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A new membrane based process to isolate immunoglobulin from chicken egg yolk

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ABSTRACT

Although the importance of eggs as a source of specific antibodies has been well recognised, the generation of egg yolk immunoglobulins (IgY) is rarely chosen due to the peculiar composition of egg yolk and the lack of specific affinity ligands. In this work, we report a novel membrane based two-stage ultrafiltration process to isolate IgY from egg yolk. The effects of solution pH, ionic strength, stirring speed and permeate flux on the transmission of proteins were quantified using the pulsed sample injection technique and parameter scanning ultrafiltration. Under optimised conditions, the purity of immunoglobulin obtained was greater than 93% after the two-stage ultrafiltration process and the recovery of immunoglobulin from the feedstock was close to 87%. The resulting immunoglobulin product was then analysed by Isoelectric Focusing (IEF), SDS–PAGE and Circular Dichroism (CD), to confirm its isoelectric point, molecular weight and molecular secondary structure.

1. Introduction

Chicken egg is widely used as food ingredients because of its nutritional and functional properties (Bragagnolo & Rodriguez-Amaya, 2003). However, chicken egg is also considered as a major source of antibodies. In the past few years, many kinds of specific antibodies have been yielded successfully from eggs of immunised hens, which include anti-HIV-1 and anti-SARS coronavirus antibodies (Devi, Bai, Lal, Umashankar, & Krishnan, 2002; Fu et al., 2006; McLaren, Prosser, Grieve, & Brissenko, 1994; Ruan, Ma, He, Meng, & Zhu, 2005; Soler, Barquet, & Lucio, 1998). Presently, IgY has been used extensively for prevention and treatment of various infections in animals and humans with mixed success (Hamada & Kodama, 1996). Other potential uses of IgY in immunotherapy and immunodiagnostics has been reported by many authors (Ambrosius & Hadge, 1987; Larsson & Sjoquist, 1988; Otake et al., 1991; Yokohama et al., 1992).

Although the importance of chicken egg as an economical and convenient source of polyclonal antibodies has been well recognised, the widespread use is often considered impractical partly due to the difficulties in isolation of IgY from yolk (Devi et al., 2002; Kuroki, 1999). The conventional techniques such as precipitation (Akita & Nakai, 1993), electrophoresis (Gee, Bate, Thomas, & Rylatt, 2003), and chromatography (Hansen, Scoble, Hanson, & Hoogenraad, 1998; Tini, Jewell, Camenisch, Chilov, & Gassmann, 2002; Verdoliva, Basile, & Fassina, 2000) have been used in the IgY separation and purification process. However, these conventional techniques are often time consuming and low yielding (Verdoliva et al., 2000). Therefore, there is a need to determine more efficient ways for IgY separation and purification, and these methods should also be economical and easily scaled up (Akita & Nakai, 1993).

Ultrafiltration (UF) is a pressure-driven, largely size based separation process in which membranes having pore sizes ranging from 1 to 100 nm are used for the concentration, diafiltration, clarification and fractionation of macromolecules (e.g. proteins, nucleic acids and synthetic polymers) (Ghosh, 2005). Recently UF has shown to be capable in separating proteins of similar sizes exploiting the difference in electrostatic charges. Extensive published papers (Fane, Fell, & Waters, 1983; Liu, Lu, Zhao, Lu, & Cui, 2007; Musale & Kulkarni, 1997; Nakao, Osada, Kurata, Tsuru, & Kimura, 1988; Palecek, Mochizuki, & Zydney, 1992; Wan, Ghosh, & Cui, 2002) have demonstrated that UF is an efficient method for protein concentration and fractionation without causing structural damage. Many protein mixtures even with close molecular weights (Wan, Vasan, Ghosh, Hale, & Cui, 2005) have been successfully separated using UF processes under optimised conditions.

In this work, once the lipids were removed from the egg yolk, the crude immunoglobulin solution thus produced was purified by two-stage UF and suitable operating parameters were selected. The effects of physiochemical conditions (pH and ionic strength) and operating parameters (hydrodynamics and permeate flux) on the transmission of all proteins were examined. The feasibility and efficiency of using UF to purify IgY from egg yolk was then assessed.

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2. Materials and methods

2.1. Materials

Fresh eggs were purchased from a local supermarket and were produced at Pingdu, Shandong, China. Bovine serum albumin (Cohn fraction V, Cat. No. A-4503) was obtained from Sigma and used as supplied. Polyethersulfone (PES) ultrafiltration membrane (100 kDa MWCO, Cat. No. PBHK09005) and regenerated cellulose (RC) ultrafiltration membrane (100 kDa MWCO, Cat. No. 14451) were purchased from Millipore. The other chemicals used in the experiment were all purchased from Sinopharm Chemical Reagent Corporation and were of analytical grade.

2.2. Buffer preparation

All buffers and carrier phases were prepared using ultrapure water (18.2 MΩ cm) produced by a Millipore water purification unit. These were further microfiltered using a membrane filter unit with a 0.22 μm cellulose nitrate membrane (Cat. No 7182-004, Whatman) and were subsequently degassed with a vacuum pump.

2.3. Preparation of crude immunoglobulin solution

To produce the crude immunoglobulin solution, the modified water dilution method was employed (Akita & Nakai, 1992). In brief, egg yolks were carefully separated from the egg white and thoroughly washed in distilled water to avoid contamination with white proteins. After removal of the yolk membrane, the yolk contents were collected and diluted 1:6 (v/v) with distilled water. The pH of the solution was adjusted to 5.0–5.2 with HCl and agitated for 15 min at 4 °C. After freezing at −20 °C (8 h) and thawing at 4 °C, the sample was centrifuged at 6000*g* for 20 min (4 °C). The supernatant collected was filtered with Whatman (No. 1) filter paper to get a clear solution.

2.4. Precipitation by sodium sulphate

To identify the target protein in crude immunoglobulin solution, the sodium sulphate precipitation method (Akita & Nakai, 1993) was employed to produce IgY from egg yolk. In brief, the egg yolk was diluted 1:9 (v/v) with distilled water and incubated for 6 h. The lipids were removed by centrifugation (10,000*g*, 4 °C, 25 min). In the second step, Na_2SO_4 was added to the supernatant to reach a final concentration of 19%. The precipitate was collected by centrifugation (8000*g*, 4 °C, 20 min) after 4 h and redissolved with distilled water. Then the second step was repeated once with a different Na_2SO_4 final concentration of 14%. The last solution was collected and stored at 4 °C for further use. All incubation and precipitation steps were performed at 4 °C.

2.5. Apparatus for ultrafiltration

Fig. 1 shows the experimental setup used for ultrafiltration in this study. The detailed description of the experimental apparatus has been given in our previous work (Lu, Wan, & Cui, 2005; Wan, Lu, & Cui, 2006). In brief, it consisted of two parts, a stirred cell for carrying out ultrafiltration and AKTA Prime Plus liquid chromatography system (GE Healthcare, USA) for process control and monitoring. The stirred cell had a working volume of 16.0 mL and could be fitted with a membrane disk having an effective diameter of 24 mm. There was a suspended bar impeller with a diameter of 20 mm inside the module, which was magnetically driven by a RCT basic stirrer (IKA, Japan). The stirring speed was monitored using a digital photo/contact tachometer (RS 163-5348, RS Components). The gap between the impeller and the membrane was about 1.5 mm. The stirred cell was integrated with the AKTA Prime Plus in which a positive displacement pump was employed to ensure constant permeate flux through the membrane. The transmembrane pressure (TMP) was monitored by a pressure transducer. The protein solution was injected into the system in the form of pulse using a sample injector. Permeate was continuously monitored at 280 nm using a flow-through UV monitor. At the same time, the pH and conductivity of permeate were also monitored on line by a flow-through conductivity cell. All these data were continuously logged into a computer for storage and then were subsequently analysed using Prime View 5.00 software (Amersham Bioscience, GE Healthcare).

2.6. Ultrafiltration experimental procedure

A fresh membrane disk was used for each experimental operating parameter examined. Before each measurement, the membrane disks to be used were immersed in the ultrapure water for at least 12 h according to manufacturer's instructions and then installed in the stirred cell to allow the used buffer to pass through it at an appropriate flow rate long enough to make sure that the wetting agent or unwanted species was removed from the membrane surface or inner pores and attain a steady baseline on line. At each parameter value two pulsed injection runs were performed. Each run lasted for 60 min after injection. The first run was done to ensure equilibrium adsorption of protein on the membrane surface at the given operating conditions and the data obtained from the second run was for the subsequent calculation. The experiment was repeated at least three times, and the data reported here are the average values. Before the second run starts, rinsing the whole system, including the membrane, are very important for getting a stable UV-absorbance baseline on line.

In both pH and salt scanning experiments, binary appropriate buffer systems were chosen to create pH or salt concentration gradients. More detailed experimental procedures including data processing were also described in our previous work (Lu et al., 2005; Wan et al., 2005). All the experiments were performed at 4 °C. The observed transmissions of all proteins were calculated using determined concentrations in the permeate and feed, as the observed transmission S_a (= C_p/C_b) is defined as the ratio of solute concentration in the permeate (C_p) to that in the bulk or retentate (C_b).

2.7. Protein concentration measurement

The concentration of different proteins in feed, permeate, and retentate streams were analysed using FPLC system (Amersham Biosciences, GE) equipped with a size exclusion chromatography column Superdex™ 200 10/60 (GE Healthcare, Sweden). The mobile phase was 50 mM sodium phosphate buffer containing 150 mM sodium chloride (pH = 7.0). The flowrate was fixed at 0.4 mL/min. A 200 μL sample loop was used for protein sample injection. Before injection, the sample was filtered using Sterile Millex® Filter Unit (Cat. No. SLGP033RB, Millipore). The sample was injected after 5 min from the start of the run. The effluent steam was monitored at 280 nm using a UV detector UV–VIS (Unicam UV-2450) and calibration curves obtained with individual proteins (area under the curve versus concentration) were used to determine the concentration of the specified proteins in the samples.

The total protein concentration of each sample was determined using the modified Bradford method (Sedmak & Grossberg, 1977). The sample absorbance values were read at 570 nm on a UV-2450

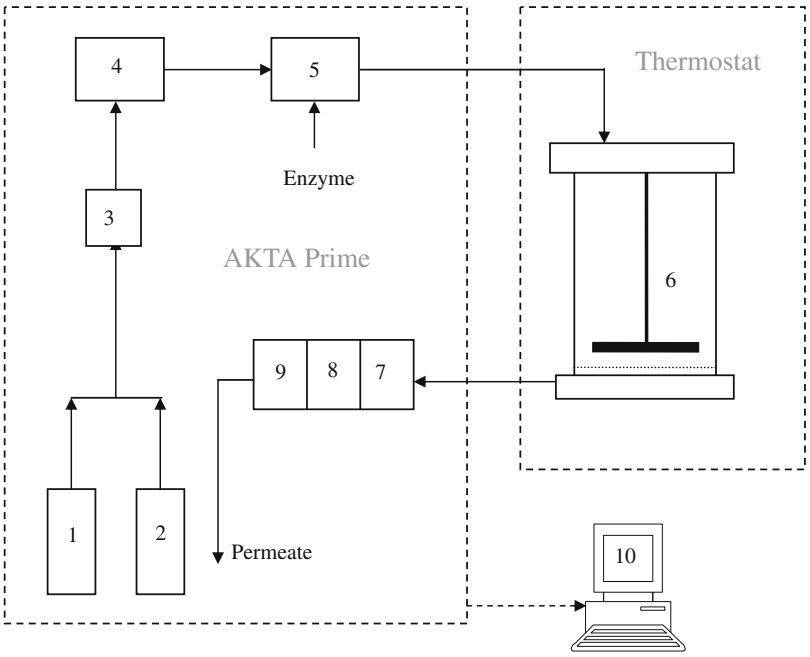

Fig. 1. Experimental setup for ultrafiltration experiments. (1) Buffer reservoir a; (2) buffer reservoir b; (3) pump; (4) buffer mixer; (5) sample injector; (6) stirred cell module; (7) UV monitor; (8) conductivity monitor; (9) pH monitor; (10) computer for data logging and processing.

(Shimadzu, Japan). A calibration curve was generated by a range of bovine serum albumin (BSA) from 0.025 to 0.5 mg/mL.

2.8. Electrophoresis

The isoelectric point of IgY in crude immunoglobulin solution was examined with Isoelectric Focusing (IEF, Bio-Rad) using Bio-Lyte ampholyte (pH 3.9–9.5, Cat. No. 163-1112) to produce the pH gradient.

The SDS–PAGE analysis was performed under reducing conditions using NuPAGE 4–10% Bis–Tris mini gels (Invitrogen, USA), following the manufacture's instructions. Detection of the protein bands was performed with the Brilliant Blue Coomassie G-250 staining method.

2.9. Circular Dichroism (CD) detection of secondary structure of IgY

Circular Dichroism experiments were preformed on a PMS 450 spectropolarimeter (Biologic, France) with a 1 mm path length cell at 25 °C. The purified protein sample was concentrated to 2.25 g/l and the solvent was replaced with super pure water. The CD spectrum was recorded from 190 to 250 nm of wavelength with 1 nm resolution and 5 s of average time. Super pure water worked as a blank to correct the baseline. Results were expressed as the molar mean residue ellipticity (θ) at a given wavelength.

3. Results and discussion

3.1. IgY identification

Fig. 2 shows the analysis results of crude immunoglobulin solution and sodium sulphate precipitated IgY sample. It can be seen that there are mainly four proteins in the crude immunoglobulin solution. IgY is the first one eluted out of the column with a carrier phase volume of 15.10 mL. The other three impurity proteins with carrier phase volumes of 17.03, 22.20 and 25.65 mL are named as impurity protein A (IPA), impurity protein B (IPB) and impurity protein C (IPC) in this work.

3.2. Separation of IgY + IPA and IPB + IPC using 100 kDa PES membrane

3.2.1. Effects of pH on protein transmission

To separate IgY + IPA from IPB + IPC, the 100 kDa PES membranes were used. The effect of pH on the transmission of four pro-

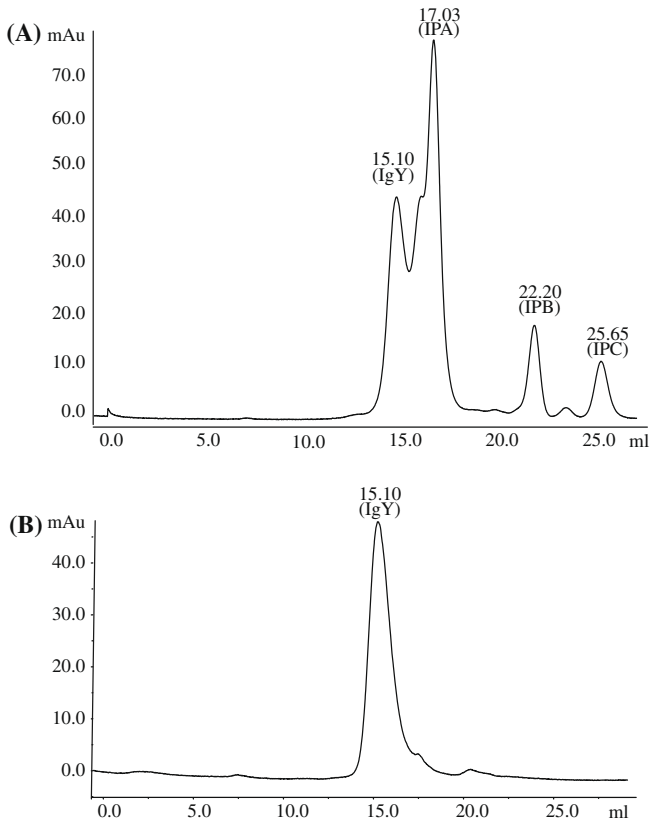

Fig. 2. Comparison of size exclusion chromatograms at 280 nm using a superdex 200 10/60 column. (A): Crude immunoglobulin solution; (B): sodium sulphate precipitated IgY sample.

teins was examined at pH 4.0–pH 10.0. This pH range was chosen because IgY is stable within this range (Akita & Nakai, 1993; Shimizu, Fitzsimmons, & Nakai, 2006). The start and end carrier phases were 10 mM sodium phosphate buffer at pH 4.0 and 10.0, respectively. Fig. 3(A) shows the transmission of four proteins obtained at different pH values. The operational flux and stirring speed were fixed at 13.3 L/m^2 h and 600 rpm, respectively.

It was found that IgY was not detected in the permeate within the experimental range. When pH increased from 4.0 to 6.0, the transmission of IPA increased at first, after reaching its maximum at pH 4.5 (~20%), and then decreased. With a further increase in pH, the IPA transmissions were kept around 4%. The transmissions of IPB were found to be pH dependent. As pH increased from 4.0 to 6.5, the IPB apparent sieving coefficient firstly increased, after reaching its maximum values at pH 5.0, and then decreased to pH 6.0, which was followed by the second and third peak at pH 7.5 and 9.0, respectively. The transmission of IPC had the same trend like IPB within the pH range from 4.0 to 7.5. When pH increased from 7.5 to 10.0, the IPC transmission firstly increased, after reaching its maximum vale at pH 8.0 (~82%), and then decreased. On the basis of the experimental data, the biggest selectivity $[(S_{a\_IPB} + S_{a\_IPC})/(S_{a\_IPA} + S_{a\_IgY})]$ was obtained at pH 8.0 (38.7). Therefore, pH 8.0 was used for other parameter's optimisation in this section.

The dependence of protein transmission on pH is obviously related to the electrostatic charge of IgY, IPA and IPB, as well as the surface charge of the membranes. The electrostatic interactions among the proteins and between the membrane and individual proteins, which are also affected by ion strength and other impurities, affect on transmission of each protein. The quantification of such effects requires detailed characterisations of each component in the feedstock and the membrane surface, which is beyond the scope of this study.

3.2.2. Effects of salt concentration

Salt concentration is known to be an important factor in the selective transmission of proteins through ultrafiltration membrane (Ehsani, Parkkinen, & Nystrom, 1997; Saksena & Zydney, 1994; van Eijindhoven, Saksena, & Zydney, 1995). Therefore, the effect of salt concentration was examined over the range of 0–400 mM sodium chloride (Fig. 3(B)). The flux and stirring speed were fixed at 13.3 L/m^2 h and 600 rpm, respectively.

It can be seen that the transmissions of IPA increase with salt concentration before salt concentration reaches 70 mM. With further increases in sodium chloride concentration, the transmissions decrease slightly. The transmissions of IPB and IPC followed the same trend of IPA with the maximum transmission happening at 70 mM. The effect of sodium chloride concentration on the separation of IgY + IPA and IPB + IPC is shown in Fig. 3(B) (with the maximum selectivity of ~19.6 at 30 mM NaCl concentration).

It was found that increasing the salt concentration helped to dissolve the aggregates because of the shielding effect of salt ions which reduces the electrostatic attraction between protein molecules. Within the experiment range studied, aggregation appeared to take place in the crude immunoglobulin solutions with salt concentrations below 70 mM. Protein solubility increased with increasing salt concentration over the low salt concentration range, which was usually regarded as the "salt-in" effect. At high ionic strength, protein solubility generally decreased with increasing salt concentration due to the reduced activity of water and the neutralisation of surface charge, which was regarded as the "salt-out" effect.

3.2.3. Effects of stirring speed

The effect of stirring speed on the transmission of proteins using 100 kDa PES membranes was shown in Fig. 3(C). These experi-

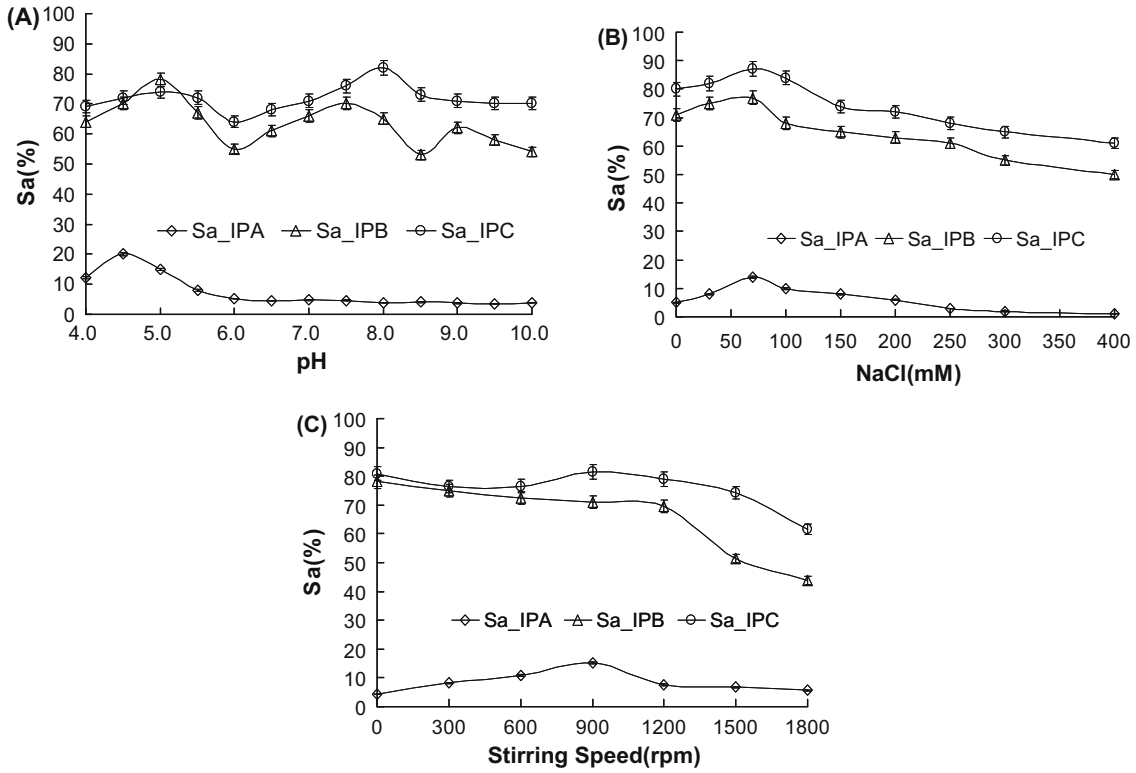

Fig. 3. Effects of operating conditions on the fractionation of IPA, IPB and IPC using 100 kDa PES membrane. (A) Start carrier phase: 10 mM Na-PO_4, pH 4.0; end carrier phase: 10 mM Na-PO_4, pH 10.0; permeate flux, 13.3 L/m^2 h; stirring speed, 600 rpm. (B) Start carrier phase: 10 mM Na-PO_4, pH 8.0; end carrier phase: 400 mM NaCl, 10 mM Na-PO_4, pH 8.0; permeate flux, 13.3 L/m^2 h; stirring speed, 600 rpm. (C) Carrier phase 10 mM Na-PO_4 + 30 mM NaCl solution at pH 8.0, permeate flux, 13.3 L/m^2 h.

ments were carried out at pH 8.0, 30 mM NaCl concentration, and a constant permeate flux of 13.3 L/m^2 h.

It can be seen that the stirring speed has a significant effect on the transmission of IPA, IPB and IPC in the experimental range. When the stirring speed increased from 300 to 1800 rpm, the transmissions of IPA and IPC firstly increased, after reaching their maximum values at 900 rpm, and then decreased to 1800 rpm. The IPB apparent sieving coefficient monotonously decreased when the stirring speed increased from 300 to 1800 rpm. Thus, the maximum selectivity $[(S_{a\_IPB} + S_{a\_IPC})/(S_{a\_IPA} + S_{a\_IgY})]$ was obtained at 1200 rpm (~19.5).

As reported previously (Liu et al., 2007; Lu et al., 2005), stirring speed has profound effect on the separation of proteins using UF as it can significantly affect the concentration polarisation and the mass transfer coefficients of proteins. In this experiment, stirring speed becomes a more important factor for IgY purification due to the serious membrane fouling at low stirring speed. For example, when the stirring speed was set to zero, the separation process had to be stopped at 12 min when the transmembrane pressure (TMP) exceeded the pressure limitation (1.0 MPa). Without stirring, ultrafiltration was carried out in a deadend operation. Sever concentration polarisation would soon lead to much increased osmotic pressure and gel layer formation. As the experiments were conducted at constant flux, the TMP would have to increase eventually to the operational limit. As expected, ultrafiltration of protein solutions would have to operate in crossflow mode, here with increased stirring speed. Concentration polarisation could be controlled, when the TMP is within an acceptable range.

3.2.4. Effects of permeate flux

The effect of permeate flux was also examined and the outcome together with other operating conditions is shown in Table 1. It can be seen that the transmission of IPA decreased when the permeate flux increased from 13.3 to 26.5 L/m^2 h while the transmissions of IPB and IPC increased. When the permeate flux increased from 39.8 to 53.1 L/m^2 h, IPA was totally rejected while the transmissions of IPB and IPC slightly decreased. According to the TMP value, the permeate flux of 26.5 L/m^2 h appears to be suitable for separation of IPA from IPB + IPC.

3.3. Fractionation of IgY and IPA using 100 kDa RC membrane

To separate IgY from IPA, the 100 kDa RC membrane was used. The operating parameters were also examined like Section 3.2 (data not shown) and the optimum operating conditions were as follows: pH 6.0, 30 mM NaCl concentration, 600 rpm, 13.3 L/m^2 h.

3.4. Electrophoresis

The isoelectric point of IgY was measured by IEF. The pI of IgY was pH 6.2–6.5, which was close to that of the literature value (6.5–7.5) (Gee et al., 2003).

Table 1
Effect of permeate flux on the fractionation of IPA, IPB and IPC using 100 kDa PES membrane.[a]

Flux (L/m^2 h)	S_{a_IPA}	S_{a_IPB}	S_{a_IPC}	TMP (MPa)
13.3	6.6	63.8	72.7	0.02
26.5	2.0	75.7	85.2	0.09
39.8	0	65.0	63.3	0.65
53.1	0	64.1	63.0	GTPL[b]

[a] Operating conditions: carrier phase 10 mM Na-PO_4 + 30 mM NaCl solution at pH 8.0, stirring speed, 1200 rpm.
[b] GTPL = greater than pressure limitation (1.0 MPa) after 15 min.

To determine the molecular structure and molecular weight of IgY, SDS–PAGE analysis was performed under reducing conditions (Fig. 4). It can be seen that the heavy chain of IgY is about 65 kDa, while the light chain is about 25 kDa. The IgY molecule consists of two identical heavy chains and two identical light chains (Hansen et al., 1998). Therefore, the molecular weight of IgY is about 180 kDa, which is equal to the literature value (Ruan et al., 2005).

3.5. Secondary structure analysis

Circular Dichroism (CD) is a valuable method for the analysis of protein secondary structure, and the far-UV CD spectroscopy from

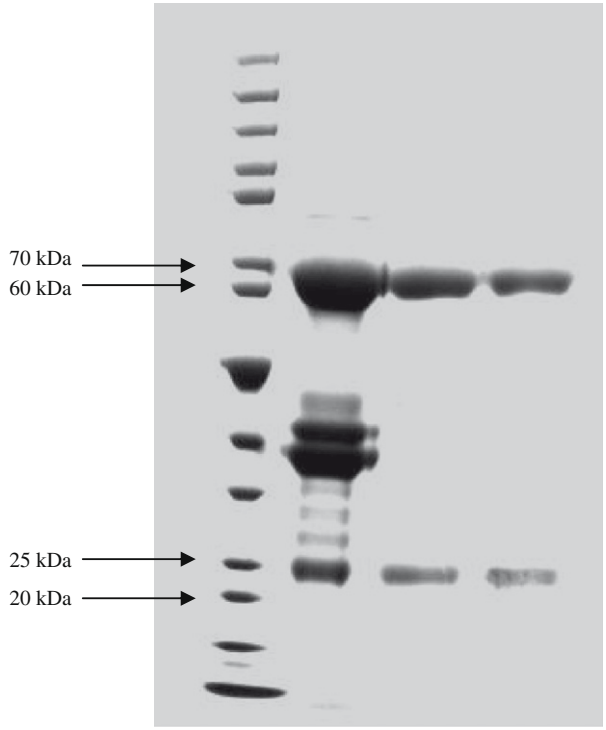

Fig. 4. SDS–PAGE analysis of protein samples under reducing condition. I, crude immunoglobulin solution; II, sodium sulphate precipitated IgY sample; III, IgY produced by two-stage ultrafiltration procedure.

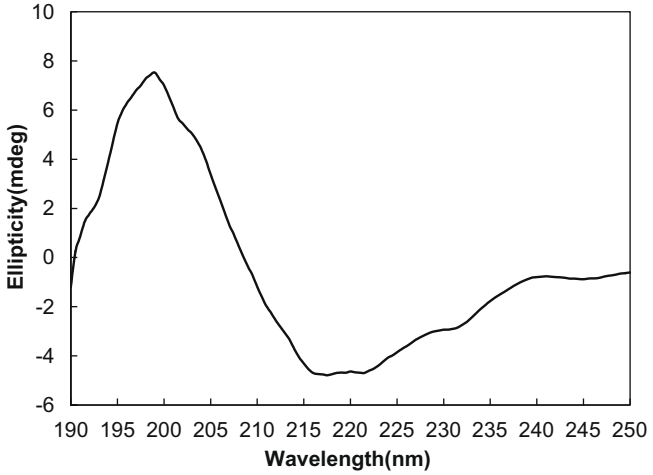

Fig. 5. Circular Dichroism (CD) spectrum of purified IgY sample.

190 to 250 nm can be used to estimate contents of secondary structures (Manavalan & Johnson, 2007). We used the UF purified IgY for CD study. The CD profile displayed a typical β-sheet curve with ~45% sheet content (Fig. 5). This value is very close to the theoretical ~48% (Whitmore & Wallace, 2004, 2008). These results suggest that the purified IgY folded with a reasonable secondary structure as calculated.

4. Conclusions

In this work, several key parameters affecting the separation and purification of IgY from egg yolk using membrane ultrafiltration were examined and the purified IgY sample was analysed with various methods. The experimental results contested that ultrafiltration was an efficient and easily scaled up means for the separation and purification of IgY from egg yolk with high purity and recovery. In addition, the final purified IgY has the right physical parameters, with an isoelectric point of 6.2–6.5, a molecular weight of 180 kDa and sheet content of ~45%.

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