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Purification of Anti-*Mycobacterium tuberculosis* MPT64 Immunoglobulin-Y from Egg-Yolk Supernatant Using Thiophilic Adsorption Chromatography

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Abstract: The significance of immunoglobulin Y (IgY) as a particular antibody equal to mammalian IgG is well understood. However, due to a lack of reliable purification procedures, producing highly pure IgY remains problematic. In this study, we aimed to optimize the recovery of pure IgY anti-MPT64 using thiophilic adsorption chromatography. The purification of IgY anti-MPT64 was achieved by initial PEG lipid precipitation, then an optimized purification by varying the gradient concentration of elution buffer into five steps gradient (0–20, 20–40, 40–60, 60–80, and 80–100%v/v) for three injection column volume (CV) each and two steps gradient (0–50 and 50–100%v/v) in eight CV for each concentration. The obtained IgY was characterized by SDS-PAGE and dot-blot then determined its content levels using the Lowry method. The results showed that the five steps gradient purification was found to provide a better purity level of IgY than the two steps gradient. However, the IgY content obtained in the two steps gradient purification (2.2632± 0.011 mg/mL) was higher than the five steps gradient purification (1.35482 ± 0.023 mg/mL). Nevertheless, both purified IgY results can recognize MPT64 protein through a dot blot test. Therefore, it can be summarized that thiophilic adsorption chromatography with five steps gradient of purification was an efficient process to obtain a higher purity of IgY anti-MPT64, especially to be targeted as a diagnostic kit component for MPT64 detection.

Keywords: immunoglobulin Y; thiophilic; MPT64; gradient step; purity; SDG 3 Good health and well-being

1. Introduction

The use of chicken immunoglobulin Y (IgY) in different research areas has risen significantly in recent years, partially due to the fact that generating antibodies in eggs proves to be economically effective and eliminates issues related to animal welfare. The primary antibody in avian blood is IgY, which is potentially transferred to egg yolk to offer passive immunization. A large amount of IgY can be generated cheaply and easily from chicken eggs[1]. IgY does not bind to human or bacterial Fc receptors, nor does it activate the complement system or interact with rheumatoid factors. Because of the evolutionary gap between birds and mammals, birds have superior immunogenicity to highly conserved mammalian proteins[2–5]. The significance of IgY as a specific antibody equal to mammalian IgG is well understood[6]. In fact, both IgY and IgG are utilized in diagnostic tests. The most common serum antibody obtained in animals bred for traditional antibody production is IgG. The quantity of antibodies that may be extracted from blood in a single setting is equivalent to the amount of IgY generated in the eggs of a commercial laying hen over the course of a year. However, the synthesis of these conventional antibodies often takes 2–6 months, and harvesting by bleeding is painful for the animal. In contrast, after being inoculated with the required antigen, chickens generate specific antibodies in their eggs in 4–6 weeks. These IgY antibodies are readily collected and employed in diagnostic tests, with less background and cross-reactivity than current industry standards. IgY is fast-acting, simple to manufacture, and inexpensive. The use of egg-laying chickens allows for the production of huge amounts of IgY antibodies with no environmental impact or infrastructure[7]. Thus, IgY encounters a lot of benefits in immunodiagnostic compared to IgG from animals.

Specific IgY generated by vaccinated chickens has tremendous therapeutic potential and can be utilized for immunodiagnostic and a wide range of procedures for IgY purification are available[8]. However, due to the lack of a specialized purification procedure, producing highly pure IgY remains problematic and limiting its use[6]. Ammonium sulfate, caprylic acid precipitation, anion exchange chromatography, immunoaffinity chromatography, fractionation on protein A gels, and other procedures are common. Unfortunately, these procedures are either time-consuming or restricted by inadequate recovery. Protein A affinity chromatography as the platform method for IgG purification, cannot be utilized for IgY purification due to differences in the Fc region[9]. Methods developed in the literature for egg yolk IgY mainly involved at least two steps: primary separation with natural gums[10], organic

solvents[11], water dilution[12], polyethylene glycol precipitation[13] or ammonium sulfate precipitation[12] to remove lipids and lipoproteins from egg yolk extract, while the fusing affinity chromatography[14–16], ion-exchange chromatography[17], thiophilic chromatography[18] or ultrafiltration for further purification[19]. Multi-step procedures typically result in low yields, increased complexity, and difficulties scaling up. A study reported the separation of IgY from chicken serum using thiophilic chromatography, and the recovery was 63.7%[20]. Consequently, developing efficient and cost-effective technologies for IgY purification is important.

However, having access to a simple and general purification approach that does not rely on the features of the antigen binding site in a certain Ig fragment is definitely preferable. Mixed-mode chromatography (MMC) with two or more interaction modes can increase separation selectivity and efficiency with selective ligands[21]. Most mixed-mode ligands contain both hydrophobic and electrostatic groups[22]. Normally, target proteins may be adsorbed on uncharged ligands by hydrophobic force at neutral pH, while desorption is driven by electrostatic repulsion between protein and charged ligands at acidic pH[23]. Fortunately, immunoglobulins have a unique affinity for thioether-substituted organic sulfone compounds, a phenomenon known as thiophilic interaction[24]. The thiophilic adsorption chromatography (TAC) approach is based on a synthetic pseudo-affinity matrix known as T-gel[25] and it allows for the selective purification of immunoglobulins from human serum[26] as well as hybridoma culture supernatants[27]. Because adsorption is only dependent on the presence of a kosmotropic salt, this technique combines the benefit of readily regulated adsorption behavior with the chemical stability of the matrix, high protein binding capacity, and affinity for several classes of antibodies[28]. Therefore, we are interested in using TAC as a simple method for the purification of our IgY anti-MPT64, which is treated by initial PEG lipid precipitation. MPT64 is one of the primary culture filtrate proteins (24 kDa) encoded by the RD2 region genes and has been demonstrated to be a particular antigen that separates the *M. tuberculosis* complex from mycobacteria other than TB (MOTT)[29,30]. Thus, the purity of the IgY anti-MPT64 is highly important for the detection of the MPT64 antigen secreted by the *M. tuberculosis* complex.

2. Materials and Methods

2.1. Chemicals

The materials used in this study were polyclonal antibody of IgY anti-MPT64, MPT64 standard protein (Mybiosource), anti-Chicken IgY (IgG) (whole molecule)−Alkaline Phosphatase antibody produced in rabbit (Sigma-Aldrich), ammonium persulfate (APS) (Vivantis), glacial acetic acid (Merck), hydrochloric acid (Merck), Bovine Serum Albumin

(BSA) (Sigma), Coomassie blue-R (Himedia), EDTA (Merck), glycerol, glycine (Merck), Whatman paper no.42, BCIP®/NBT solution (Himedia), 0.9% saline NaCl solution (PT. Widatara Bhakti), nitrocellulose membrane, methanol (Merck), mercaptoethanol (Merck), sodium phosphate (Merck), sodium sulfate (Merck), n-propanol (Merck), phosphate-buffered saline (PBS) (Vivantis), superflow thiophilic resin (G-Biosciences), skim milk, sodium dodecyl sulfate (SDS) (Vivantis), tris base (Vivantis), tris-HCl (Merck), TEMED (Bio-Rad), and tween-20 (Vivantis).

2.2. Purification of IgY

The system preparation process begins with setting the system to manual mode using the UNICORN software. Purification of polyclonal antibody of IgY anti-MPT64 was first carried out with a five steps gradient variable. The purification process begins by inserting a centrifuge tube into the fraction collector component. The end of the hose was moved to the hose holder in the fraction collector, ensuring that the hose did not interfere with the rotation of the fraction collector. Then, UNICORN software was set in automatic mode before the process was running. Setting the automatic mode in the UNICORN software was divided into five stages. The first stage, method settings, was carried out by adjusting the column volume (CV) variable, column type, and flow rate. For this study, settings were made for a variable column volume of 5 CV, and flow rate of 1 mL/min. The second stage was equilibration to balance the system with the volume equilibration variable in units of CV. In this study, a 4 CV balancing buffer was used but the resulting fraction was not collected, so the results of this phase will come out through the drain hose. The third stage was the IgY anti-MPT64 sample application with a variable number of samples entered into the system through the sample valve. In the 5-step gradient method, 1 mL of the sample was used which will bind IgY to the resin matrix. Then the next stage was to wash out the unbound IgY by washing the resin column with 5 CV of balancing buffer, then the resulting eluate was collected. The last stage was elution and fractionation to remove IgY bound to the matrix. Prior to use, manual mode is used for washing the FPLC system with distilled water to remove residual impurities from previous processing. The thiophilic matrix was packed onto the chromatography column manually outside the Fast Protein Liquid Chromatography (FPLC) system. The T-gel was activated using a balancing buffer (20 mM sodium phosphate, 0.5 M sodium sulfate, pH 7.5) and allowed to solidify in a column of about 5 mL as the CV. Then, the column was installed in the FPLC system which was washed using distilled water. Then FPLC is operated with the run method as shown in Table 1.

The elution was carried out in gradient steps with the percentage of eluting buffer (%B) constantly changing. The 5 gradient steps with %B of 0–20%, 20–40%, 60–80%, and

80–100% in each of 3 CV. Each fraction produced at each step was accommodated for further concentration with Amicon ultrafilter. The last stage was the column regeneration process with a regeneration buffer of 5 CV and the resulting fraction was accommodated. The 2 steps gradient purification variable has the same processing steps. The difference was in the elution and fractionation stages for this method which was carried out in 2 levels. The 2 gradient steps using %B of 0–50% and 50–100% with 8 CV each. After completing the setting, the running process was carried out and the resulting chromatogram was observed. The fraction of IgY anti-MPT64 which was detected at the absorbance of 280 nm UV light in the elution process was collected for further characterization. The fractions in the 5 steps gradient elution stage which had absorbance were combined into one and 1 mL was taken to be characterized as the second peak before concentration. The combined fractions were concentrated by placing 12 mL of the fractions into a 15 mL 100,000 MWCO ultrafilter Amicon column and centrifuging for 15 min at 5,000 x g at 4°C. The filtrate was separated and the obtained concentrate in the concentrate tube on the ultrafilter was re-added to the sample as much as the lost volume. The centrifugation process was repeated until all samples were concentrated. The concentrated sample in the ultrafilter was added with PBS 1x pH 7.4 for 2 times the volume of the sample. The sample was again centrifuged for 15 min at 5,000 x g at 4°C. The same steps were carried out for the 2 steps gradient elution fraction.

Table 1. Process flow of IgY anti-MPT64 purification.

Setting	Quantity and Unit	Note
Flow rate	1 mL / min	During operation
Pressure	0.3 mPa	During operation
Fractionation	3 mL / fraction	During operation
Column/matrix volume (CV)	5 mL	
System balancing	4 CV	• Balancing buffers (20 mM sodium phosphate, 0.5 M sodium sulfate, pH 7.5) • The eluate was discarded
Sample application	1 mL	Through the sample valve
Sample washing	5 CV	• Balancing buffers (20 mM sodium phosphate, 0.5 M sodium sulfate, pH 7.5) • The eluate was fractionated
Elution of protein analytes 5 steps gradient method (%B)	3 CV	• Elution Buffer (20 mM sodium phosphate pH 7.5) • The eluate was fractionated

Setting	Quantity and Unit	Note
		• Concentration gradient step method 0-20% buffer B
	3 CV	• Elution Buffer (20 mM sodium phosphate pH 7.5) • The eluate was fractionated • Concentration gradient step method 20-40% *buffer* B
	3 CV	• Elution Buffer (20 mM sodium phosphate pH 7.5) • The eluate was fractionated • Concentration gradient step method 40-60% *buffer* B
	3 CV	• Elution Buffer (20 mM sodium phosphate pH 7.5) • The eluate was fractionated • Concentration gradient step method 60-80% *buffer* B
	3 CV	• Elution Buffer (20 mM sodium phosphate pH 7.5) • The eluate was fractionated • Concentration gradient step method 80-100% *buffer* B
Elution of protein analytes 2 steps gradient method (%B)	8 CV	• Elution Buffer (20 mM sodium phosphate pH 7.5) • The eluate was fractionated • Concentration gradient step method 0-50% *buffer* B
	8 CV	• Elution buffer (20 mM sodium phosphate pH 7.5) • The eluate was fractionated • Concentration gradient step method 50–100% *buffer* B
Column regeneration	5 CV	• Regeneration buffer (20 mM sodium phosphate, 30% n-propanol, pH 7.5) • The eluate was fractionated

2.3. Determination of IgY purity

In this study, the SDS PAGE analysis was used to characterize the purified IgY anti-MPT64. A separating gel (15%) and a stacking gel (4%), respectively, made up the gel electrophoresis. The SDS PAGE gel was prepared by the standard procedure[31]. The prepared

gel was subsequently dipped in the SDS electrophoresis buffer. The prepared sample (30 µL sample, 5 µL sample buffer, heated at 95°C for 5 min) and the protein marker were put into the well on the gel. The electrophoresis process was performed at 100 V and 400 A for 90 min. After being rinsed three times with running water, the gel was colored with Coomassie blue solution at room temperature and gently swirled for 1–2 h. The stained gel was also rinsed with distilled water before being submerged in the destaining solution for 18 to 24 h, until the background of the gel turned colorless and the bands of separated proteins turned blue. In theory, IgY reduced by β-mercaptoethanol will give rise to a band at 65 kDa (for 2 identical heavy chains) and a band at 25 kDa (for 2 identical light chains).

2.4. Determination of IgY concentration

The concentration of purified IgY anti-MPT64 was performed using the Lowry method. The UV spectrophotometer was set at a wavelength of 750 nm and left for the equilibration process for 15 min. Then the absorbance reading was made to zero by using a blank solution. After the blank solution, the absorbance reading of the BSA standard solution was carried out with 5 different concentrations, between 10–100 µg which diluted from 1 mg/mL BSA stock solution. The readings of the BSA standard solution were then plotted to create a standard curve. For the analysis, a 0.4 mL protein sample was added with 0.4 mL of Lowry's reagent and then incubated for 10 min at room temperature. The sample solution was then added with 0.2 mL of Folin's reagent 0.2 N and mixed using a vortex. After mixing, the solution was allowed to stand for 30 min, and then the absorbance was read at a wavelength of 750 nm. The protein concentration of the sample was calculated by inserting the absorbance value of the sample into the equation from the standard curve graph[32].

2.5. Determination of binding activity

The IgY anti-MPT64 recognition of the MPT64 protein was carried out using the dot blot method. A volume of 10 µL MPT64 protein standard and the extracellular protein extracts containing MPT64 were dropped onto the nitrocellulose membrane. The extracellular MPT64 was isolated from the secretion of bacterial suspension of *Escherichia coli* BL21 (DE3) transformant [pD861-SR: 319895]. The membrane was then dried in open air. Then the nitrocellulose membrane was blocked with 5 mL of PBS blocking solution (PBS, 0.5% skim milk, and 0.5% tween 20) and shaken with a rocker-shaker for 30 min at 4°C. After that, 10 µL of IgY anti-MPT64 was added to the mixed-blocking solution. The incubation process was carried out using a rocker-shaker for 2 h at 4ºC. After the incubation process was complete, the blocking solution was discarded and the membrane was washed with 5 mL PBS for three times, in 5 min of each process. Then the membrane was re-washed

with 5 mL of TBS for 5 min. The blocking process was repeated using 5 mL of TBS blocking solution (TBS, 5% skim milk, and 0.5% tween 20). The blocking process was carried out using a rocker shaker for 5 min at 4°C. Then a secondary antibody (anti-rabbit alkaline phosphatase) was added to the blocking solution in the amount of 10 µL and incubated for 2 h at room temperature. After the incubation process was complete, the blocking solution was discarded and the membrane was washed with 5 mL TBS for three times, for 5 min each. Then each nitrocellulose membrane was dripped with NBT/BCIP solution and incubated in the dark room for 1 min. Upon a blue-purple color change occurring on the membrane, the reaction was stopped by adding 0.5 M EDTA solution.

3. Results

Figure 1 shows five different groups of peaks. The first peak (1) is the washout of proteins that did not bind to the column with a binding buffer. The second to four peaks (2-4) reflect the elution of column-bound IgY with elution buffer, while the third peak (5) represents other non-IgY that are strongly bonded to the column that elute following the addition of 30% n-propanol elution solution. The figure also shows the fractions in tubes T18-T22 (second peak), T26-27 (third peak) and T36-37 (fourth peak) collected during the runs. Meanwhile, T40-41 (fifth peak) was not collected due to n-propanol which was used to elute column-bound proteins that remained strongly attached to the column after elution with elution buffer, which was likely to denaturize the proteins.

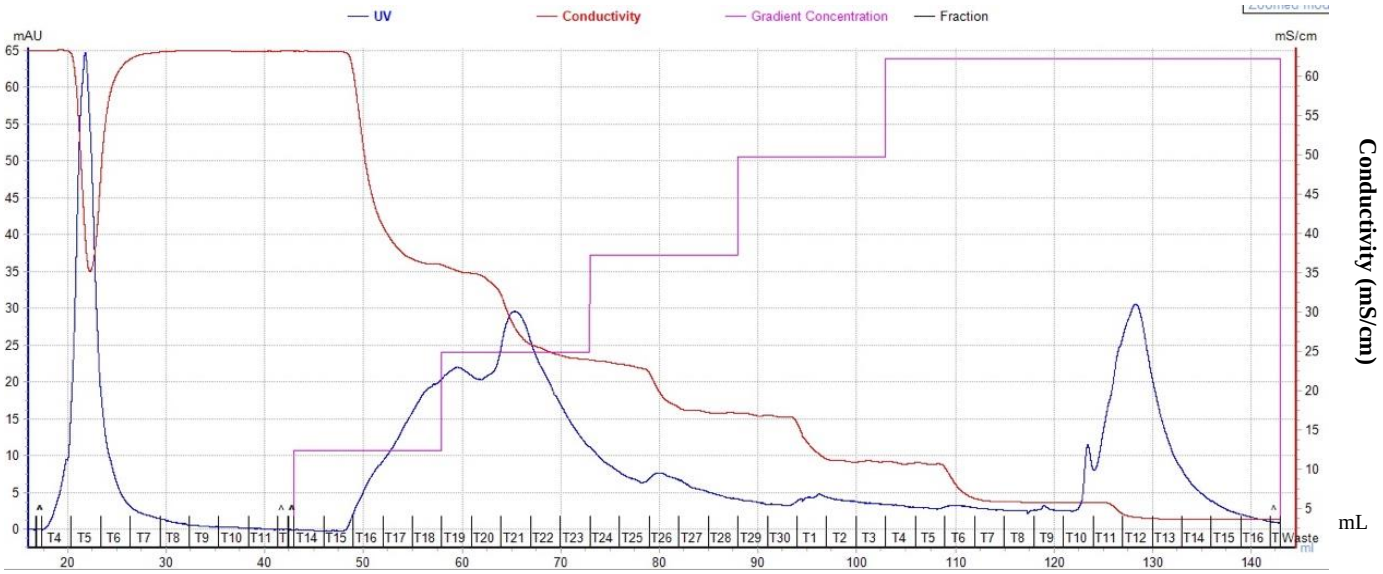

Figure 1. Chromatogram of 5 step gradient elution

The profile of the 2-step gradient purification chromatogram is shown in Figure 2. The first peak (T4–12) yielded a low IgY concentration. The second peak was divided into two significantly separated peaks, namely T 21–25 and T34–37. Meanwhile, the third peak (T48 – 51) has many serrations at its peak.

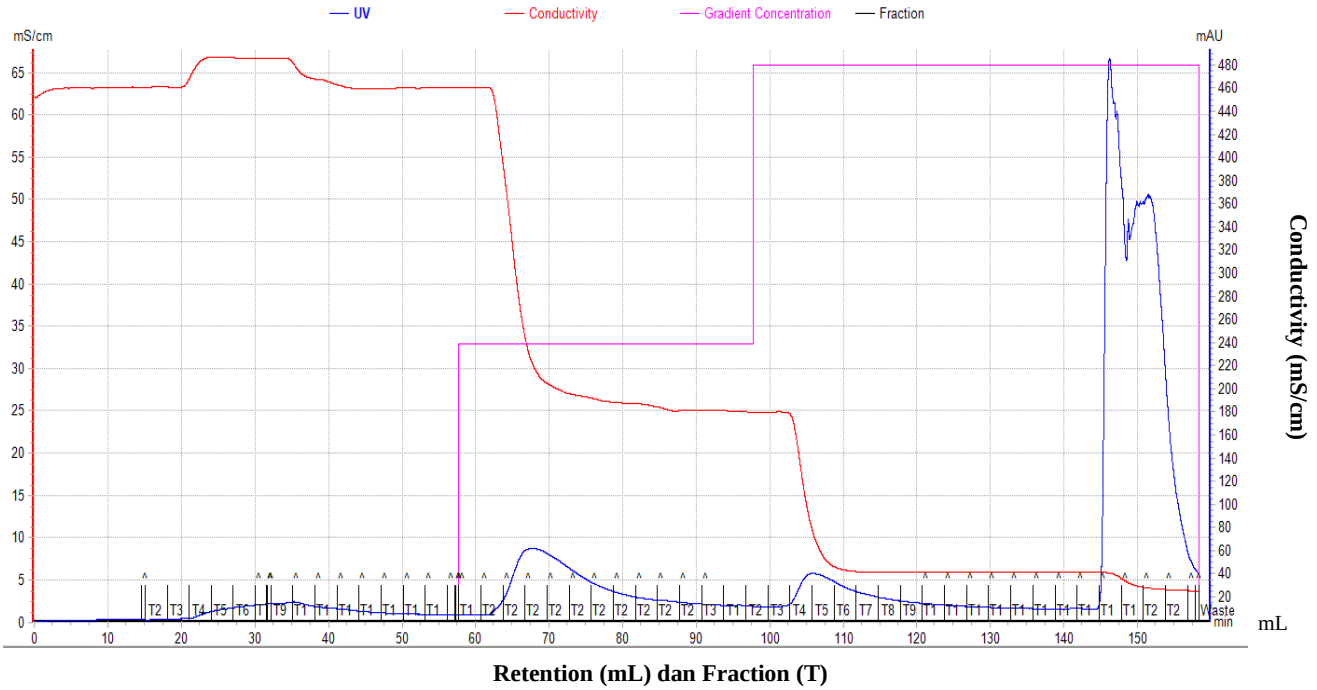

Figure 2. Chromatogram of 2 step gradient elution.

The high level of purified IgY can be predicted from the absorbance value displayed on the chromatogram. This absorbance value affects the IgY concentration, the smaller the absorbance value, the smaller the measured IgY concentration[33]. The highest absorbance values of the peaks of the two-phase gradient 5-step purification and 2-step gradient purification were in the range of 30 mAU and 9 mAU, respectively. The obtained volume of each gradient purification step is shown in the Table 2.

Table 2. The volume of purified IgY.

IgY	Volume (mL)	Concentration (mg/mL)
IgY from 5 step gradient elution after concentrated	0.6	1.35482
IgY from 2 step gradient elution after concentrated	0.9	2.2632

The peak fractions of those phases before and after concentration were subsequently analyzed using SDS-PAGE, as shown in Figure 3. The reductive approach was utilized for the SDS-PAGE characterization, employing β-mercaptoethanol as a reducing agent. In principle, β-mercaptoethanol will diminish IgY, causing the light and heavy chains to split into two distinct bands on SDS-PAGE, recognized as the IgY heavy (65 kDa) and IgY light

chain (25 kDa)[34]. The IgY concentration was then determined using the Lowry technique and there was a substantial difference between the two outcomes, with the 2-step gradient concentration containing higher total protein. However, SDS PAGE revealed that a 5-step gradient concentration yielded higher IgY purity.

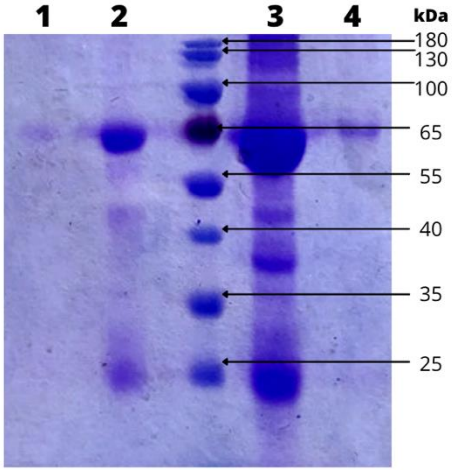

Figure 3. Reductive SDS-PAGE characterization.

Notes: 1= IgY from 5 step gradient elution before concentrated, 2 = IgY from 5 step gradient elution after concentrated, 3 = IgY from 2 step gradient elution after concentrated, 4 = IgY from 2 step gradient elution before concentrated.

The IgY anti-MPT64 antibody developed in this work is intended to be the major component of a TB detection test kit that includes the MPT64 protein as a biomarker. As a consequence, the purification findings that demonstrated the highest degree of IgY purity were further characterized. The target of this study is the level of purity of the conjugated antibody that must also be considered to increase the sensitivity of the lateral flow immunoassay (LFIA) and the quality of its performance. In order to enhance the sensitivity and performance quality of the lateral flow immunoassay (LFIA), the sensitivity of the conjugated IgY must also be investigated. The standard MPT64 protein and extracellular protein extracts containing MPT64 can be recognized by the purified IgY, presented in Figure 4.

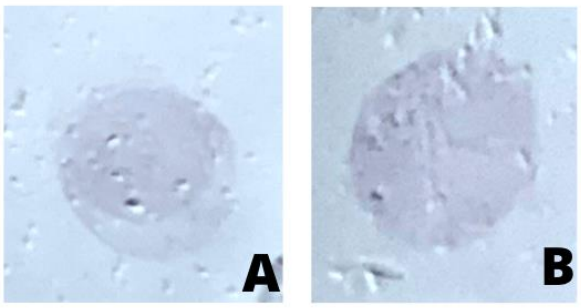

Figure 4. Sensitivity of IgY anti-MPT64. Notes: A: extracellular protein extracts containing MPT64, B: standard MPT64 protein.

4. Discussion

In this study, we aimed to optimize the recovery of pure IgY anti-MPT64 using thiophilic adsorption chromatography**.** New matrix technology for large-scale protein separations based on naturally occurring polysaccharides has recently been developed with the goal of achieving process savings[35,36]. The recovery of research costs is one of the most prevalent thiophilic mechanisms. Thiophilic adsorption chromatography is an effective and simple method for the purification of proteins—in particular, immunoglobulins. Thiophilic adsorption has been shown to selectively purify IgG's from complex biological fluids like serum, cell culture supernatant, and colostrum milk under mild elution conditions[28,36,37]. We believe that when the advantages of large bead technology are coupled with new and improved immobilization approaches, true advances in bioprocessing throughput and productivity will emerge. This method depends on the affinity of the sulfur-containing ligands (thiols). Ligands are immobilized in a suitable matrix for immunoglobulin by eluting with a solvent containing high concentrations of sodium sulfate salts and their release is carried out by reducing or removing these salts[38]. The most popular agarose-based adsorbents for thiophilic chromatography are "T-gels" that comprise a linear ligand with two sulfur atoms and are generated via the reaction of divinyl-sulphone with 2-mercaptoethanol. In the presence of high concentrations of structure-forming salts, such as ammonium sulfate or sodium sulfate. These compounds demonstrated a slight preference for binding to immunoglobulins.

Thiophilic adsorption chromatography is analogous to hydrophobic interaction chromatography since high salt concentrations enhance adsorption and elution will occur when the salt concentration is reduced or eliminated. When the thioethylsulfone structure lacks specified hydrophobicity and ionic charges, hydrophobic associations and ionic interactions between IgY and thiophilic adsorbents will not occur. As a result, the type of salt utilized influences the strength of the interaction. Salts in the form of sulfates and phosphates are frequently used in thiophilic mediated chromatography, despite sodium chloride, even at high concentrations, does not increase protein adsorption[39]. The salt concentration in the balancing buffer provided a significant effect when immobilizing the column. The higher the UV absorbance value on the chromatogram indicates the higher the protein concentration in that fraction. It can be seen that the more gradual the addition of balancing buffer, the lower the total IgY concentration is obtained, compared to the gradient step of adding less balancing buffer. In Figure 1, it can be seen that more protein is washed through from the column than the 2 steps gradient treatment shown in Figure 2. According to the theory, at the first peak of

the 5 steps gradient method, the eluted protein is not IgY but another protein, because the IgY needs thiophilic interactions to be adsorbed on the thiophilic matrix.

The presence of a high concentration of salt (0.5 M sodium sulfate) in the mobile phase disrupts the hydrogen bonding between water and IgY which causes the hydrophobic sites in the IgY structure to open to the surface so that it is adsorbed on the matrix, while other proteins that do not have hydrophobic interactions will elude and separate from IgY. In its normal phase, proteins (IgY) dissolved in water have hydrophilic sites on the surface of their structure. These IgY hydrophilic sites interact directly with water molecules while the hydrophobic IgY sites are on the inside of their structure and do not interact directly with water molecules. Salt at a certain concentration can disrupt the hydrogen bond between water and the hydrophilic sites of IgY so that the hydrophobic sites on the inside of IgY which have functional groups that act as nucleophiles and also other functional groups that act as electrophiles can appear on the surface of the IgY structure so that they can form an interaction. IgY is first eluted with a solution containing a certain salt concentration so that IgY can be adsorbed on the matrix. Compounds that do not have this interaction will elute and separate from IgY. IgY adsorbed on the matrix can be released by reducing or eliminating the salt concentration in the solvent. In theory, at this stage, IgY is released from the matrix after being eluted with a buffer that does not contain salt so that it can be estimated that the protein contained in these fractions is IgY. This process causes the thiophilic interaction to be weakened because the IgY hydrophobic site will re-enter the interior of the IgY structure then IgY can be eluted[24]. Separation with a high range in the 5 steps gradient produced a higher acquisition of purer IgY compared to the 2 steps gradient method. These results suggest that one step of affinity chromatography with the optimized condition is enough to purify the IgY costly and efficiently. It also generates IgY with high specificity against the MPT64. The efficiency of this purification method is also reported in comparison to other IgY purification methods. SDS-PAGE and gel filtration revealed that IgY purity after one-step chromatography was 70%, lower than using thiophilic chromatography[40]. As a result, we can infer that a fast method for purifying IgY from egg yolk has been developed utilizing thiophilic chromatography.

5. Conclusions

This optimized purification of thiophilic adsorption chromatography by varying the gradient concentration of elution buffer into five steps gradient (0–20, 20–40, 40–60, 60–80, and 80–100%v/v) for three injection column volume can be applied to purify IgY anti-MPT64 efficiently. To our knowledge, the optimization of IgY purification conditions has never been done in other studies.

Author Contributions: Conceptualization, SAFK and TS; methodology, SAFK, SAK, and MY; software, MF and ITM; validation, SAFK, TS and SAK; formal analysis, SAFK and YR; investigation, SAK, ITM, and MF; resources, SAFK; data curation, SAFK; writing—original draft preparation, SAFK; writing—review and editing, SAFK.

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