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Purification of IgY Anti-SARS-CoV-2-Chicken Nucleocapsid and Elimination of Its Low-temperature Storage-induced Aggregates

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Abstract

Even though the COVID-19 pandemic has ended, the virus is still circulating. It is important to detect it, as its symptoms are similar to other endemic diseases, e.g. dengue fever. Detection can be done using birds' specific immunoglobulin Y, IgY Anti-SARS-CoV-2-Chicken Nucleocapsid (IgY anti-N), obtained by immunising chickens with the SARS CoV2 nucleocapsid protein. However, IgY purification requires further research, as commonly used ligands are unsuitable. Another common problem with IgY anti-N is aggregation, even when stored at low temperatures. Thus, this research aims to purify IgY anti-N and eliminate low-temperature storage-induced aggregates. In this study, polyethene glycol (PEG) was used for delipidation. The resulting IgY was divided into 2 parts, one of which was stored at 4 °C and purified within the next 1 or 2 days (labelled as IgY 4 °C), while the other was stored at −20 °C and purified within a period of 1 or 2 years (labelled as IgY −20 °C). The next step involved purifying both samples using hydrophobic chromatography and sequential anion exchange. Peaks resulting from the hydrophobic column were analyzed using High-Performance Liquid Chromatography (HPLC) and Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE). Another purification result was analysed using PAGE. The migration of each separation step result was observed using native, denaturing and non-reducing PAGE. The study showed, qualitatively, that using two-step chromatographic techniques, hydrophobic interaction and ion exchange chromatography significantly increased the purity of IgY during purification. Additionally, the results demonstrated that hydrophobic chromatography purifies IgY and removes aggregates that may arise from storage. Moreover, similar to immunoglobulin G, IgY anti-N was found to have an uncommon migration in native PAGE compared to other proteins.

Keywords: IgY anti-N-SARS CoV2, Protein purification, Hydrophobic interaction, Ion exchange chromatography, Aggregate, Chicken IgY, Denaturation

Introduction

Specific immunoglobulin Y (IgY) can be created by immunisation of chickens with specific antigens; therefore, it has broad application prospects in scientific, diagnostic, prophylactic, therapeutic purposes, immunochemical reagents, and food formulation or supplements [1-9]. IgY anti-SARS-CoV-2-Nucleocapsid (IgY anti-N) is obtained similarly and plays a role as a detection antibody in the COVID-19 rapid detection tool. Even though the COVID-19 pandemic has ended, the SARS-CoV-2 virus is still circulating, so this rapid detection tool is essential, especially in tropical countries, in making a diagnosis, considering that some of the symptoms of COVID-19 are similar to dengue fever [10-12].

IgY anti-N must be purified from other components in egg yolk before it can be employed as an antibody-detecting agent. IgYs make up roughly 3 - 5 % of the proteins in egg yolks, which are spread in a lipid emulsion with lipoproteins and glycoproteins. As a result, in most situations, IgY isolation begins with the removal of lipids to create a water-soluble fraction ("delipidation" phase) [8,13-15]. This lipid removal can be performed using acidified water, acid, organic solvents, natural gums, and polysaccharides. Following delipidation, several IgY extraction methods that may be used were precipitation, chromatographic, and filtration [14]. Based on research [13,16], organic solvents (chloroform or phenol) were found to reduce the lipid ratio in egg yolk; the water dilution method, PEG precipitation, and caprylic acid extraction produced high yields; and antibodies purified with PEG and carrageenan demonstrated high purity [16]. In the purification of immunoglobulin IgG, affinity chromatography with protein ligands A, G, and L is commonly used. However, since there are differences in its Fc structure, this method does not apply to IgY. [13,17,18]. Contrary to proteins A, G and L, IgY can bind to protein M, a human mycoplasma protein. Some studies use it as a ligand in affinity chromatography for IgY purification. However, there appeared new protein in protein-M purified IgY, so it still needs further investigation whether protein M could introduce the exogenous proteins in the final products [13]. Therefore, there is an open possibility to develop a purification method for IgY.

Another problem commonly encountered by proteins (including IgY anti-N) is denaturation, which can occur due to decreased solubility, decrease in biological activity, depletion of crystallisation properties, an increased reactivity of the component groups, altered shape of protein molecule and susceptibility to enzyme hydrolysis [19-21]. Denaturation is a chemical process that can be defined as any change in the secondary structure, tertiary structure or quaternary structure of a protein molecule which leads to disruption of covalent bonds [20]. Denaturation may occur in the storage phase, even though stored at a cold temperature. According to studies, proteins lose activity when exposed to low temperatures or stored in the refrigerator. Salt concentration, pH, and cold temperature seem to affect the rate and extent of denaturation [20].

Hence, our current study aims to isolate IgY anti-N by PEG, hydrophobic interaction chromatography, and anion exchange chromatography subsequently. The goal is to obtain a pure IgY anti-N. Additionally, hydrophobic chromatography will remove aggregates that may arise from storing IgY at low temperatures.

Materials and methods

Extraction of IgY anti-SARS-CoV-2-Nucleocapsid

IgY anti-N was extracted with the PEG precipitation method [13,22-27] and described briefly below. The yolk of the egg was separated from the egg white using an egg separator. The egg yolk was gently rolled on the filter paper to remove the egg white as much as possible. The yolk membrane was torn and held by a spatula, and then the yolk was flowed into a graduated cylinder. Two volume of phosphate buffer saline (PBS) (pH = 7.5) was added to one volume of egg yolk, followed by the addition of a pulverised solid of PEG-6000 to give a final concentration of 3.5 % (w/v). At room temperature, the mixture was stirred, and after all PEG-600 dissolved, the solution was centrifuged at 13,000xg; 20 min; 4 °C. The resulting supernatant was collected and filtered through a coarse filter paper. Subsequently, it was added by PEG-6000 to a concentration of 8.5 % (w/v), stirred, and centrifuged as described above. The precipitate was resuspended, and another precipitation step was performed with 12 % of PEG-6000 (w/v). The precipitate from 12 % of PEG-6000 was solubilised in sufficient PBS buffer until completely dissolved. The solution was stored at 4 - 8 °C to be purified in the next few days and labelled as IgY 4 °C. Whereas some of it was stored at −20 °C to be purified the next year or 2 and labelled as IgY −20°C. The extracted and unextracted IgY anti-SARS-CoV-2-Nucleocapsid were characterised by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) [28].

Purification of IgY anti-SARS-CoV-2-Nucleocapsid using butyl toyopearl resin and its characterisation

Butyl toyopearl resin was resuspended in an equilibrium buffer 1 (20 mM sodium phosphate, 0.5 M potassium sulfate (pH 7.5)) to prepare the butyl chromatographic column. This slurry was then packed into an XK 16/20 chromatography column and was left to stand overnight, and the resin volume was 5 - 6 bed volume/column volume (CV). The prepared column was then attached to the chromatography system, AKTA Start instrument.

IgY 4 °C was dialysed using dialysis tubing cut-off 14 kDa against equilibration buffer 1 and injected into the AKTA Start chromatography system. Purification was carried out using the “method run” program, which was arranged in 4 steps that were prime and equilibration with 3 CV equilibrium buffer 1, washing with 7 CV equilibrium buffer 1, first elution using 7 CV of concentration gradient of 0 - 100 % elution buffer (20 mM Sodium phosphate, pH7.5), second elution using 4 CV of 100 % elution buffer and regeneration using 3 CV of regeneration buffer (20 mM sodium phosphate, 30 % n-propanol, pH 7.5). All processes were run at a flow rate of 1.0 mL/min, and fractions were collected as much as 3 mL each. The peak containing IgY was concentrated using Amicon® Ultra-15 Centrifugal Filter Devices. The same procedures were performed on the IgY −20 °C sample.

SDS-PAGE characterised the purified and unpurified IgY anti-N. In addition to SDS PAGE, HPLC is used to identify the IgY peaks. The Agilent HPLC system was used and run under the following conditions: Agilent SEC 3 300A (70.8×300 mm) column at 25 °C; flow rate of 1.0 mL/min; photodiode array detector at 280 nm. Elution was carried out in several steps, i.e. linear gradient of acetonitrile (ACN) containing 0.1 % trifluoroacetic acid (TFA), linear gradient elution increased from 5 to 80 % ACN in 15 min, decreased to 5 % ACN in 1 min, then remained at a constant concentration for 4 min [29].

Purification of IgY anti-N using DEAE toyopearl resin and its characterisation

DEAE column preparation and its attachment to the AKTA Start instrument were carried out in the same steps as butyl column preparation, except the resin was DEAE toyopearl.

The concentrated peak containing IgY resulting from butyl purification was dialysed using dialysis tubing cut-off 14 kDa against equilibration buffer 2 (Britton Robinson buffer, pH 7.0). It was subsequently injected into the AKTA Start chromatography system.

Purification was run under the following conditions: Equilibration, washing, elution 1 and elution 2 were performed respectively by using 1 CV of equilibrium buffer 2, 2 CV of equilibrium buffer 2, 5 CV of a concentration gradient of 0 - 100 % elution buffer 2 (1 M NaCl in Britton Robinson pH 7,0) and 6 CV of 100 % elution buffer 2. All processes were run at a flow rate of 1.0 mL/min, and fractions were collected as much as 3 mL each.

Purified and unpurified IgY anti-N were characterised by SDS-PAGE [28].

Migration of purified IgY anti-N on PAGE

Migration of IgY anti-N was characterised by polyacrylamide gel electrophoresis (PAGE) in native, denaturing and non-reducing conditions. The 3 factors that differentiate the PAGE methods are denaturing agents like SDS, reducing agents such as beta-mercaptoethanol, and heating. The native (also known as non-denaturing) PAGE method does not use any of these 3 factors. The denaturing PAGE method (SDS-PAGE or denaturing PAGE) applies these 3 factors. On the other hand, non-reducing PAGE is the PAGE method that doesn’t involve reducing agents and heating.

Results and discussion

IgY anti-N was successfully isolated from egg yolks using the PEG precipitation method. This method was carried out by adding PEG gradually to the sample. According to Ren *et al.* [16], the delipidation process, precipitating the yolk lipid, occurred when adding 3.5 % of the PEG-6000. Then, increasing the PEG concentration to 8.5 and 12 %, IgY precipitated, and some other proteins remained soluble (about 40 kDa molecular weight). As a result, IgY purity was increased, and SDS PAGE evaluated its purity. Due to the reduction of the IgY disulfide bridge by SDS, the IgY targeted bands arose approximately 65 kDa (thick band) and 25 kDa (thin band) (

Figure *1*),

IgY anti-N resulting from 12 % PEG precipitation, which was then stored at a temperature of 4 °C, was labelled as IgY 4 °C.

IgY 4 °C was further purified by column chromatography with a butyl toyopearl resin. The protein interacts with the butyl groups of the resin at hydrophobic sites on its surface; a water mantle would typically cover this area. In this study, K2SO4 was used, where this salt served to attract the water mantle that coats the hydrophobic sites of the protein so that the hydrophobic sites become open and can interact with the butyl groups. **Figure 2(a)** shows that in IgY resulting from PEG precipitation, there are 3 groups of proteins with significantly different hydrophobicity. The first group (peak 1) was low hydrophobicity proteins, characterised by weak attachment to the resin. Then, the second group was the moderate hydrophobicity protein, which was read on the chromatogram as the second peak. Finally, group 3 exhibited a very strong interaction with butyl. This group was only released when eluted with a regeneration buffer containing a nonpolar compound, namely isopropanol. The characterisation results using SDS-PAGE showed that the second and third peaks had bands that matched the target (**Figure 2(b)**).

The IgY-targeted peaks were confirmed by analysing the peaks using an HPLC with a size-exclusion chromatography column. It was observed that IgY 4 °C was mainly eluted in peak 2 (**Figure 3(c)**), with a small amount present in peak 1 (**Figure 3(b)**), and none found in peak 3 (**Figure 3(d)**), based on the retention time of the IgY standard (**Figure 3(a)**). Combining these results with the SDS PAGE data, it can be concluded that IgY was eluted at peak 2 (**Figure 2(a)**).

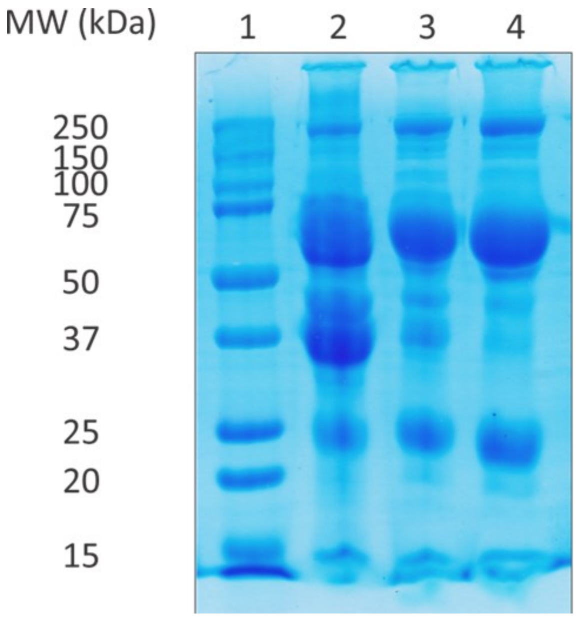
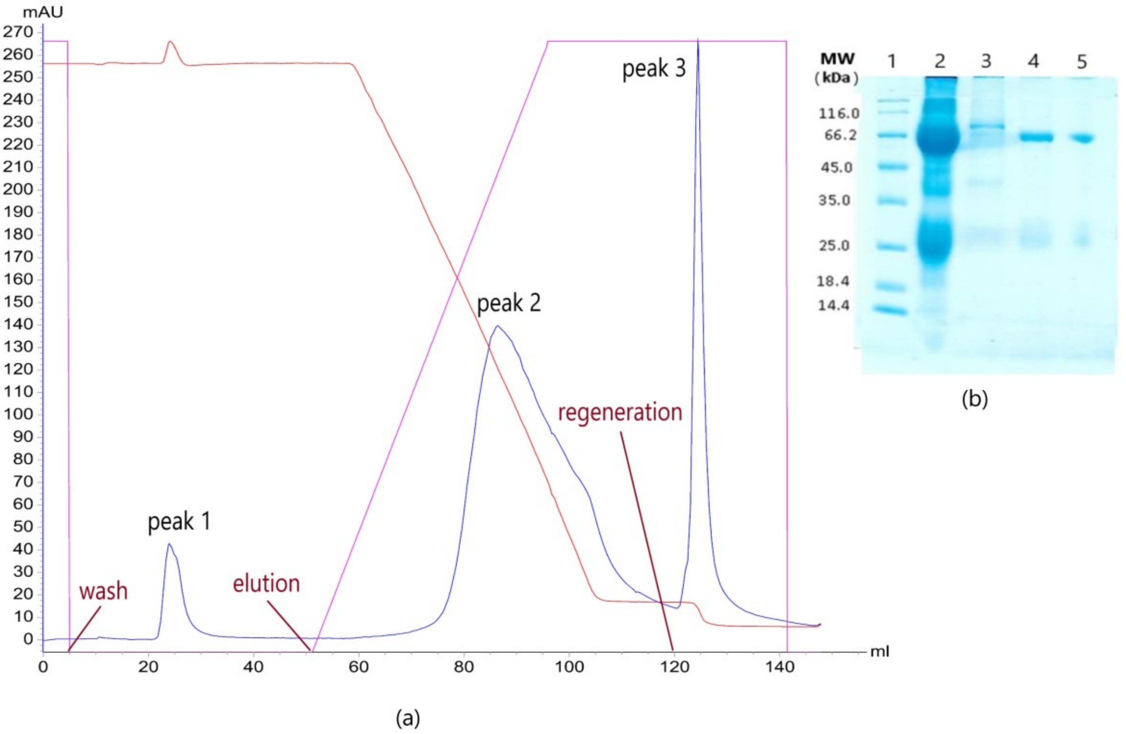


Figure 1 Migration pattern of IgY anti-N extracted by various concentrations of PEG-6000 on SDS-PAGE. Where PEG-6000 concentration was 3.5 % (lane 2), 8.5 % (lane 3), and 12 % (lane 4) which were compared with Bio-Rad Precision Plus Pre-stained (Protein Standards) (lane 1).



(a)

(b)

Figure 2 (a) Chromatogram of the purification process of IgY 4 °C using butyl Toyopear, (b) the results of the SDS PAGE analysis: Lane 1 shows the Pierce™ Unstained Protein MW Marker, while Lanes 2-5 respectively display the unpurified IgY 4 °C, Peak 1, Peak 2, and Peak 3.

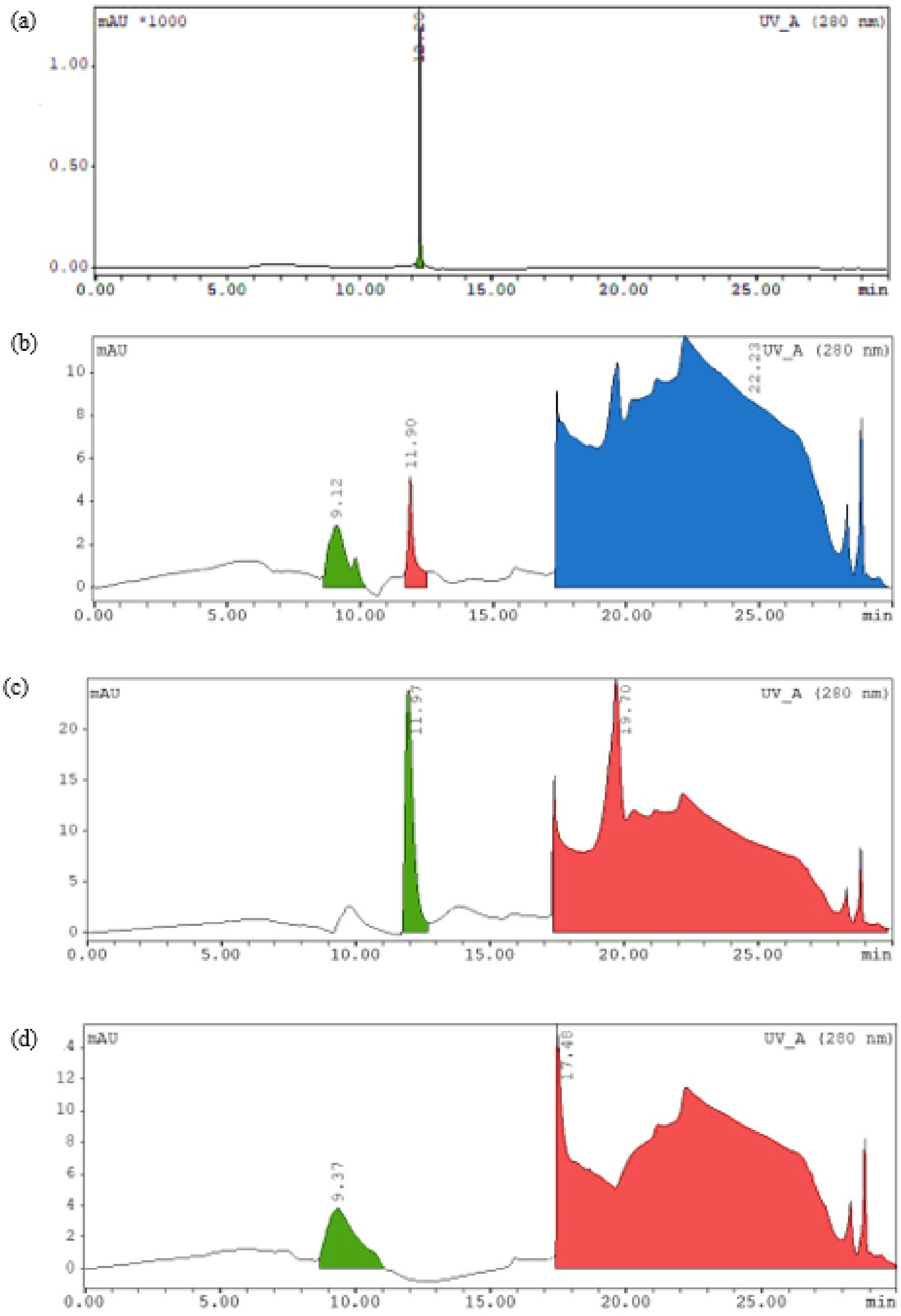


Figure 3 The IgY peaks of butyl Toyopearl were analysed using HPLC and were identified as peak 1 (B), peak 2 (C), and peak 3 (D) in comparison to standard IgY (A).

In addition to IgY 4 °C, there was IgY −20 °C, an IgY anti-N precipitated with 12 % PEG and stored at −20 °C. Then, the IgY −20 °C sample was purified using the same procedure as IgY 4 °C. **Figure 4(a)** shows that IgY −20 °C has a similar chromatogram profile to IgY 4 °C, producing 3 peaks. The difference was seen in peak one, where the IgY −20 °C sample has a greater intensity than the 2nd peak. It seems that the high intensity of peak one was caused by protein aggregation during storage. Physically, the sample looked cloudy, and there was sediment if it stood for a long time. It has been proven that the presence of aggregates in IgY can be detected through SDS PAGE characterisation results obtained from PEG precipitation. The proteins remain at the top of the well without passing through the gel, as seen in lane 2 of **Figures 4(b)** and **4(c)**. It should be noted that this SDS-PAGE profile is different from that of the unaggregated IgY 4 °C sample, as observed in lane 2 of **Figure 2(b)**.

IgY −20 °C is known to cause aggregation, but why does it happen? This is because phosphate buffer saline (PBS) is not the best buffer for preserving proteins at low temperatures. This is because when the sodium phosphate buffer freezes, it forms dibasic sodium salt precipitates, which in turn causes a significant drop in pH. For example, the pH level of a 50 mM sodium phosphate solution may drop from 7.00 at 25 °C to as low as 3.36 at −20 °C. Additionally, the formation of $Na_2HPO_4 \cdot 12H_2O$ crystals increases protein concentration due to the sequestration of water from the solution. When this happens in combination with low pH levels and a fluid-solid interface on the surface of dibasic sodium phosphate crystals, it can result in protein unfolding and aggregation [30].

Butyl Toyopearl effectively separated aggregates of the IgY −20 °C sample, resulting in the emergence of peak 1. When aggregate was present, SDS-PAGE analysis didn't reveal a clear band (**Figure 4(b)** lane 2,3,2*), but one emerged after allowing peak 1 to settle and analysing the supernatant (**Figure 4(c)**).

As with the previous findings, the IgY target of the IgY −20 °C sample was detected at peak 2 (lane 4 in **Figures 4(b)** and **4(c)**). When it was concentrated using an Amicon cut-off of 50 kDa, resulting in a concentrated sample, the SDS-PAGE band profile was similar to that of an aggregate. It was due to a large amount of protein accumulating in the stacking gel section and the top of the resolving gel. However, in the concentrated sample, the bands are still clearly visible (lane 5 in **Figures 4(b)** and **4(c)**).

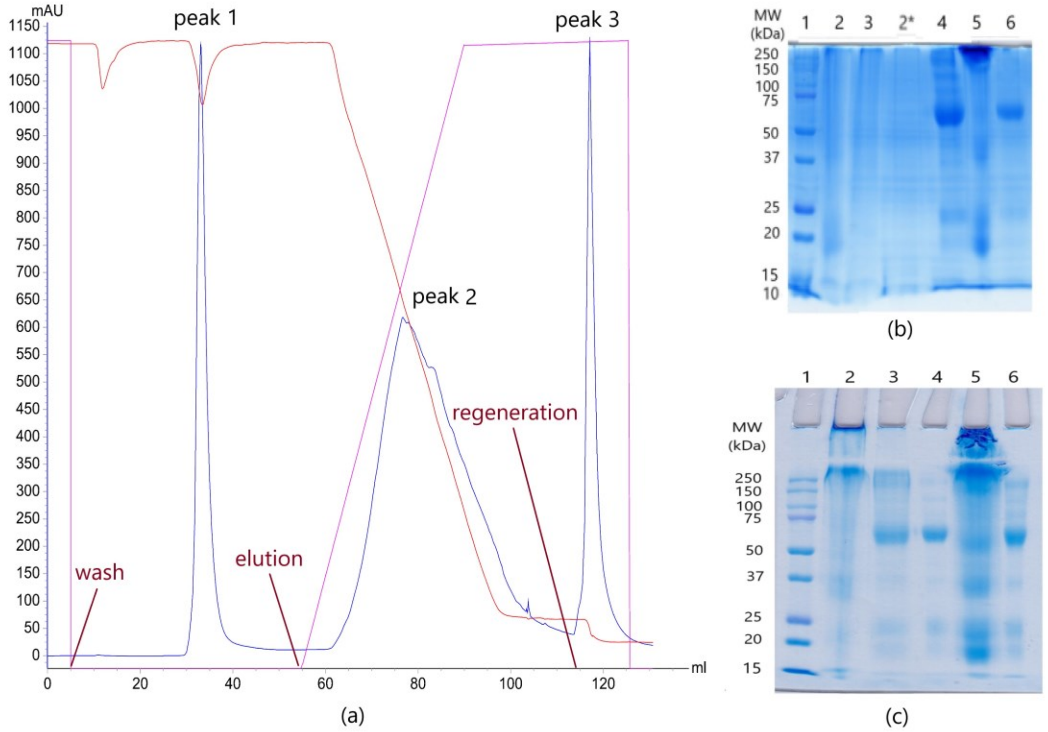
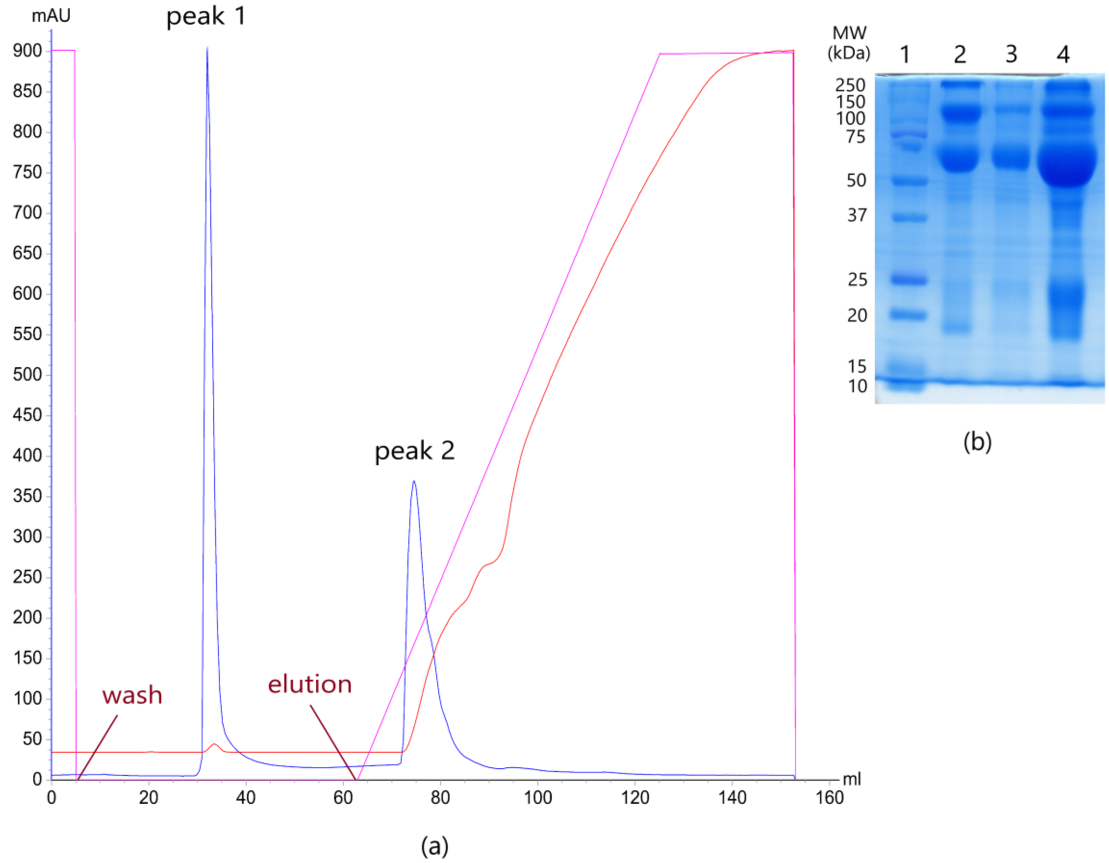


Figure 4 (a) Chromatogram of purification of IgY −20 °C with butyl toyopearl, (b) Analysis of the results with SDS PAGE at 12 % gel concentration, and (c) 10 % gel concentration where Lane 1-6 and 2* contained, Bio-Rad Precision Plus Pre-stained Protein Standards, unpurified IgY at −20 °C, peak 1, peak 2, concentrated peak-2, peak 3, and equilibrium-buffer dialysed IgY, respectively.

It can be concluded that peak 2 (IgY anti-N-containing) contains a more diverse range of protein types. Therefore, further separation is required due to the broadened peak. Thus, the next step in the purification process involved using anion exchange chromatography, specifically DEAE Toyopearl resin. The sample utilised at this stage was peak 2, which was the outcome of the concentrated purified Butyl Toyopearl. As a result, 2 peaks emerged (**Figure 5(a)**), wherein both peak 1 (lane 2 **Figure 5(b)**) and peak 2 (lane 3 **Figure 5(b)**) provided target bands when analysed using SDS-PAGE. However, preliminary research showed that the target IgY was released after elution with the addition of salt. Therefore, it can be inferred that the target IgY was present at peak 2 (**Figure 5(a)**).



(b)

(a)

Figure 5 (a) A Chromatogram of IgY resulting from butyl purification was further purified by DEAE Toyopearl. (b) SDS PAGE analysis, where: Lane 1 - Bio-Rad Precision Plus Pre-stained Protein Standards; Lane 2 - peak 1; Lane 3 - peak 2; Lane 4 - concentrated peak 2.

The purified IgY's molecular weight was analysed through 3 PAGE types: Native, denaturing, and non-reducing. Native PAGE determines the protein molecular weight proportional to the Slope Rf of protein migration at various gel concentrations. However, this method is not applicable for determining the molecular weight of IgY anti-N because IgY does not follow the typical migration pattern of most proteins. **Figure 6** shows the native PAGE profile of peak 2 of butyl toyopearl purification (lanes 4), peak 2 and peak 1 resulting from DEAE Toyopearl purification at lanes 5 and 6, respectively. They were identified to have a molecular weight above 250 kDa, similar to the pattern observed in mammalian immunoglobulin IgG (lanes 3). In comparison, BSA (lanes 2) represents proteins that migrate commonly under native conditions.

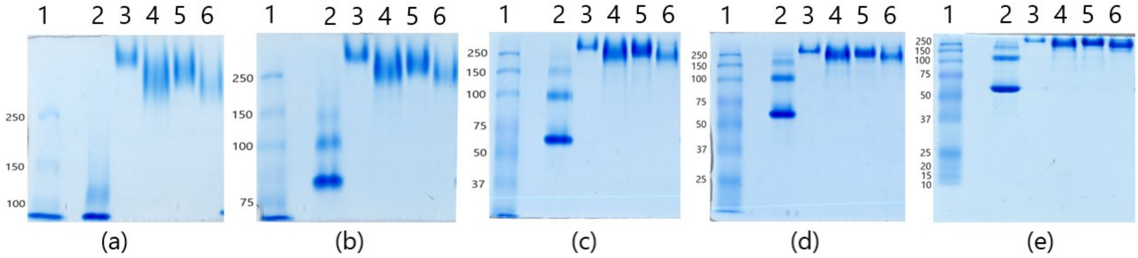


Figure 6 Native PAGE profiles of Bio-Rad Precision Plus Pre-stained Protein Standards (lane 1), bovine serum albumin/ BSA (lane 2), IgG (lane 3), peak 2 of butyl toyopearl purification (lane 4), peak 1 of DEAE toyopearl purification (lane 5), peak 2 of DEAE toyopearl purification (lane 6) which were run in gel concentrations of respectively, (a) 4 %, (b) 6 %, (c) 8 %, (d) 10 %, and (e) 12 %. All samples run 1 µg/mL in concentration, except protein standards.

Figure 7 shows 2 PAGE gels with concentrations of (a) 8 % and (b) 10 %, where each gel is filled with IgY anti-N samples in denaturing and non-reducing conditions. The same lane numbering is filled with the same sample concentration, except lane 3, where 3(b) has a more concentrated sample concentration than 3(a). Unlike its behaviour under native conditions, IgY anti-N could migrate along with standard proteins in non-reducing conditions. The study observed that IgY anti-N had a molecular weight of approximately 180 kDa (as indicated by the arrow in **Figure 7(a)**).

After undergoing successive purification processes such as PEG precipitation, hydrophobic interaction chromatography, and anion exchange chromatography, there was a significant improvement in the purity of IgY anti-N. **Figure 7(b)** lanes 2, 3, and 4 demonstrate the gradual removal of impurity proteins.

Accurately assessing the IgY anti-N purity requires quantitatively determining its specific activity. One reliable method for determining specific activity is using the ELISA method. However, it should be noted that there is currently no commercially available test kit for this purpose. As such, further research is necessary to develop a reliable means of quantifying its specific activity.

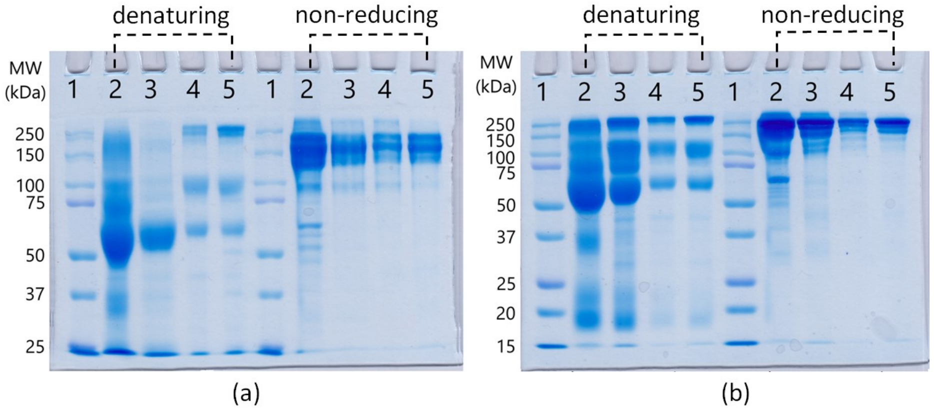


Figure 7 Denaturing and non-reducing PAGE of Bio-Rad Precision Plus Pre-stained Protein Standards (lane 1), IgY of 12 % PEG-6000 precipitation (lane 2), peak-2 of butyl toyopearl purification (lane 3a), concentrated peak-2 of butyl toyopearl purification (lane 3b), peak-2 of DEAE toyopearl purification (lane 4) and peak-1 of DEAE toyopearl purification (lane 5) which were run in gel concentrations of respectively, (a) 8 % and (b) 10 %.

Conclusions

In conclusion, the present study qualitatively demonstrated that the purity of IgY has increased significantly by the two-step chromatographic method: hydrophobic interaction and ion exchange. Furthermore, it was shown that hydrophobic chromatography may remove aggregates from storage besides purifying IgY anti-N.

There were limitations in the research. First, to accurately assess the purity of IgY anti-N, we need to quantify its specific activity. ELISA is a reliable method, but no commercial test kit is available. Further research is required to determine how to quantify its specific activity. Second, the purification method still involved using general resin like hydrophobic and DEAE. This method couldn't guarantee that the purified samples were particular IgY. However, since IgY has various applications, this research can be utilised in the future to extract IgY from yolk impurities, resulting in high-purity IgY. Perhaps a specific resin can be designed based on the target's affinity.

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