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Purification of Egg Yolk Immunoglobulin (IgY) by Ultrafiltration: Effect of pH, Ionic Strength, and Membrane Properties

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Immunoglobulin Y (IgY) was purified from hen egg yolk water-soluble protein fraction by ultrafiltration−diafiltration with different membranes. The effect of changing solution properties (pH and ionic strength) on purification factor (P), process selectivity (Ψ), and IgY recovery (R_{IgY}) was studied. Salt presence (150 and 1500 mM) decreased the selectivity and purity factor. This effect was more evident at pH values closer to or higher than the IgY's isoelectric point. The best results were obtained in the absence of salt at pH values of 5.7 and 6.7 using poliethersulfone (PES) and modified PES (MPES), respectively. Process selectivity was doubled, and IgY's purification factors were increased in more than 1 order of magnitude when diafiltration was used. Results from this work show the potential of membrane technology for the purification of IgY from hen's egg yolk.

KEYWORDS: Ultrafiltration; diafiltration; immunoglobulin; ionic strength; membrane selectivity

INTRODUCTION

Hen's egg yolk is an interesting source of immunoglobulin (IgY), a specific antibody with potential applications in several fields including therapeutic (*1*, *2*), immunodiagnosis (*3*, *4*), and food analysis (toxins detection) (*5*). IgY competes with mammalian antibodies with some advantages since it presents no interaction with Fc human serum factor, it promotes higher antibody production, and it has compatibility with animal protection and ethical laboratory handling regulations (*6*). Despite these advantages, IgY is commercially produced by fermentation in small quantities (≈100 kg/year) and high production costs (908 usd/g), which are mainly attributed to the several steps required to purify it (*7*). Purification schemes of IgY from egg yolk involve extraction of the water-soluble protein fraction (WSPF) followed by several purification steps to separate it from other water-soluble proteins. Separation of the WSPF from lipoproteins is carried out by simple water dilution. At this step, optimization of environmental conditions (pH, temperature, dilution factor, etc.) has allowed the increase of IgY recovery up to 90% (*8*, *9*). However, purification of IgY from the WSPF requires several separation steps including salt, polysaccharides, or cryoethanol precipitation followed by centrifugation (*10*) and/or ion exchange (*9*, *11*) or affinity chromatography (*12*, *13*). The many steps involved in IgY purification trends generally resulted in low yields and high costs. In addition, limitations for scaling up some of the purification methods used have been pointed out (*14*). In this context, membrane technology (i.e., ultrafiltration) seems more suitable for industrial applications because of lower operation costs and direct scale up (*14*, *15*). Ultrafiltration (UF) has been generally viewed only as a size-based separation process; however, there is considerable evidence that electrostatic interactions may also increase process separation efficiency. This has been achieved through changes on solution properties and/or by modifying membrane surface charge. Saksena and Sydney (*16*) increased the selectivity of polyehtersulfone membranes (100 kDa) from 2 to about 50 when separating mixtures of bovine serum albumin (BSA) and IgG, by lowering pH from 7 to 4.8 and NaCl concentrations from 0.15 to 0.0015 M. This selectivity increase was explained in terms of electrostatic contributions (protein−protein and protein−membrane) to both bulk and membrane transports, which resulted in higher BSA transport through the membrane. Similar results were obtained for model solutions of myoglobin−cytochrome *C* (*17*) and HSA-lgG (*18*). Otherwise, membrane surface modification has been used to reduce protein−membrane interactions and fouling phenomena, as well as to increase solute mixture separation and permeate flux values (*17*, *19*, *20*). Kim et al. (*21*) increased the hydrophilicity of polyvinyl chloride (PVC) membranes by using different concentrations of *N*-vinyl-pirrolidinone and UV light irradiations. They reported the rising of water flux values up to 50% because of the reduction of membrane resistance and up to twice the increments on flux with protein solution (not defined by the author) which was mainly attributed to lower specific resistance of the protein layer. Similar work has been done by other authors using different membranes and protein model solutions (*17*, *22*, *23*). In all cases, authors agree that under certain conditions (pH and ionic strength solution) the hydrophilic modified membranes gave by far better results (cleaning included) than unmodified membranes. As an example, Lucas et al. (*22*) reported a drop on

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selectivity (from 10 to 4) when NaCl concentration increased from 0.02 to 1.0 mol/L in the ultrafiltration of α-lactalbumin−β-lactoglobulin solution (pH 7.0) using inorganic membranes chemically modified by polyethyleneimine coating (positive charge), while in unmodified membranes, selectivity values were almost constant (3.0 to 2.5). The authors explained that this drastic decrement was caused by the increase in β-lactoglobulin transmission. Nakao et al. (*17*) separated a mixture of myoglobin and cytochrome *C* using different polysulfone membranes: unmodified and negatively (sulfonated) and positively (chloromethylated) modified. They found the best separation results with the modified membranes by setting the solution's pH near the isoelectric point of one of the proteins. This phenomenon was attributed to a preferential permeation of the electrical neutral protein and rejection of the charged protein due to the electrical repulsion acting between this protein and the membrane surface.

Unfortunately, reports on the application of UF for fractionating proteins from real mixtures are scarce in the literature (*23*, *24*). Eshani et al. (*24*) fractionated egg-white proteins at different pH values (4.5, 6.5, and 8.5) and NaCl concentrations using 50 kDa molecular weight cutoff (MWCO) polysulfone membranes. They found lower protein retention at pH 4.8 in the absence of salt. However, when 0.05 M NaCl was added protein retention increased at pH 4.8 and decreased at the highest pH tested. A permeate assay in the absence of salt showed higher ovalbumin (pI ≈ 4.8) concentration, whereas lysozyme (pI ≈ 11.1) and conalbumin (pI ≈ 6.5) were retained by electrostatic and molecular exclusion effects, respectively. When NaCl was added and the pH of the solution was set to 6.5 or 8.5, ovalbumin retention and lysozyme permeation increased, affecting membrane selectivity. These authors repeated these experiments with a model solution of ovalbumin, lysozyme, and conalbumin, the three main proteins of egg white, and could not reproduce the results found with natural egg-white solutions. However, they did observe the negative effect in protein fractionation when salt was added at both pH values 6.5 and 8.5. Such results were attributed to the interaction of proteins and/or salts present in the natural egg-white solutions, which could not be modeled by the main protein components.

Otherwise, Kim and Nakai (*25*) fractionated IgY from the WSPF of egg yolk by diafiltration with a 100 kDa membrane and obtained up to 99% purity and 85% recovery at pH 9.0 and a NaCl concentration of 1.5 M. These purity and recovery levels were higher than those reported for other authors when combining different purification methods (*8*−*13*). They suggested that purity improvements were the consequence of increased IgY aggregation and enhanced molecular exclusion of IgY due to the presence of salt (salting-out). However, UF conditions reported by these authors (pH far-off IgY isoelectric point and high salt concentration) are opposite to the behavior reported for UF protein fractionation in other studies (*16*, *17*, *22*, *24*). Later, these authors (*26*) compared the performance of different ultrafiltration systems (Amicon, Harp, A/G, and Koch) using similar conditions of pH (9.0) and NaCl concentration (1.5 M) and reported purity and recovery of IgY between 74 and 99%, and 72−85%, respectively. They have also tested sodium carbonate solution (10 mM and pH 9.0) as a diafiltration buffer instead of water (pH 9.0), but remotion of undesired protein was only 25%.

From the above, it is clear that the effect of adding salt on protein fractionation by UF is complex and depends not only on the pH and the concentration of salt present in the mixture but also on the membrane surface properties. However, most of the work found in the literature has been carried out with model solutions (*23*, *24*, *27*), and there is a lack of data obtained with real protein mixtures where the presence of other constituents may bias results. Therefore, the aim of this work was to evaluate the effect of a wide range of pH and ionic strength as well as membrane type, on membrane selectivity, purification, and recovery of IgY from the water-soluble fraction of hen's egg yolk in order to further improve UF resolution.

EXPERIMENTAL PROCEDURES

Water-Soluble Protein Fraction Extraction from Egg Yolk. Water-soluble protein fraction was obtained according to Akita and Nakai (*15*). Hens' eggs were obtained from a local supermarket with packing dates no more than two weeks previous to their use and stored at 4−6 °C. Egg yolk was separated from egg-white and dried with paper towels. Membrane was punctured and the yolk allowed to flow into a graduated cylinder without the membrane. Egg yolk was diluted in 6-fold deionized water (Milli-Q Academic, Millipore Inc.); pH was adjusted between 5.0 and 5.2 with HCl (0.1 N) and stored at 4 °C for 6 h. After storage, the solution was centrifuged (10,000*g* for 15 min), and the supernatant was filtered. According to the literature (*3*, *14*, *25*), the main components of WSPF are IgY (170 kDa, pI = 5.7); α-livetin (70 kDa, pI = 4.7); β-livetin (42 kDa, 5.6); low density lipoproteins (20−135 kDa); and albumin (45 kDa, pI = 4.7−5.0). This composition was verified by poliacrylamide gels.

Total Protein Contents. Protein concentration was determined using the Bradford reagent (Sigma; St. Louis MO). Absorbance measurements were taken at 595 nm in a Smartspec 3000 spectrophotometer (Bio Rad) after room incubation for 15 min. A standard curve was obtained using BSA (Sigma; St. Louis MO) solutions at different concentrations (0.0−1.2 mg/mL).

IgY Quantification by Radial Immunodiffusion (RID). IgY quantification was carried out by the method described by Fukumoto et al. (*28*) with modifications. Agarose type IV from Sigma Chemical Co. (St. Louis, MO) at 1% w/v in 0.01 M PBS (0.138 M NaCl; 0.0027 M KCl; 20−0.05% Tween; pH 7.4; 0.02% sodium azide) was boiled and cooled to 55 °C. Then, 0.15 mL of rabbit antichicken IgG (whole molecule, 2.5 mg/mL) from Sigma Chemical Co. (St. Louis, MO) was incubated at 55 °C in 1.9 mL of PBS. This solution was later mixed with 6 mL of agarose solution, poured into glass plates ($10 \times 8 \times 0.1$ cm^3), and cooled for 20 min. Twelve evenly distributed 2 mm diameter wells were made in each gel. Then, 2.5 μL volumes of samples and standards were added to wells before incubating the RID gel in a moisture chamber for 48 h at 37 °C. Immunodiffusion ring sizes were measured after incubation, and a standard curve was obtained by plotting precipitation ring diameter versus IgY concentration of standards.

Sodium Dodecyl Sulfate−Polyacrylamide Gel Electrophoresis (SDS−PAGE). Sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE) was done under nonreducing conditions using Mini-PROTEAN III (Bio-Rad) following the instructions of the manufacturer. An 8% acrylamide separating gel and a 4% stacking gel were used. Staining with Coomassie Brilliant Blue R-250 and distaining procedures were according to Bio-Rad's instructions manual. Broad-range molecular weight standards (12−450 kDa) of SERVA were used as molecular weight markers.

Particle Size Distribution (PSD) Analysis. In order to evaluate the effect of pH and ionic strength on properties of the WSPF in terms of PSD, NaCl, and HCl (0.1 N) or NaOH (0.1 N), solutions were used to attain every tested condition. PSD analysis was carried out using a DELSA 440-SX particle analyzer (Coulter). The operation basis of the equipment is photon correlation spectroscopy applied to suspended particles (in liquid) with Brownian movement.

Ultrafiltration. UF experiments were carried out following a 3^3 factorial experimental design (three membrane types, three pH levels, and three salt concentrations). Millipore (100 kDa MWCO) membranes of regenerated cellulose (RC) and polyethersulfone (PES) were tested. In addition, PES membrane surface properties were modified by UV light exposure (20 min at $\lambda = 254$ nm) using a UV lamp darkroom Cole Parmer 9818 series (Chicago, Ill). According to Nyström and Järvinen (*19*), UV released negatively charged radicals in the membrane surface and increased the pore contact angle. UF experiments were carried out at 25 °C

and ΔP_{TM} = 10 psi in an unstirred cell (Amicon 8200). Aliquots of WSPF (80 mL) were adjusted at the pH tested (4.7, 5.7, and 6.7) using HCl or NaOH (both 0.1 N). Salt (NaCl) concentrations tested were 0.0, 150, and 1500 mM. UF time was set to 3 h, and automatic data acquisition was done using Winwedge 32 software.

Data Analysis. Ultrafiltration process efficiency can be evaluated by means of parameters such as selectivity and purification factor (*29*). Selectivity (Ψ) is a dimensionless value defined as follows:

$$\Psi = S_1/S_2 \quad (1)$$

where S_1 and S_2 are the dimensionless observed sieving coefficients for undesired proteins and IgY, respectively.

$$S_x = C_f/C_s \quad (2)$$

where C_f is the filtrate concentration of a specific protein and C_s the feed concentration of the same protein. Purification factor (P) for IgY in the retentate is defined as follows:

$$P = \frac{(VC_2)_F}{(VC_2)_i}/\frac{(VC_1)_F}{(VC_1)_i} \quad (3)$$

where V, is work volume, C_1 and C_2 are the concentration of undesired proteins and IgY, respectively.

IgY recovery (R_{IgY}) is defined as:

$$R_{IgY} = (C_{IgY})_F/(C_{IgY})_i$$

RESULTS AND DISCUSSION

Particle Size Distribution (PSD) Analysis. PSD analysis was carried out in order to detect protein aggregation in the WSPF solutions under the different pH and ionic strengths tested. Results indicate the presence of large size protein aggregates even in the absence of salt (**Figure 1A**). This might be attributed to micelles formed by the interaction between proteins, phospholipids, and triglycerides. In **Figure 1A** and **B**, no significant differences could be observed between particle size distributions obtained when no salt was added and when 150 mM NaCl was added to WSPF solutions at the three pH values tested. However, when NaCl concentration was increased to 1500 mM (**Figure 1C**) an important increment in PSD at both 4.7 and 5.7 pH values were observed, suggesting a strong protein aggregation effect. However, at pH 6.7 (**Figure 1A**, **B**, and **C**) this effect was less significant, probably because of a higher electrostatic repulsion effect caused by an increment in the number of proteins (IgY, α and β livetins, and albumin) which have a similar charge at this pH value, decreasing protein aggregation. **Figure 1C** shows the PSD analysis for WSPF using conditions (pH 9.0 and 1500 mM of NaCl) similar to those reported by Kim and Nakai (*25*). It can be seen that the particle size of proteins in the WSPF was lower (< 500 nm) and smaller than the particle size distribution obtained under different pH and similar NaCl concentration. These results contradict Kim and Nakai's assumptions of molecular exclusion enhancement.

Ultrafiltration. UF results obtained with all membranes and the different levels of pH tested showed in general a decrease in membrane selectivity (Ψ) and IgY purification factor (P) (**Figures 2** and **3**, respectively) when NaCl was added. This effect was more evident at pH values close to or higher than the IgY isoelectric point (5.7). These results agree with those reported by other authors (*16*−*18*, *24*) with different protein systems, and it has been explained in terms of protein aggregation induced by increased ionic interactions between proteins and ions from the dissolved salt. This explanation is also in agreement with results from the PSD analysis obtained at 1500 mM (**Figure 1C**) and with the SDS−PAGE separation profiles for the retentate (**Figure 4A** and **B**, line 6). However, at the lower salt concentration tested (150 mM NaCl) aggregation may not be the phenomenon affecting Ψ and P, as can be seen from **Figure 1B**. At this salt concentration and pH 6.7, all main proteins from the WSPF probably have a net charge (IgY included) and might be covered by surrounding ions in solution forming an electrostatic shield. This will decrease electrostatic rejection between proteins in bulk solution and those deposited onto the membrane surface (*16*−*18*, *24*), improving permeation. Otherwise, at pH 5.7 most proteins may carry a net charge, while IgY is near the isoelectric point; therefore, when 150 mM NaCl is added, a considerable improvement in Ψ and P might be expected, which did not happen. A possible explanation to this phenomen could be the interaction between ions and hydrophilic groups of low density lipoproteins (LDL) which might form a clot on gel forms (*30*) (gel layers), reducing protein separation. This effect is suggested by the

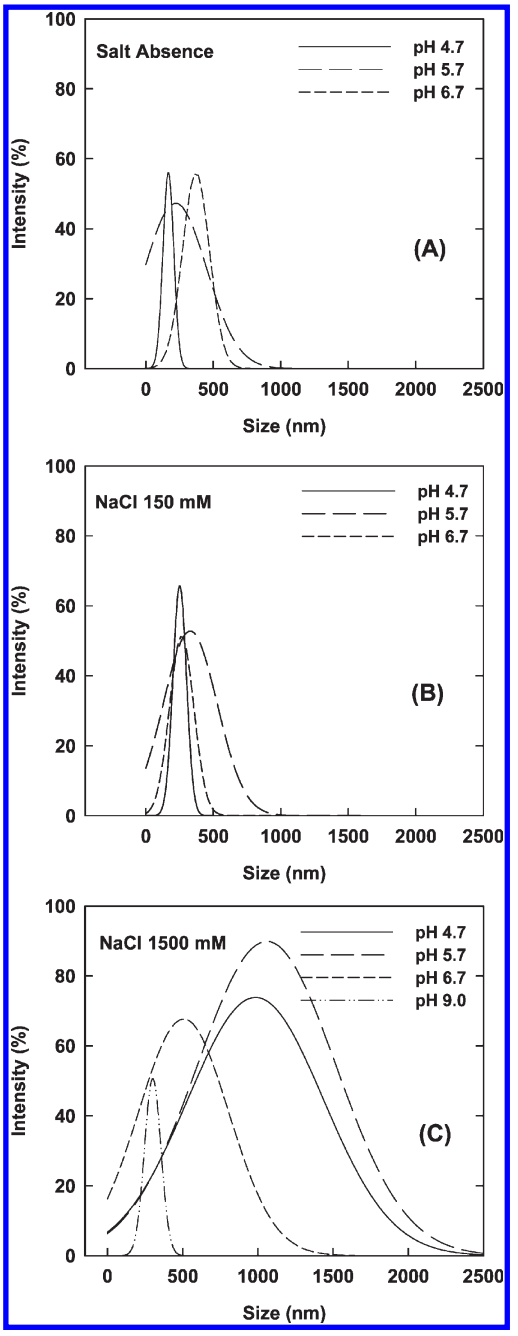

Figure 1. Particle size distribution observed in WSPF at the different pH and ionic strength values analyzed.

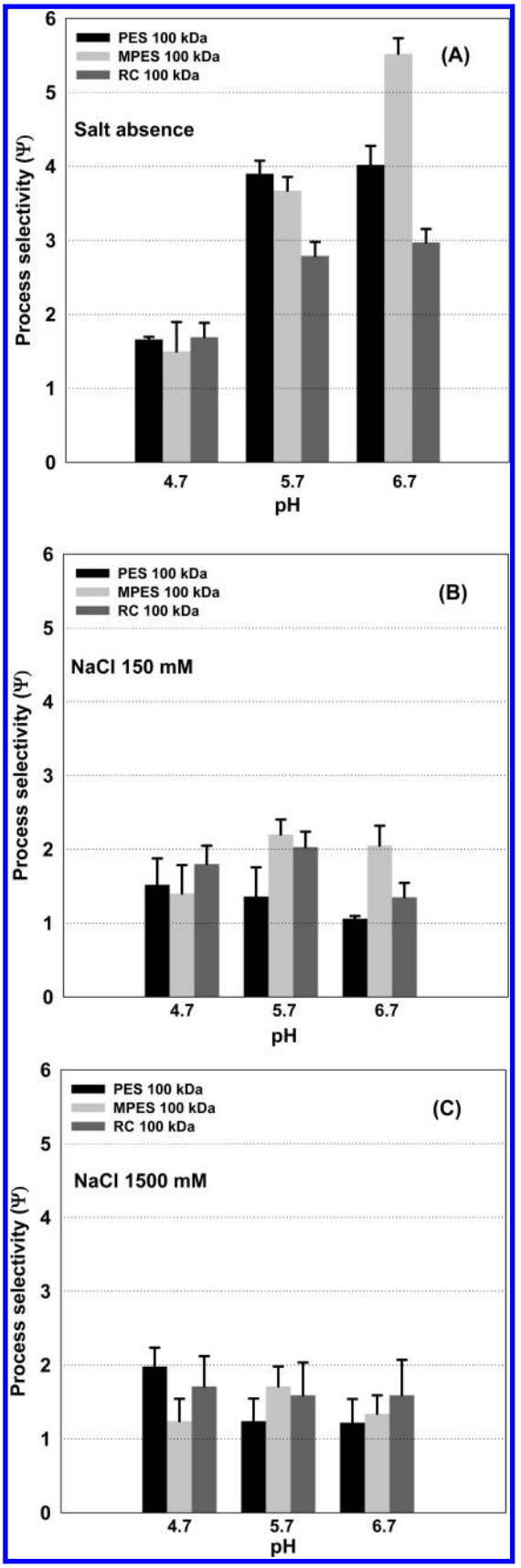

Figure 2. pH and ionic strength effect on process selectivity (Ψ) using different membrane types during IgY ultrafiltration from WSPF. Membranes used: PES, polyethersulfone; MPES, modified PES; RC, regenerated cellulose.

SDS−PAGE separation profile (**Figure 4A**, line 9), where the presence of β-livetin, albumin, and IgY in the permeate was

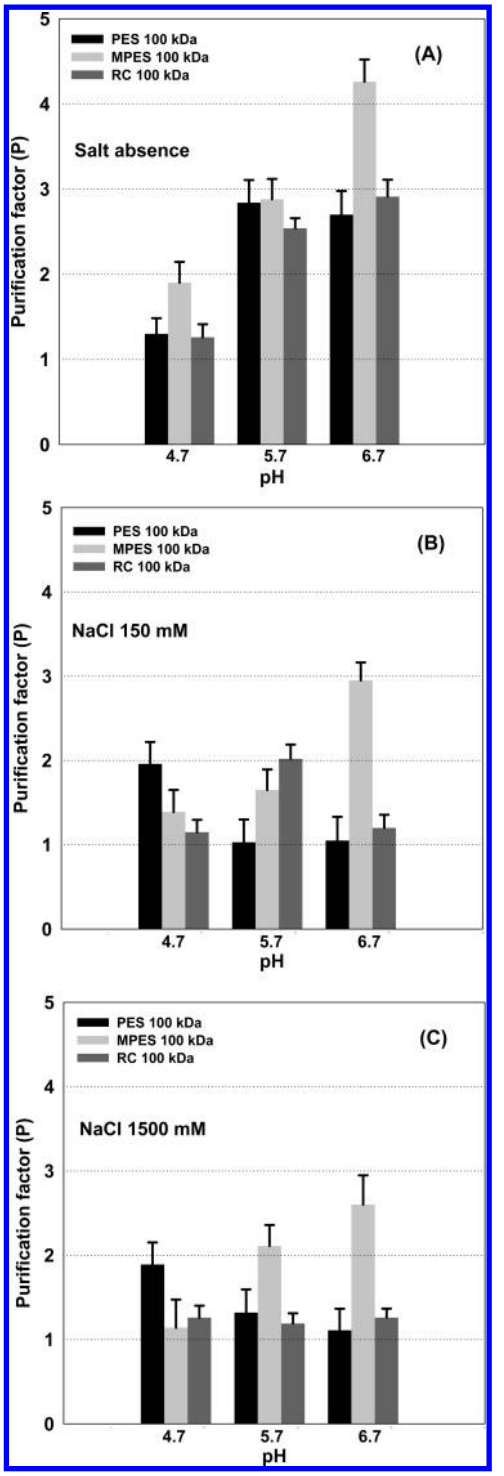

Figure 3. pH and ionic strength effect on purification factor (*P*) using different membrane types during IgY ultrafiltration from WSPF. Membranes used: PES, polyethersulfone; MPES, modified PES; RC, regenerated cellulose.

observed. When the negatively charged modified membrane was used (MPES), the presence of electrostatic interferences in the protein−salt ion-membrane (*16*) occurring by means of the ion−bridge union could be an additional phenomenon acting to reduce protein fractionation at this salt concentration (150 mM). At a pH value of 4.7, α-livetin and the albumin fraction have no net charge because its structure is less compact, increasing its retention; this effect combined with the IgY retention produced a decrement in Ψ values.

In the absence of salt, Ψ (**Figure 2A**) and *P* (**Figure 3A**) improved significantly at pH values of 6.7 and 5.7 for all membranes. However, with the RC membrane, lower IgY recoveries (**Table 1**) were obtained (56−73%) with respect to the unmodified (76−90%) and surface modified (90−94%) PES membranes. It can be observed in the SDS−PAGE separation profile (**Figure 4A** and **B**, line 5) that the concentration of undesired proteins (mainly β-livetin and albumin) in the permeate was high, while IgY losses were low. These results agree with observed values of Ψ and *P*, when using PES and MPES membranes. The best results obtained with PES membranes at the pH conditions tested are summarized in **Table 2**. The increased selectivity and IgY retention (≈94% recovery) obtained with the negatively charged modified membrane (MPES) at pH 6.7 were probably due to the combination of electrostatic exclusion (between membrane surface and IgY) and molecular exclusion phenomena. Molecular exclusion was the separation phenomenon responsible for the permeation of proteins carrying

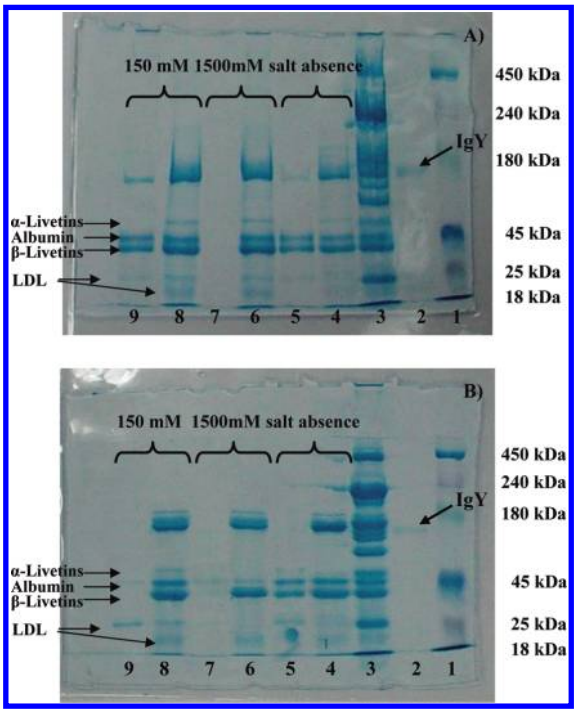

Figure 4. SDS−PAGE (nonreducing) separation profiles obtained with PES (**A**) and MPES (**B**) membranes at pH values of 5.7 and 6.7, respectively. Line 1, Molecular weight standard; line 2, IgY control; line 3, whole egg yolk (diluted but not centrifuged); lines 4, 6, and 8, retentate; lines 5, 7, and 9, permeate.

Table 2. Best Results Obtained from Fractionation of the WSPF Carried out with Different Membranes and pH Conditions, and in the absence of salt[a]

	Conditions			
	PES		MPES	
pH	5.7	6.7	5.7	6.7
Ψ	3.90	4.02	3.67	5.52
P	2.84	2.70	2.88	4.26
R_{PT} (%)	60.26	77.42	54.61	50.68
R_{IgY} (%)	75.90	90.0	90.13	93.70
J_{SS} (L-m^{-2}-h^{-1})	2.76	2.71	2.90	2.79

[a] R_{PT}, total protein recovery; R_{IgY}, IgY recovery; J_{SS}, stationary flux permeate.

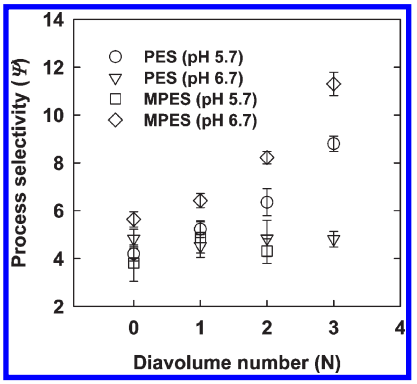

Figure 5. Diavolume number effect (*N*) on process selectivity (Ψ) when purifying IgY from the WSPF of hen's egg yolk by diafiltration.

Table 1. pH and Ionic Strength Effect on IgY Recovery (R_{IgY}) and Flux Permeate (J_{SS}) Using Different Membrane Types during IgY Ultrafiltration from WSP[a]

Poliethersulfone (PES)									
pH	4.7			5.7			6.7		
NaCl (mM)	1500	150	0.0	1500	150	0.0	1500	150	0.0
R_{IgY} (%)	80.14	80.03	76.08	84.78	75.90	82.73	83.15	70.10	90.00
J_{SS} (L-m^{-2}-h^{-1})	2.51	1.80	2.59	3.11	2.52	2.76	2.65	3.04	2.71
Modified PES (MPES)									
pH	4.7			5.7			6.7		
NaCl (mM)	1500	150	0.0	1500	150	0.0	1500	150	0.0
R_{IgY} (%)	83.56	77.55	72.45	84.52	79.35	90.13	86.00	88.21	93.70
J_{SS} (L-m^{-2}-h^{-1})	2.86	1.81	2.29	3.74	2.51	2.90	2.69	3.13	2.79
Regenerated Cellulose (RC)									
pH	4.7			5.7			6.7		
NaCl (mM)	1500	150	0.0	1500	150	0.0	1500	150	0.0
R_{IgY} (%)	75.10	73.40	68.00	68.90	70.97	72.70	59.52	56.95	55.68
J_{SS} (L-m^{-2}-h^{-1})	3.12	2.56	2.94	2.97	2.68	2.92	2.71	3.04	3.12

[a] R_{IgY}, IgY recovery; J_{SS}, stationary flux permeate.

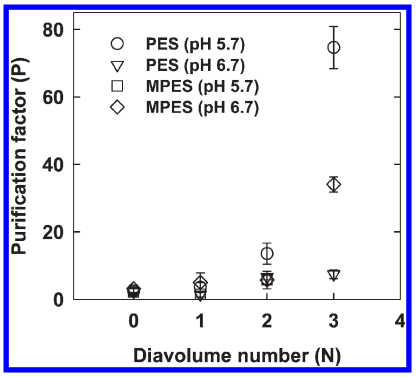

Figure 6. Diavolume number effect (*N*) on purification factor (*P*) when purifying IgY from the WSPF of hen's egg yolk by diafiltration.

Table 3. Diavolume Number Effect (*N*) on IgY Recovery (R_{IgY}) and Flux Permeate (J_{SS}) for IgY Separation from WSPF[a]

conditions		*N*	R_{IgY} (%)	J_{SS} (L-m^{-2}-h^{-1})
PES	5.7	0	78.54	2.76
		1	77.64	3.92
		2	77.63	4.46
		3	76.70	5.47
	6.7	0	92.17	2.71
		1	89.97	4.23
		2	80.00	5.16
		3	71.30	6.03
MPES	5.7	0	84.72	2.90
		1	78.10	5.33
		2	64.12	5.77
		3	90.13	7.76
	6.7	0	91.92	2.79
		1	91.01	3.57
		2	90.68	4.82
		3	90.48	5.24

[a] *N*, diavolume number; R_{IgY}, IgY recovery; J_{SS}, stationary flux permeate.

a charge similar to that of IgY (i.e., β-livetin) at pH 6.7 because of the membrane molecular weight cutoff (*18*, *24*).

In order to increase resolution in the fractionation of proteins from the WSPF of hen's egg yolk, diafiltration was carried out with pure water at two pH conditions (5.7 and 6.7) with the modified and unmodified PES membranes. Results show that Ψ (**Figure 5**) and *P* (**Figure 6**) increased with increments on the diavolume number used when diafiltration was performed with both PES and MPES membranes at pH values of 5.7 and 6.7, respectively. On the basis of IgY recovery (R_{IgY}) (**Table 3**), most of the undesired proteins were removed with a minimum of IgY losses (less than 3%) with the PES membrane at pH 5.7 (**Figure 6**). Under these conditions, electrostatic exclusion and molecular exclusion phenomena were combined because the charge of the membrane surface and that of the IgY molecule was similar. As a result, process selectivity was doubled, and increments on purification factor of IgY by more than 1 order of magnitude were achieved. These results are significantly higher than those found when using experimental conditions reported previously (*25*) and demonstrate the potential that membrane technology offers for large scale purification of IgY from hen's egg yolk.

ABBREVIATIONS USED

IgY, immunoglobulin Y; MPES, modified poliethersulfone; MWCO, molecular weight cutoff; PES, poliethersulfone; RC, regenerated cellulose; UF, ultrafiltration; WSPF, water-soluble protein fraction; LDL, low density lipoprotein; *C*, concentration; *N*, diavolume; *P*, purification factor; R_{IgY}, IgY recovery; *S*, sieving coefficient; *V*, volume; Ψ, selectivity; *1*, undesired proteins; *2*, filtrate; *f*, IgY; *F*, final; *i*, initial; *s*, feed; *x*, specific protein.

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