a fresh silica glass conjugated with Protein A to purify monoclonal antibody. Furthermore, the silica can also be regenerated (Esser *et al*., 2005), hence the protocol would be quite helpful, especially for a small laboratory for their bench-top experiments. But it is certain that further comprehensive studies need to be done.

CONCLUSION

Monoclonal antibody against hepatitis B virus has been successfully isolated from mouse ascites using "homemade Protein A spin mini-column" developed in this study. The spin mini-column can be used to purify monoclonal antibody to the needs of the laboratory-scale researches.

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