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Separation and flux characteristics in cross-flow ultrafiltration of bovine serum albumin and bovine hemoglobin solutions

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Abstract. The flux behavior in the separation of equimolar bovine serum albumin (BSA) and bovine hemoglobin (HB) in aqueous solutions by cross-flow ultrafiltration (UF) was investigated, in which polyacylonitrile membrane with a molecular weight cut-off (MWCO) of 100 kDa was used. BSA and HB have comparable molar mass (67,000 vs. 68,000) but different isoelectric points (4.7 vs. 7.1). The effects of process variables including solution pH (6.5, 7.1, and 7.5), total protein concentration (1.48 and 7.40 μM), transmembrane pressure (69, 207, and 345 kPa), and solution ionic strength (with or without 0.01 M NaCl) on the separation were examined. It was shown that the ionic strength had a negligible effect on separation performance under the conditions studied. Although BSA and HB are not rigid bodies, the flux decline in the present cross-flow UF did not result from the mechanism of cake filtration with compression. In this regard, the specific cake resistance when pseudo steady-state was reached was evaluated and discussed.

Keywords: separation; bovine serum albumin; bovine hemoglobin; cross-flow ultrafiltration; specific cake resistance.

1. Introduction

Ultrafiltration (UF) is a size exclusion-based pressure driven process that has been widely applied in food and biomedical industries. It has been generally recognized as one of the most effective methods for concentration and purification of macromolecular biochemicals from aqueous mixtures due to its advantages of low energy consumption, no phase change, and no additive requirement (Charcosset 2006, Sexena *et al*. 2009). Typically, UF processes can be performed in either dead-end or cross-flow mode (Becht *et al*. 2008, Kumar and De 2010). The major problem with dead-end UF is significant decline in the permeate flux caused by concentration polarization and fouling (Ghosh 2002); concentration polarization results from solute accumulation near the membrane surface whereas fouling results from the deposition of solutes onto the membrane surface. Both phenomena are regarded as the drawbacks that greatly limit the application of UF. To alleviate concentration polarization and fouling behaviors, cross-flow UF that provides a tangential flow to the membrane surface has been

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developed (Keskinler *et al*. 2002, Stopka *et al*. 2001, Tseng *et al*. 2006).

Besides the filtration mode, factors relating to membrane characteristics such as pore size, surface area, material, and conductivity are known to be influential on the flux during UF. Salehi and Madaeni (2010) have studied the effect of the conductivity on adsorptive behavior of UF membrane using BSA as the feed. Traditionally, UF has been applied for the separation of protein mixtures that have assorted sizes. However, many proteins in biochemical industry nowadays may have comparable sizes or molar masses. To achieve better purification of such protein mixtures, considerable research has taken place focusing on "fine tuning" operating and physicochemical conditions including transmembrane pressure (TMP), solute concentration, solution pH, ionic strength, and temperature (Bhattacharjee *et al*. 2006, Feins and Sirkar 2005). Hwang and Sz (2010) have studied the effect of TMP on the filtration characteristics and membrane fouling in cross-flow microfiltration of bovine serum albumin (BSA)/ dextran. They found that an increase in TMP leads to higher steady flux due to less membrane fouling or higher driving force. In general, the steady flux does not linearly depend on TMP indefinitely; there exist a limiting flux for a UF system. Wang and Rodgers (2008) have applied the free solvent model to demonstrate the factors that determine the limiting flux in protein UF. On the other hand, the steady flux is inversely proportional to feed concentration since solute deposition on the membrane surface tends to increase with solute concentration; also, varying the temperature will change the properties of the feed, such as viscosity, that thus affects the flux (Chen *et al*. 2007, Chollangi and Hossain 2007). Musale and Kulkarni (1997) have examined the separation of binary proteins with similar sizes, BSA and bovine hemoglobin (HB), using acrylonitrile homopolymer and its copolymer with acrylamide as the separating membrane at various pH values. They indicated that protein-protein and protein-membrane electrostatic interactions vary upon the change in pH, hence, affect the separation of proteins. Avramescu *et al*. (2003) have also studied the separation of BSA and HB using a new type of ion-exchange mixed-matrix membrane adsorber and examined the effect of solution ionic strength on protein separation. Their results suggested that increasing ionic strength will decrease the separation factor due to the enhanced screening of protein-protein and protein-membrane interactions (Becht *et al*. 2008).

Several factors affecting flux decline in dead-end UF of BSA and HB over polyethersulfone (PES) and polyacrylonitrile (PAN) membranes including solution pH, protein concentration, TMP, and ionic strength have been systematically studied in our earlier study (Lin *et al*. 2008a). It was shown that flux decline strongly depends on hydrophobic characteristics of the membranes, concentration and charge

Table 1 Physical characteristics of BSA and HB (Avramescu *et al*. 2003)

Protein	BSA	HB
Molar mass (Da)	67,000	68,000
Equivalent radius (nm)	3.61	3.10
Ellipsoidal diameters (nm)	14×4×4	7×5×5
Area occupied by each spherical molecule (nm^2)	52	38
Area occupied by each ellipsoidal molecule (nm^2)	16-56	30-39
Isoelectric point	4.7	7.1
Protein charge at pH 7.4 (mV)	–20.5	–4.0
Hydrophobic amino acid content (g/100 g protein)	44.07	54.16
Hydrophilic amino acid content (g/100 g protein)	56.16	43.13

of the proteins, as well as pH and ionic strength of the solution. In this work, the conditions for separating equimolar BSA and HB solutions by one-stage cross-flow UF process was evaluated. The two proteins were selected because most of their physical characteristics such as molar mass, molecular size, and steric structure are very similar, except for their hydrophilicities and isoelectric points (p*I*), as summarized in Table 1 (Christel *et al*. 2001). PAN membrane with a molecular weight cut-off (MWCO) of 100 kDa was chosen for this purpose because it has strongly hydrophilic character and charged surface (Lin *et al*. 2008a). The effect of process variables including solution pH, total protein concentration (C_{tot}), TMP, and ionic strength on the flux and separation performance was investigated. In addition, the modified fouling index (MFI) and specific cake resistance was determined to characterize the filtration process under various conditions (Schippers and Verdouw 1980).

2. Materials and methods

2.1 Sample preparation

BSA and HB were purchased from Merck Co. and used as received. The solutions were prepared by adding equimolar BSA and HB into buffered solutions with gentle agitation for 20 min, and then pre-filtered through a 0.45-μm Durapore membrane (Millipore, Bedford, MA) to remove any undissolved proteins and large particulates. Three buffers with pH 6.5, 7.1, and 7.5 were prepared by mixing appropriate volume of 10 mM NaH_2PO_4 and 10 mM CH_3COONa. The total protein concentration C_{tot} was fixed at 1.48 or 7.40 μM. The ionic strength of protein solution was adjusted by adding NaCl. All inorganic chemicals used were of analytical grade and the deionized water used was produced by a Millipore-Q plus purification unit.

2.2 UF apparatus and experiments

Fig. 1 shows the schematic diagram of experimental setup. A two-parallel-plate cross-flow UF cell (Mieshin Biotechnology Co., Taiwan) that had a channel dimension of 2 mm (H) × 138 mm (W) × 150 mm (L) with a geometric area of 270 cm^2 was used here. PAN membrane with a MWCO of 100 kDa was purchased from Osmonics Co. Prior to filtration, the membrane was soaked in 10 vol%

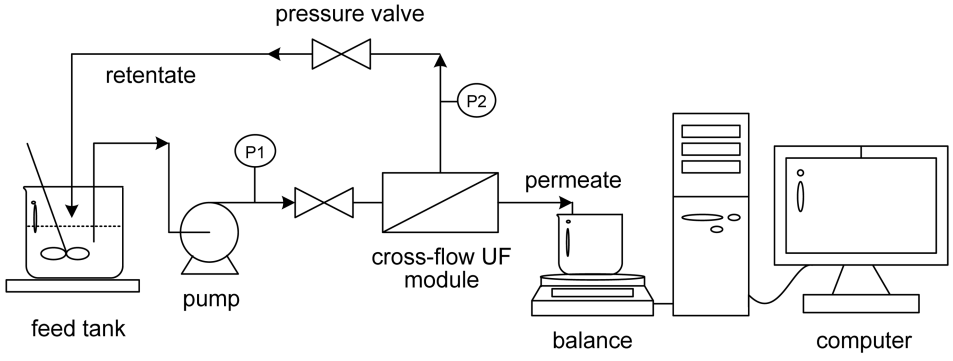

Fig. 1 Schematic diagram of the experimental setup involving cross-flow UF system

ethanol for 30 min to remove its protective layer and then soaked in deionized water for 10 min to rinse out any residual solvent and impurities. The pure water flux of virgin membrane (J_w) was subsequently measured. The membrane was finally soaked overnight in protein solution to ensure the attainment of equilibrium between the membrane and protein molecules before performing UF experiments.

As shown in Fig. 1, this system was equipped with two pressure gauges (at the inlet and outlet of the retentate). The solution was fed to the module using peristaltic pump (Masterflex Co., Model 7518-00 and 7518-10). Experiment was performed at 25°C with an initial feed volume of 250 mL, and at a fixed cross-flow velocity of 0.12 m s^{-1}. The effect of cross-flow velocity on the permeate flux was negligible under the conditions studied, thus a cross-flow velocity of 0.12 m s^{-1} was fixed. The flow velocity and TMP were adjusted by manual valve and pump controller, where TMP was calculated by (Chen *et al*. 2007)

$$TMP = \left(\frac{P_{inlet} + P_{outlet}}{2}\right) - P_{permeate} \tag{1}$$

where P_{inlet}, P_{outlet}, and $P_{permeate}$ are the pressures in the feed, retentate, and permeate, respectively (kPa).

After filtration, the concentrations of proteins in the permeate were analyzed by HPLC (Jasco, Japan) with an UV detector at 254 nm, following the method of William *et al*. (1995). To explore the electrical charge variations at the double layer of protein molecules at different pH values, zeta potentials were measured by a Malvern Zetasizer (Model 3000HS) with laser Doppler electrophoresis. In the measurement, BSA or HB was placed in a 100-mL plastic vessel filled with 50 mL of CO_2-free aqueous solution. It was accepted that the effect of hydrophilic interaction was maximized at the pI of protein molecule, because electrostatic interaction could be minimized at this point (Kimberly and Charles 2000). Thus, the effect of pH on the flux and separation factor was performed. The pH of the buffer for preparing the binary-protein solution was measured by a pH meter (Horiba F-23, Japan). In addition to the charge variation, the particle sizes of BSA and HB in the absence and presence of salt were also determined by zeta potential analyzer.

The permeate flux (J_v) at each run was obtained in time intervals t_1 and t_2 as follows

$$J_v = \frac{V_2 - V_1}{A(t_2 - t_1)} \tag{2}$$

where V_1 and V_2 are the permeate volumes collected at t_1 and t_2, respectively (m^3), and A is the effective membrane area (m^2). The separation factor (β) of BSA over HB was calculated based on the following expression at pseudo steady-state

$$\beta = \frac{(C_{f,BSA} - C_{p,BSA})/C_{f,BSA}}{(C_{f,HB} - C_{p,HB})/C_{f,HB}} \tag{3}$$

where C_f and C_p are the concentrations of proteins in feed and in permeate, respectively (μM).

After the completion of each experiment, the membrane used was cleaned in ultrasonic cleaner with 0.1 M NaOH for 30 min once and then with deionized water twice. The pure water flux J_w was re-checked. If J_w was within 95% of that for the virgin membrane, the performance and integrity of membrane was considered to be maintained. The cleaned membranes were stored in 0.05% sodium azide solution at 4°C.

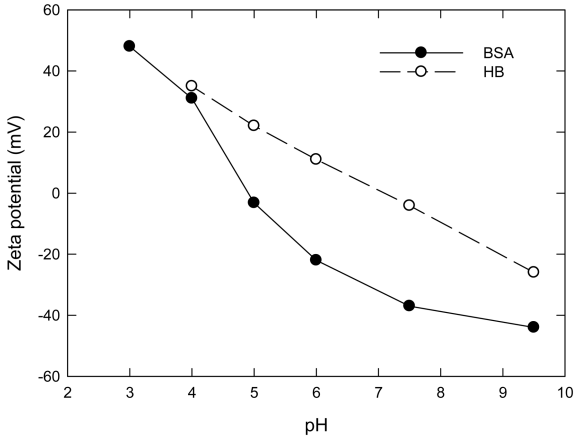

Fig. 2 Zeta potentials of BSA and HB at different pH values

3. Results and discussions

3.1 Zeta potential measurements

Fig. 2 shows the pH dependence of zeta potentials in single protein solution. As expected, zeta potentials change from positive to negative value when pH increases. The isoelectric points for BSA and HB are about 4.8 and 7.1, respectively, which are identical to literature values (Christel *et al*. 2001).

3.2 Effects of TMP and solution pH on the permeate flux

Fig. 3 shows the effects of solution pH on the time changes of normalized flux, J_v/J_w, at different C_{tot} (1.48 and 7.40 μM) and TMP (69, 207, 345 kPa). It is seen that increasing TMP or C_{tot} enlarges the decline in permeate flux regardless of the pH. This can be explained by the fact that increasing TMP will enhance the concentration polarization effect, leading to fouling phenomena; and, increasing C_{tot} increases the chance of solute blocking within the membrane pores (Becht *et al*. 2008, Lin *et al*. 2008a).

On the other hand, at a given TMP and C_{tot}, it is apparent that reducing pH would magnify the decline of flux. This can be realized by the change of charge polarity on the surface of protein molecules. For instance, at pH 6.5 (below the p*I* of HB and above the p*I* of BSA), HB is likely to be positively charged and BSA is negatively charged. At the same time, the surface of PAN membrane is negatively charged (Lin *et al*. 2008b); hence, HB would be adsorbed on the membrane surface. However, such behavior is not observed at solution pH 7.1 and 7.4. The higher steady fluxes obtained at pH 7.4 than at pH 7.1 is likely due to the fact that both proteins are negatively charged at pH 7.4, which would be repelled by the membrane surface.

3.3 Critical flux behavior

The plots of permeate flux versus TMP at different pH values are shown in Fig. 4 when C_{tot}=1.48

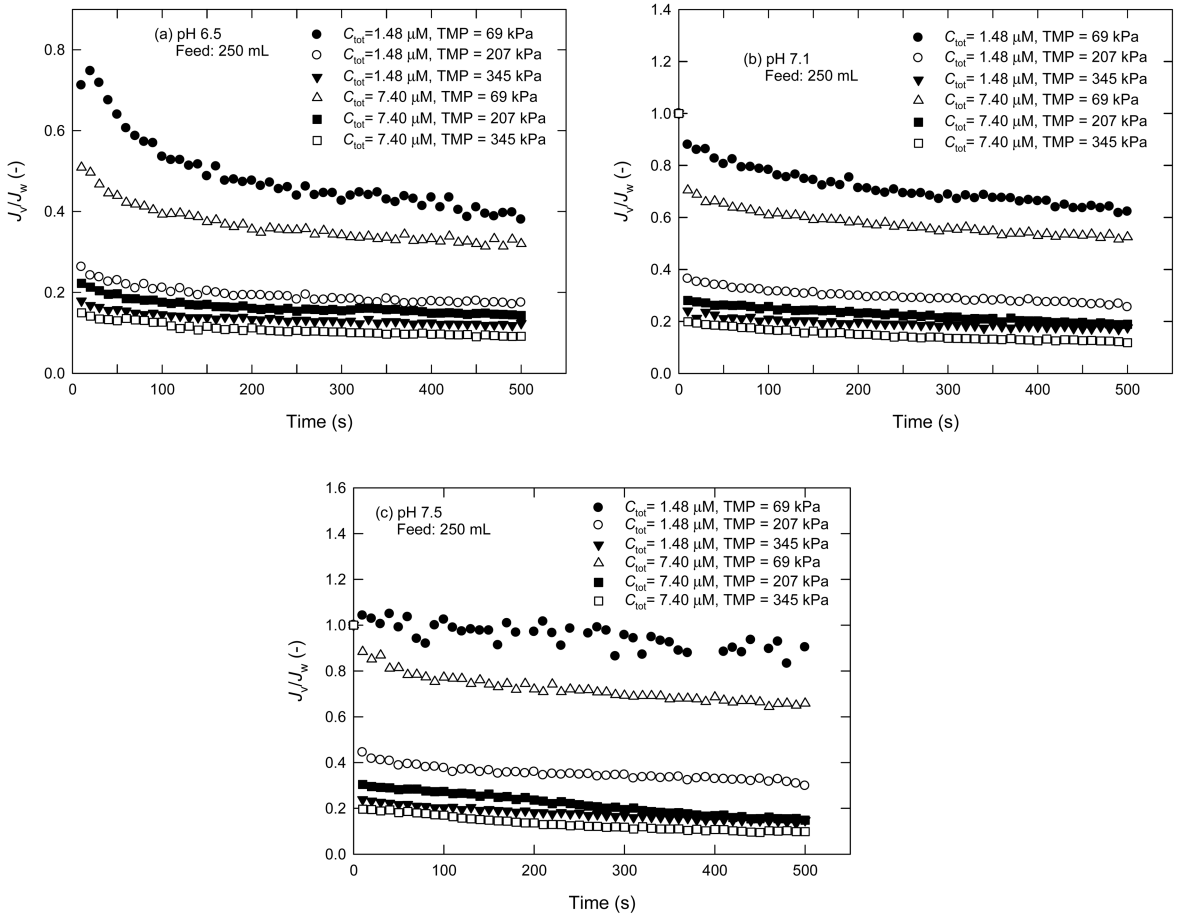

Fig. 3 Time changes of normalized flux at the pH value of (a) 6.5, (b) 7.1, and (c) 7.5

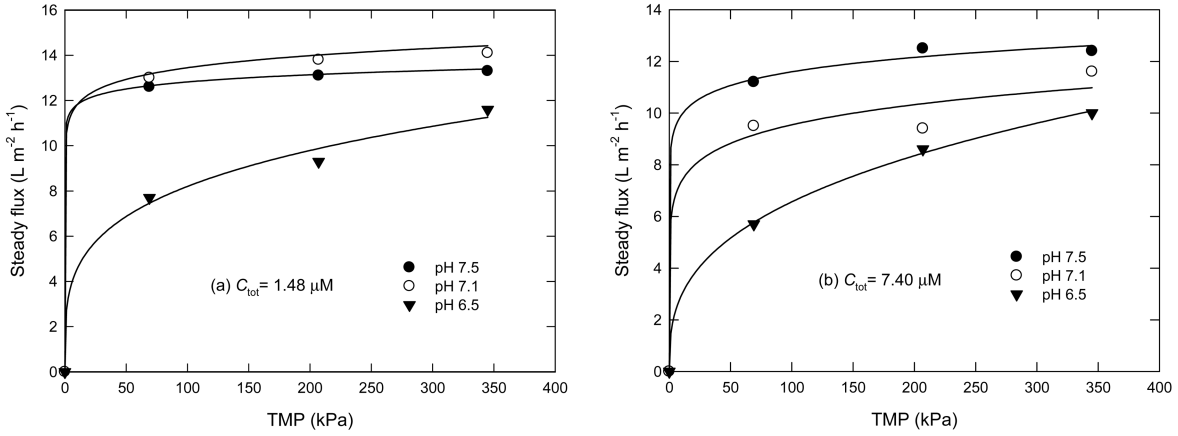

Fig. 4 Effect of TMP on steady-state flux at the total protein concentration of (a) 1.48 and (b) 7.40 μM

and 7.40 μM, respectively. Apparently, there exists a TMP in the curve that further increase in TMP can no longer improve the flux; the corresponding flux is the so-called limiting flux. The limiting flux appears to decrease with decreasing pH and increasing C_{tot} (Song 1998). This is because decreasing pH will increase the amount of positively charged proteins adsorbed on membrane surface, and increasing C_{tot} will increase the chance of solute blocking within the membrane pores as mentioned earlier; consequently, leads to the adverse effect of flux decline. It should be noted that the TMP at which limiting flux appears at pH 6.5 (*i.e.*, 207 kPa) is higher than those at pH 7.1 and 7.5 (*i.e.*, 69-105 kPa).

3.4 Separation factor

Fig. 5 shows the correlations between separation factor (β) and TMP at different C_{tot} and pH values. From the definition of β (Eq. 3), it is perceived that the larger the deviation of β from 1, the more effective the separation of binary proteins. As clearly seen in the plots, increasing TMP moves β closer to 1 regardless of C_{tot} and pH. The possible reason is that increasing TMP promotes concentration polarization, therefore, preventing both proteins from passing through the membrane. Also, the solution with low C_{tot} seems to show better separation compared to that with high C_{tot}. More concentrated solutions are likely to undergo fouling that is detrimental to separation. It is noticed that a maximum

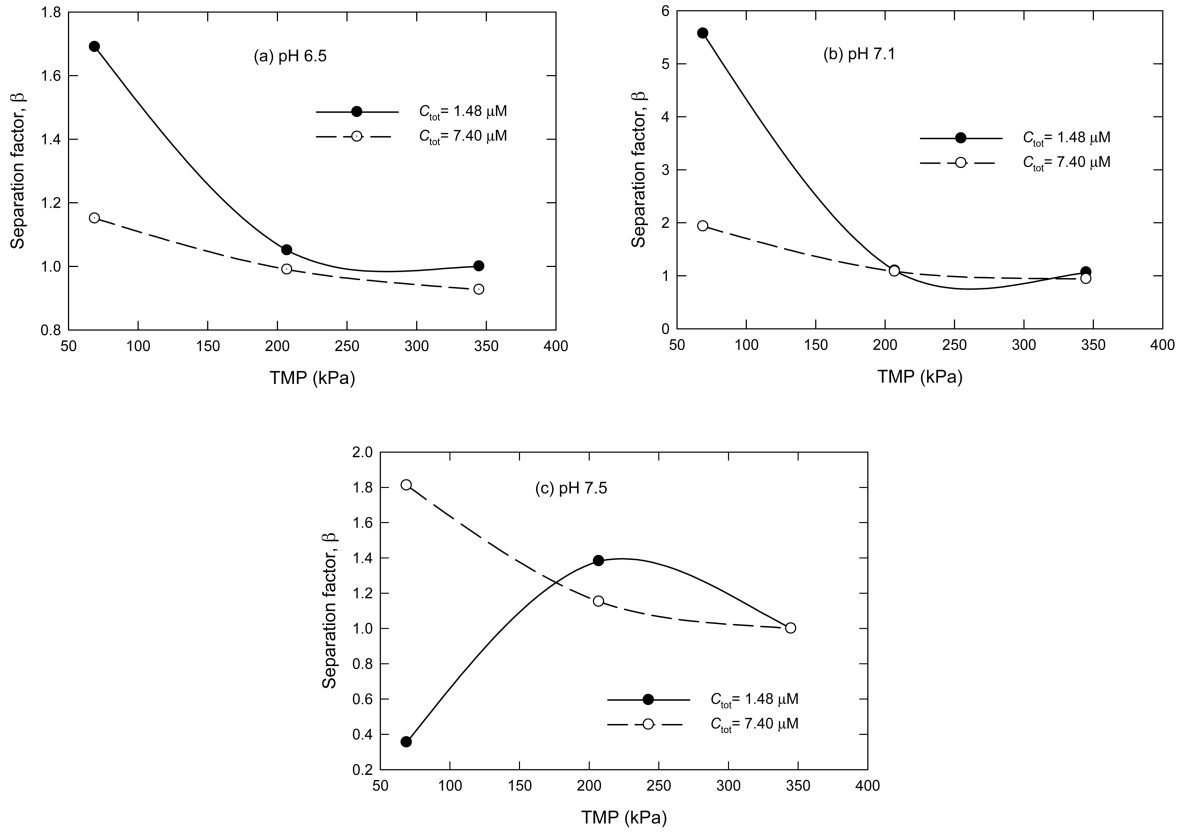

Fig. 5 Effect of TMP on separation factor at the pH value of (a) 6.5, (b) 7.1, and (c) 7.5

Table 2 Separation factor (β) of equimolar BSA-HB mixtures by cross-flow UF at a fixed TMP of 69 kPa

pH	Separation factor	
	C_{tot} = 1.48 μM	C_{tot} = 7.40 μM
6.5	1.1	1.8
7.1	2.0	5.3
7.5	1.9	0.2

β of 5.3 is obtained when pH = 7.1 and C_{tot} = 1.48 μM. Such a substantial increase in β is due to the fact that HB molecule has zero net charge at this pH while BSA molecule is negatively charged. The charge difference allows HB to readily pass through the membrane more easily than BSA, therefore, resulting in more effective separation.

On the other hand, BSA molecules are negatively charged at pH 6.5 while HB molecules are positively charged. As a result, the permeation of both BSA and HB is greatly hindered, as evidenced by the β value of near unity, owing to the charge repulsion of BSA molecules as well as the charge attraction of HB molecules with the negatively charged PAN membrane (Sexena *et al*. 2009). Table 2 compiles the separation factors of binary BSA-HB solutions obtained at a fixed TMP of 69 kPa under different pH and C_{tot} conditions.

Musale and Kulkarni (1997) have used poly(acrylonitrile) and poly(acrylonitrile-co-acrylamide) UF membranes to separate the BSA-HB mixtures, and indicated a maximum separation factor of 3.2 at low pH. Eijndhoven-van *et al*. (1995) have applied the electrostatic interactions to effectively separate the BSA-HB mixtures by UF. The high initial separation factor (up to 70) was achieved at a pH of 7, an ionic strength of 2.3 mM, and a filtration velocity of 11 L m^{-2} h^{-1}. After reaching a HB recovery of 60%, the overall selectivity dropped to 10. Moreover, Causserand *et al*. (2001) have proposed a selective protein separation process using montmorillonite clay adsorption and microfiltration. Once the adsorption equilibrium had been reached, the mixture of BSA-HB-clay particle was filtered through a polyvinyldifluoride membrane with a nominal pore size of 0.1 μm. Free BSA passes through the membrane, while the HB adsorbed on the clay particles was retained by the membrane. The maximum separation factor was 6.4 at a flux of 50 L m^{-2} h^{-1}, an ionic strength of 1 mM and the use of low protein fiber membranes. Avramescu *et al*. (2003) have also studied the separation of BSA from HB using a new type of ion-exchange mixed-matrix adsorber membranes. Selective adsorption of HB into the cationic adsorber membrane with an average separation factor of 40, calculated over the filtration until the HB breakthrough for a feed containing 10% HB at an ionic strength of 50 mM and a flow rate of 10 L m^{-2} h^{-1}. Although a maximum separation factor of 5.5 is obtained in this work, the moderately high flux and easy-to-operate nature makes it still promising.

3.5 Effect of ionic strength

It was reported that an increase in ionic strength of protein solution will reduce the thickness of electric double layer of protein molecules (Choi *et al*. 2003), thus decreases their sizes. Previous work has indicated that ionic strength of the solution affects the extent of charge interactions in terms of protein-protein and protein-membrane interactions, and has demonstrated the dependence of flux on ionic strength (Becht *et al*. 2008). Even though a decrease in particle sizes of BSA and HB is observed here in the presence of NaCl (Table 3), the addition of NaCl up to 0.01 M shows a negligible effect on the flux as shown in Figs. 6 and 7. The unchanged flux in the presence of NaCl is likely a result

Table 3 Molecular sizes of BSA and HB in an equimolar mixture (C_{tot} = 1.48 μM)

Protein	Condition of aqueous solution	Molecular size (nm)
BSA	Absence of NaCl	13.1
BSA	Presence of 0.01 M NaCl	11.2
HB	Absence of NaCl	8.07
HB	Presence of 0.01 M NaCl	7.02

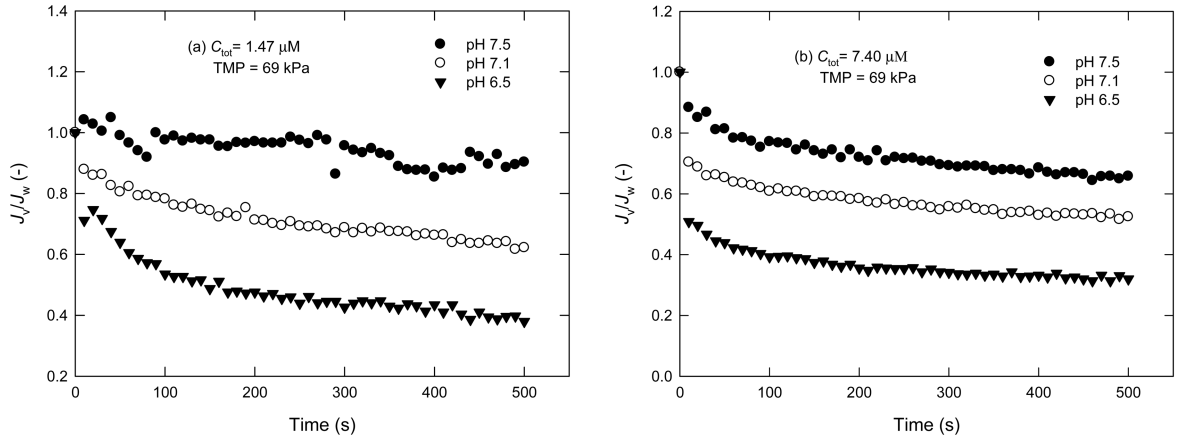

Fig. 6 Time changes of normalized flux in the absence of NaCl at the total protein concentration of (a) 1.48 and (b) 7.40 μM

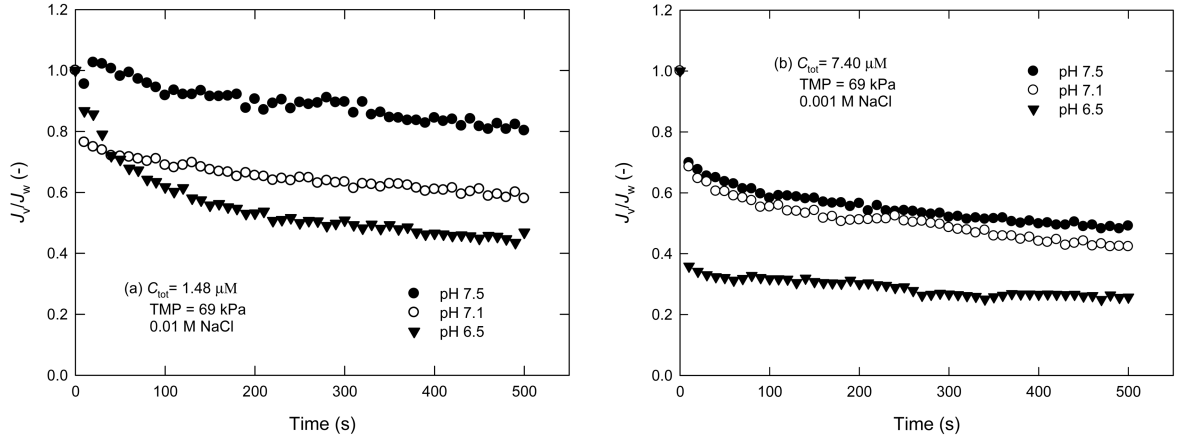

Fig. 7 Time changes of normalized flux in the presence of 0.01 M NaCl at the total protein concentration of (a) 1.48 and (b) 7.40 μM

of the low NaCl concentration present as observed in other studies (Choi *et al*. 2003, Becht *et al*. 2008). Although further increase of ionic strength may improve the flux, it will adversely create an environment undesirable in practical applications such as the occurrence of salting-in effect or

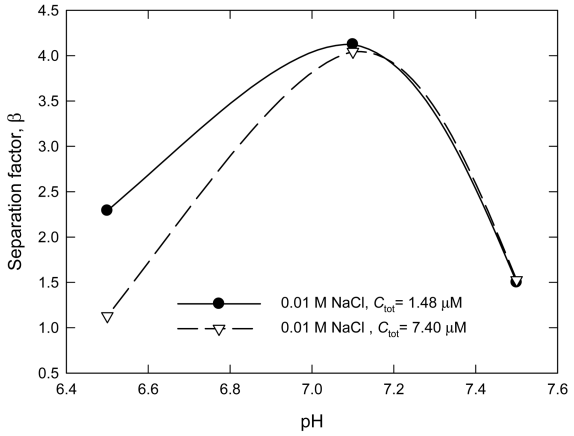

Fig. 8 Effect of pH on the separation factor in the presence of 0.01 M NaCl under different total protein concentrations

denaturation (de Wit and van Kessel 1996).

As for the relationship between separation factor and pH in the presence of NaCl (Fig. 8), the trend is similar to that observed in the absence of NaCl; maximum separation factor can be obtained at the pI of HB (7.1). In other words, the effect of molecular sizes of protein molecules on the flux and separation ability is not significant compared to that of the nature of the charges of proteins.

3.6 Evaluation of specific cake resistance

In principle, typical filtration has three regions, that is, pore blocking, cake filtration, and cake filtration with compression, take place consecutively (Schippers and Verdouw 1980). In the first region, the deposition of particles blocking the entry to a pore or inside membrane pore causes a sharp increase in slope. This is followed by a minimum linear slope where particles deposit on the

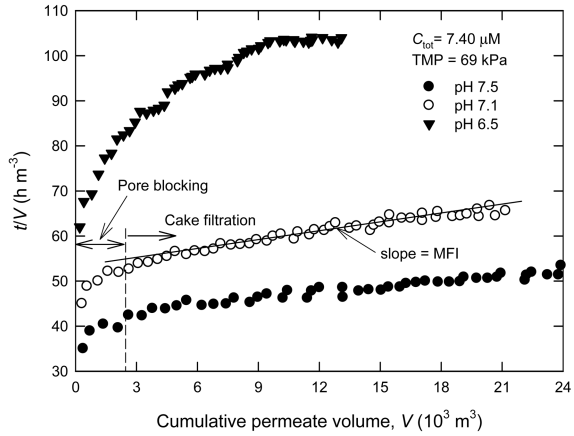

Fig. 9 Typical plot for the determination of modified fouling index (MFI) in the present UF process

membrane surface. The modified fouling index (MFI) is based on cake filtration (region 2); particles are retained on membrane surface as a cake. This can be demonstrated in Fig. 9. The cake adds additional resistance (R_c) to the resistance of membrane (R_m), and flux decline under constant pressure filtration is described as follows (Schippers and Verdouw 1980)

$$\frac{dV}{Adt} = \frac{\Delta P}{\mu(R_m + R_C)} \tag{4}$$

where ΔP denotes the TMP (kPa) and μ is the viscosity of the permeate (Pa s).

Graphical tests indicate that there is no cake compression (the last region) in these cases. Hence, the resistance of the cake, assuming the retention of particles is constant, is proportional to the amount of cake deposited at the membrane and the fouling tendency of feed water expressed as the fouling index, αC_{tot}

$$R_c = \frac{V\alpha C_{tot}}{A} \tag{5}$$

where α is the specific cake resistance (m kg^{-1}). Combining Eqs. (4) and (5), followed by integration at constant pressure gives the known cake filtration equation

$$\frac{t}{V} = \frac{\mu R_m}{\Delta P A} + \left(\frac{\mu\alpha C_{tot}}{2\Delta P A^2}\right) V \tag{6}$$

Therefore, a plot of (t/V) versus V should give a straight line with slope equal to ($\mu\alpha C_{tot}/2\Delta P A^2$), which is referred to the MFI, as shown in Fig. 9. The specific cake resistance is often used to characterize the hydrodynamic resistance of cake during the filtration of particulate suspensions (Keskinler *et al*. 2002).

The calculated MFI and a values are listed in Table 4. It is found that a increases with increasing C_{tot}. This result is expected because more concentrated solutions promote the fouling behavior. On the other hand, a decreases with increasing solution pH, likely due to the occurrence of charge attraction of HB-membrane at low pH. With regard to the effect of ionic strength, it is seen that specific cake resistance slightly reduces with the addition of 0.01 M NaCl whenever at pH 6.5, 7.1, and 7.4, although the effect of ionic strength on the flux is negligible (Fig. 7). Similar results on ionic strength

Table 4 Variations of modified fouling index (MFI) and specific cake resistance (α) in cross-flow UF of equimolar BSA and HB solutions at a TMP of 69 kPa

Matrix	C_{tot} (μM)	pH	MFI (h m^{-6})	α (10^7 m kg^{-1})
without NaCl	1.48	6.5	790.9	10.9
	1.48	7.1	206.1	2.86
	1.48	7.5	59.5	0.81
without NaCl	7.40	6.5	867.4	45.9
	7.40	7.1	261.7	18.0
	7.40	7.5	159.4	11.0
with 0.01 M NaCl	1.48	6.5	663.9	9.15
	1.48	7.1	197.4	2.73
	1.48	7.5	93.9	0.76

effect have been reported; *e.g.*, Keskinler *et al*. (2002) have found that specific cake resistance decreases in the presence of metals while steady-state flux increases in examining the effect of ionic environment on cross-flow microfiltration of yeast suspensions.

4. Conclusions

A hydrophilic polyacylonitrile (PAN) membrane with a MWCO of 100 kDa was used in cross-flow UF for the separation of equimolar bovine serum albumin (BSA) and bovine hemoglobin (HB) solutions at a fixed cross-flow velocity of 0.12 m s^{-1}. It was shown that the addition of 0.01 M NaCl had little effect on the flux. Moreover, increasing transmembrane pressure (TMP) or total protein concentration magnified the flux decline while increasing pH had an opposite effect. On the other hand, lowering TMP as well as total protein concentration appeared to give better separation; and the most effective separation was achieved near the pI of HB, the relative hydrophobic one of both proteins. The specific cake resistance increased with increasing total protein concentration but decreased with increasing solution pH. The highest separation factor of BSA over HB of 5.3 was obtained under the conditions of pH 7.1, total protein concentration of 1.48 μM, and a TMP of 69 kPa.

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CC

Notation

A = effective membrane area (m^2)
C_f = protein concentration in the feed (μM)
C_p = protein concentration in the permeate (μM)
C_{tot} = total protein concentration (μM)
J_v = permeate flux of protein solution (L m^{-2} h^{-1})
J_w = pure water flux (L m^{-2} h^{-1})
MFI = modified fouling index defined in Eq. (6) (h m^{-6})
R_c = resistance of the cake (m^{-1})
R_m = resistance of the membrane (m^{-1})
t = filtration time (s)
TMP = transmembrane pressure defined in Eq. (1) (kPa)
V = cumulative permeate volume (m^3)

Greek letters

α = specific cake resistance defined in Eq. (5) (m kg^{-1})
β = separation factor of BSA over HB defined in Eq. (3)
μ = viscosity of the permeate (Pa s)