Gradient Formation in Membrane Unit for Differential Precipitation of Proteins

Terasut Sookkumnerd and James T. Hsu
Biopharmaceutical Technology Institute, Dept. of Chemical Engineering, Lehigh University, Bethlehem, PA 18015

Yoichiro Ito
Laboratory of Biophysical Chemistry, National Heart, Lung and Blood Institute, National Institutes of Health, Bethesda, MD 20892

A new protein fractionation technique presented transfers ammonium sulfate to create a gradient of ammonium sulfate inside a membrane unit for differential precipitation of proteins called ''centrifugal precipitation chromatography.'' Because it does not require any solid support, it should provide a better alternative to the conventional chromatography with solid stationary phase. To understand the phenomena and achieve a better separation, a mathematical model explaining the ammonium sulfate gradient formation inside the stationary membrane unit is investigated. The model is extended with empirical correlation to the centrifugal membrane unit — a new approach for protein purification. The model agreed well with the experiments for both stationary and centrifugal units. Upon using the model to calculate the ammonium sulfate gradient formation in a membrane unit, this new technique can be useful in separating a mixture of proteins whose solubility in ammonium sulfate solution differs. To demonstrate the technique experimentally, mixtures of proteins are loaded into the column; the partial resolution between proteins is achieved with the step concentration switch of the inlet ammonium sulfate solution.

Introduction

Although several new techniques in protein purification have evolved over the past three decades, ammonium sulfate precipitation is still the preferable method for crude protein purification, especially in large-scale separation (Englard and Seifter, 1990, and Bell et al., 1983). Ammonium sulfate precipitation is, perhaps, the most inexpensive technique available, the simplest one to operate, and yet does not harm most proteins and enzymes (Scopes, 1994). These advantages, combined with a relatively high degree of recovery, make the technique very favorable. Unfortunately, because the solubility of proteins is slightly different, a common batch-process ammonium sulfate precipitation usually does not yield a high-purity protein.

Several groups of researchers thus attempted to improve the protein purity by performing a differential ammonium sulfate precipitation in a batch, continuous stirred-tank (Foster et al., 1976) and continuous tubular mixer (Virkar et al., 1982) [for a review of the subject, consult Rothstein (1994)]. Although, in principle, performing the precipitation in this manner could yield high-purity proteins, it was very tedious and complicated, especially when it was desirable to purify several proteins, which had similar solubility, from crude mixtures. In order to overcome the complexity, Porath (1962) and King (1972) developed a method of ammonium sulfate precipitation in a chromatographic column, sometimes called ''zone precipitation.'' In general, a concentration gradient of ammonium sulfate was first formed in the column by retarding salt with a solid support, for example, Sephadex or Celite. When proteins were loaded into the column, they precipitated according to their solubility. Then a gradient of ammonium sulfate was shifted toward the outlet by lowering the ammonium sulfate concentration fed into the column. As a gradient was shifted, proteins dissolved, reprecipitated, and were finally eluted from the column in the order correspond-

Correspondence concerning this article should be addressed to J. T. Hsu.
This article was presented at the ''Membrane Separations in Food and Pharmaceutical'' session of the AIChE Meeting, Dallas, TX (1999).

ing to their solubility. This procedure, however, had the drawback that proteins were adsorbed on the solid support. In addition, the efficiency of the separation in this procedure was reduced by the difference in the ability of small and large precipitates to travel in a column (Porath, 1962).

Recently, Ito (1999) proposed the alternative technique of inducing the ammonium sulfate gradient inside the membrane unit by using the transfer of ammonium sulfate across the membrane. Because the membrane's surface area was much smaller than the surface area of the Sephadex or Celite used previously, the problem of interaction between the solid phase and the proteins would be minimized. Moreover, the ammonium sulfate gradient inside a membrane unit was easier to predict and control, compared to that in the packed column; hence, the results became more reproducible. In addition, it was possible to apply an additional force, such as the centrifugal force, to improve the sedimentation and fractionation of smaller precipitates.

Conceptually, the technique, which is called ''centrifugal precipitation chromatography'' by Ito, depends on the transfer of ammonium sulfate across a membrane. As shown in Figure 1, a parallel-flow membrane unit is used to form a gradient inside the water channel. The membrane unit is mainly composed of two channels, separated by a piece of semipermeable membrane, which allows the ammonium sulfate to pass through from the salt channel to the water channel, but retains proteins in the water channel. The salt channel contains an ammonium sulfate solution at high flow rate, while the water and protein solution is supplied to the water channel. The flow rate in the salt channel is much greater than the flow rate in the water channel in order to enhance the mass transfer of ammonium sulfate across a membrane. Due to the transfer of ammonium sulfate from the salt channel to the water channel, the ammonium sulfate gradient in the water channel is formed, with the salt concentration increasing as away from the inlet of the water channel. After the ammonium sulfate gradient is formed, proteins are injected into the water channel. Proteins travel in the water channel until the salt concentration in the water channel reaches the critical solubility of each protein; then the proteins precipitate. To improve the sedimentation of precipitates, centrifugal force is applied. After the proteins precipitate, the ammonium sulfate concentration at the inlet of the salt channel decreases, causing the ammonium sulfate gradient and protein precipitates in the water channel to shift toward the outlet, just as is described in Porath (1962) and King (1972) for packed column. With repetitive dissolution and precipitation, proteins will be fractionated and a high purity of the protein products can be achieved.

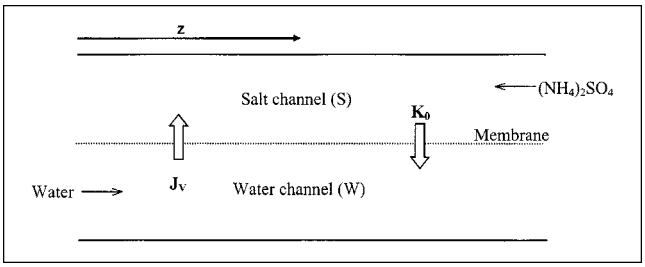

Figure 1. Parallel flow membrane unit to form an ammonium sulfate gradient in the water channel to facilitate the precipitation of the protein mixture.

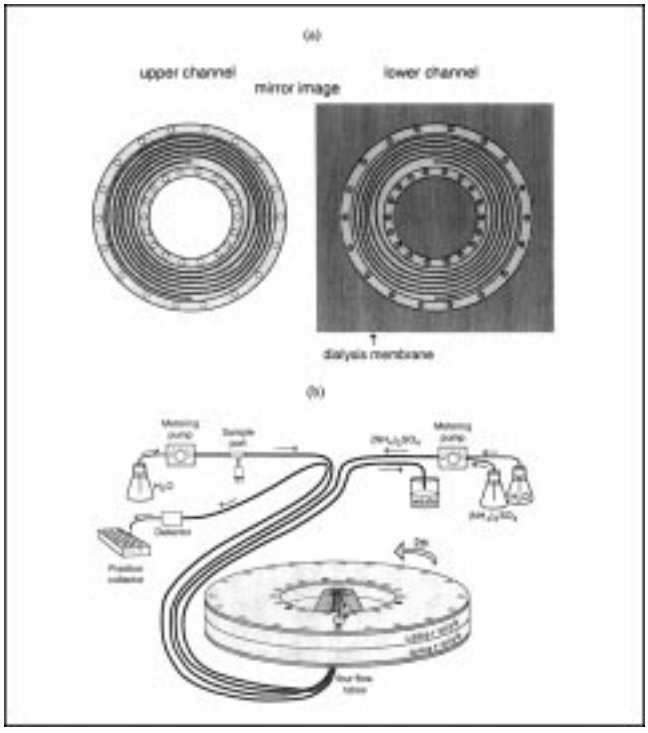

Figure 2. Instrument for centrifugal precipitation chromatography.

(a) The actual column. (b) Chromatographic system for centrifugal precipitation chromatography (after Ito, 1999).

The actual instrument consists of a separation column made of a pair of flat disks (high-density polyethylene, 13.5 cm in diameter and 1.5 cm in thickness) with a spiral-shaped groove (1.5 mm wide and 2 mm deep) at the periphery, as shown in Figure 2a. The spiral groove in the left disk is the mirror image to that in the right disk, so that with the proper alignment these two spiral grooves can be made to form a single spiral channel. The regenerated-cellulose dialysis membrane sheet is sandwiched between them to form two channels, as shown in Figure 2a. The disks are tightly pressed in order to seal against leakage. The column assembly is mounted on a sealless continuous flow centrifuge that allows continuous elution through the multiple flow lines of the rotating column without the use of rotary seals (Ito et al., 1970). The column is connected to pumps and the detector, as shown in Figure 2b.

Because the ammonium sulfate gradient profile inside the water channel is crucial to success in this technique, formation of the gradient is simulated numerically in this article. In the first part of the article, we discuss the mathematical model of the steady-state stationary membrane unit. Then, by using the model developed here, we estimate two important parameters involved in the mass transfer, mass-transfer coefficient (k_m), and coefficient of the convective flux (L_P) across a membrane by fitting the numerical results with the experimental data for the stationary membrane unit. After having

established the model for the stationary membrane unit, we propose an empirical correlation to extend the model to the centrifugal membrane unit, which is the unit used for protein fractionation by precipitation. Then the examples of gradients forming in the membrane unit under the actual operating condition are provided along with a discussion on using the gradients to facilitate the separation of proteins. Finally, we use the technique to separate the myoglobin, hemoglobin, and γ-globulin mixture, and the myoglobin, hemoglobin, and fibrinogen mixture with a step concentration switch of the inlet ammonium sulfate solution.

Method and Materials

Apparatus

An HPLC gradient pump was used to provide a step concentration switch of ammonium sulfate solution fed into the salt channel. The centrifugal precipitation chromatography was performed in the unit described by Ito (1999), as shown in Figure 2. A UV monitor and a strip-chart recorder were used to measure the absorbance of the eluent at 280 nm.

Materials

Ammonium sulfate, monobasic and dibasic potassium phosphates were all of reagent grade (Mallinckrodt Baker, Paris, KY). Water of a chromatographic grade (Fisher Scientific, Fair Lawn, NJ) was used for preparing 50 mM potassium phosphate buffer, with a pH 6.8. Myoglobin (horse heart), human serum albumin (HSA), fibrinogen (bovine plasma), and bovine γ-globulin were obtained from Sigma (St. Louis, MO).

Centrifugal precipitation chromatography of proteins

A 50-mM potassium phosphate, pH 6.8, and 95% saturated ammonium sulfate aqueous solution was prepared. Proteins were weighed and dissolved in a potassium phosphate buffer. The desired concentrated ammonium sulfate solution was initially fed into the salt channel by mixing the 95% saturated ammonium sulfate solution and water with a HPLC gradient pump, while the 50-mM potassium phosphate buffer, pH 6.8, was fed into the water channel under centrifugation at 2,000 rpm. After supplying the desired concentrated ammonium sulfate to the salt channel for 4 h, 1 mL of a protein solution was injected into the water channel in order to establish the gradient formation for protein precipitation. After 30 min, the centrifugation was terminated and the precipitates in the water channel were flushed out by switching from the ammonium sulfate solution in the salt channel to water. The chromatogram was recorded using a UV monitor and a chart recorder. The eluent was collected by the fraction collector every 10 min. To analyze the fractionated eluent, the desired fractions were desalted by ultracentrifuge. After that, the desalted samples were analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) on a 4–20% gel.

Model Development

The mathematical model discussed here follows the approach of Jaffrin et al. (1990) for simultaneous hemodialysis and ultrafiltration, with some modifications. We incorporated the pressure variation, velocity, and the effect of frictional force, which were not mentioned in the original model. The model is described as follows.

In general, the transfer of ammonium sulfate across the membrane comes from the net results of the convective flux of water and the diffusion flux of ammonium sulfate. The convective flux of water is mainly a result of the differences in osmotic pressures between the salt and water channels, while the diffusion flux of ammonium sulfate is a result of the differences in ammonium sulfate concentration in the salt and water channels. Mathematically, the mass transfer of ammonium sulfate across a membrane from the salt channel to the water channel for small convective flux (J_V), in which concentration polarization is considered, can be expressed as (Jaffrin et al., 1990)

$$K_0(C_{SS}-C_{SW})-J_VK_0\left[\left(\frac{1}{2k_m}+\frac{1}{k_S}\right)C_{SS}+\left(\frac{1}{2k_m}+\frac{1}{k_W}\right)C_{SW}\right], \quad (1)$$

where K_0 is the overall mass transfer coefficient across the membrane; k_m is the mass-transfer coefficient of ammonium sulfate in the membrane; k_S and k_W are the mass-transfer coefficients of ammonium sulfate in the salt and water channels, respectively; C_{SS} and C_{SW} are the salt concentration in the salt and water channels, respectively; and J_V is the convective flux of water across the membrane. Note that J_V is positive when the net convective flux is from the water channel to the salt channel and is negative when the net convective flux is from the salt channel to the water channel.

Based on Eq. 1, the mass balance of salt and the equation of continuity in both channels are as follows.

Mass Balance of Salt in Salt and Water Channels.

$$\Phi\frac{\partial}{\partial z}(C_{SS}\nu_S)=-\frac{K_0}{h_S}(C_{SS}-C_{SW})+\frac{J_VK_0}{h_S}\left[\left(\frac{1}{2k_m}+\frac{1}{k_S}\right)C_{SS}+\left(\frac{1}{2k_m}+\frac{1}{k_W}\right)C_{SW}\right] \quad (2a)$$

$$\frac{\partial}{\partial z}(C_{SW}\nu_W)=\frac{K_0}{h_W}(C_{SS}-C_{SW})-\frac{J_VK_0}{h_W}\left[\left(\frac{1}{2k_m}+\frac{1}{k_S}\right)C_{SS}+\left(\frac{1}{2k_m}+\frac{1}{k_W}\right)C_{SW}\right]. \quad (2b)$$

Equation of Continuity.

$$\Phi\frac{\partial}{\partial z}(\rho_S\nu_S)=\Phi\nu_S\left(\frac{\partial C_{SS}}{\partial z}\right)\left(\frac{\partial\rho_S}{\partial C_{SS}}\right)+\Phi\rho_S\frac{\partial}{\partial z}\nu_S=\frac{J_V\rho_m}{h_S} \quad (3a)$$

$$\frac{\partial}{\partial z}(\rho_W\nu_W)=\nu_W\left(\frac{\partial C_{SW}}{\partial z}\right)\left(\frac{\partial\rho_W}{\partial C_{SW}}\right)+\rho_W\frac{\partial}{\partial z}\nu_W=-\frac{J_V\rho_m}{h_W}. \quad (3b)$$

Note that, because of the very high Peclet number in both

channels ($Pe \gg 1{,}000$), dispersion is neglected in Eqs. 2a and 2b. Also, in order to make the model work in both the cocurrent and countercurrent membrane units, we introduce the new parameter Φ. This parameter depends on the direction of the flow: Φ is 1 for cocurrent and -1 for countercurrent, respectively. Moreover, it should be pointed out that the Cartesian coordinate could be used, because the width of the channel is much smaller than the radius of the disk; hence, the effect of curvature can be neglected.

Besides the mass balance of salt and the equations of continuity, the equations of motion have to be considered in order to explain the effect of friction and the variation in pressure along the channels. Assuming that the transfer of momentum due to the mass transfer across the membrane is small when compared to the frictional losses and the convection, as suggested by Bird et al. (1960), the equations of motion can be written as follows.

Equations of Motion.

$$\rho_S \Phi \nu_S \frac{\partial}{\partial z} \nu_S + \Phi \frac{\partial}{\partial z} P_S = -\tau_w \frac{P_f}{A_{xS}} - \frac{\nu_S J_V \rho_m}{h_S} \tag{4a}$$

$$\rho_W \nu_W \frac{\partial}{\partial z} \nu_W + \frac{\partial}{\partial z} P_W = -\tau_w \frac{P_f}{A_{xW}} + \frac{\nu_W J_V \rho_m}{h_W} \tag{4b}$$

Boundary conditions

Because the pressures at the outlets were the atmospheric pressure, Eqs. 2 to 4 can be solved numerically, since the inlet concentrations and the inlet volumetric flow rate in both channels were operating variables, which were usually set by the operator. In order to solve the equations, however, we needed to know the correlation of the overall mass transfer of ammonium sulfate across the membrane (K_0), the volumetric flux across the membrane (J_V), and the momentum losses due to frictional force (τ_w).

Correlation for K_0, J_V, and τ_w

The following correlations, obtained from the literature, were used to calculate K_0 (Jakob, 1949), J_V (Kessler and Klein, 1992), and τ_w (Bird et al., 1960).

$$\frac{1}{K_0} = \frac{1}{1/k_S + 1/k_m + 1/k_W} \tag{5a}$$

$$J_V = -L_P\left[(P_S - P_W) - \sigma(\Pi_S - \Pi_W)\right] \tag{5b}$$

$$\tau_w = \rho_i \nu_i^2 \frac{(w_m + h_i)}{w_m h_i} f, \quad \text{subscript } i \text{ is } S \text{ and } W, \tag{5c}$$

where σ is the Staverman reflection coefficient, and k_S (mass transfer coefficient in the salt channel) and k_W (mass transfer coefficient in the water channel) can be calculated from Jakob (1949) as suggested by Jaffrin et al. (1990)

$$k_i = A \cdot \frac{D_{sw}(w_m + h_i)}{2 w_m h_i} Gz^n \tag{5d}$$

$$Gz = \frac{4\nu_i}{D_{sw} L}\left(\frac{w_m h_i}{w_m + h_i}\right)^2 \tag{5e}$$

where

$$\begin{array}{llll} A = 2.2 & \text{and} & n = 0.32 & \text{for } Gz > 330 \\ A = 4.4 & \text{and} & n = 0.20 & \text{for } 15 \le Gz \le 330 \\ A = 7.6 & \text{and} & n = 0 & \text{for } Gz < 15. \end{array}$$

Numerical Methods

The fourth-order Runge-Kutta method with a step size of 0.01 and the shooting method were used to solve Eqs. 2 to 4 simultaneously. First, for the countercurrent membrane unit, we guessed the salt concentration and the volumetric flow rate at the outlet of the salt channel and the pressure at the inlet of the water channel. Then we calculated the inlet concentration and the inlet volumetric flow rate of the salt channel, and the pressure at the outlet of the water channel. After that, if the calculated variables did not match those specified in the boundary conditions, we iterated until the calculated inlet concentration and inlet flow rate of the salt channel and the pressure at the outlet of the water channel matched those specified in the boundary conditions. Normally, no more than 10 iterations were needed. The tolerance was set at 10^{-5} for all calculations.

The numerical method was similar for the cocurrent membrane unit. However, in that case, we iterated for the salt and water channel outlet pressures by guessing the inlet pressure of the salt and water channels instead.

Results and Discussions

Estimation of k_m and L_P for stationary membrane unit

Using the mathematical model just discussed with the parameter values listed in Table 1, two important parameters, k_m (mass-transfer coefficient of ammonium sulfate in a membrane) and L_P (coefficient of the convective across a membrane), were obtained by fitting the computed concentration of ammonium sulfate and the computed flow rate at the outlet of the water channel with the experimental data, reported by Ito (1999). From the computation, k_m and L_P that gave the best agreement between the numerical and experimental results for the fed water flow rate of 0.2 mL/min and the feed ammonium sulfate flow rate of 1.0 mL/min for the regenerated cellulose membrane were shown in Table 2. As seen in Table 2, k_m and J_V depended on the location of the water and salt channels and also on the molecular weight cutoff (MWCO) of the membrane. The differences in k_m and L_P due to the MWCO were expected since different membranes had different average pore sizes.

In order to evaluate the estimated k_m and L_P, the volumetric flow rate and ammonium sulfate concentration at the exit of the water channel were calculated at different fed-water flow rates by using the estimated k_m and L_P from Table 2. As shown in Figures 3 and 4, the numerical results agreed well with the experimental results. Hence, the model and the estimated k_m and L_P can be used to explain the transport processes across the membrane and the transport processes in both ammonium sulfate and water channels for a stationary membrane unit.

Estimation of k_m and L_P for centrifugal membrane unit

In order to facilitate the protein precipitation in water (lower) channel, centrifugal force is normally applied. With

Table 1. Parameters and Physical Properties Used in the Numerical Analysis

Parameters	Values	Remarks
μ_S (kg·m^{-1}·s^{-1})	1.00×10^{-3}	Bird et al. (1960)
		Assume that μ_S is equal to μ of water
μ_W (kg·m^{-1}·s^{-1})	1.00×10^{-3}	Bird et al. (1960)
		Assume that μ_W is equal to μ of water
ρ_S (kg·m^{-3})	Function of salt	Perry and Green (1973)
ρ_m (kg·m^{-3})	Function of salt	Perry and Green (1973)
ρ_W (kg·m^{-3})	Function of salt	Perry and Green (1973)
σ	0.03	Approximated from Bailey and Ollis (1986)
D_{SW}(m^2·s^{-1})	0.92×10^{-9}	*Int. Critical Table,* Vol. 5, p. 65 (1930)
h_S (m)	0.002	Ito (1999)
h_W (m)	0.002	Ito (1999)
L(m)	1.70	Ito (1999)
w_m (m)	1.50×10^{-3}	Ito (1999)

Table 2. Mass-Transfer Coefficient in the Membrane, k_m, and Coefficient of the Convective Flux Across a Membrane, L_P, for Different Molecular Weight Cutoff of the Membrane and Flow Configuration*

MWCO	Upper Channel	Lower Channel	k_m (m·s^{-1})	L_P (m^2·s·kg^{-1})
6,000–8,000	Ammonium sulfate	Water	6.505×10^{-7}	1.716×10^{-12}
6,000–8,000	Water	Ammonium sulfate	4.347×10^{-7}	1.090×10^{-12}
12,000–14,000	Ammonium sulfate	Water	1.110×10^{-6}	2.060×10^{-12}
12,000–14,000	Water	Ammonium sulfate	5.925×10^{-7}	1.373×10^{-12}

*L_P and k_m were obtained by fitting the numerical result with experimental data of the stationary membrane unit.

the introduction of centrifugal force, both the mass transfer of salt and the momentum transfer of fluid flowing in the channel would be influenced. Therefore, there is a need to extend the model discussed earlier to include the effect of the centrifugal force.

While a rigorous analysis of the effect of centrifugal force on the membrane unit in 2-D or 3-D is extremely difficult, we can deduce some important observations from the experiments. With the introduction of centrifugal force, it can be seen from a comparison of Figures 3 and 4 with Figures 5 and 6 that both diffusion flux and convection flux were en-

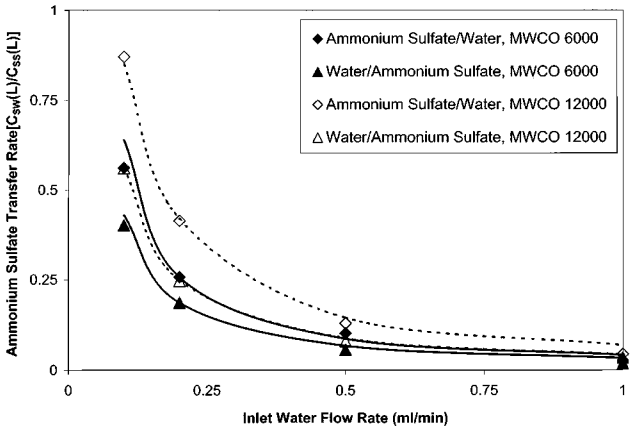

Figure 3. Experimental (symbols) and calculated (lines) ammonium sulfate transfer rate through the membrane, defined as the ratio of the outlet concentration of ammonium sulfate in the water channel to the feed concentration of ammonium sulfate in the salt channel as a function of feed flow rates in the water channel.

Ninety-five % saturation ammonium sulfate solution was fed into the salt channel. No rotation was applied. The length of the channels (L) is 1.7 m. The legend indicates the solution fed in the upper/solution fed in the lower channel and membrane MWCO. The solid lines represent the numerical results of membrane with 6,000 MWCO, and the dashed lines represent the numerical result of membrane with 12,000 MWCO. Experimental data were obtained from Ito (1999).

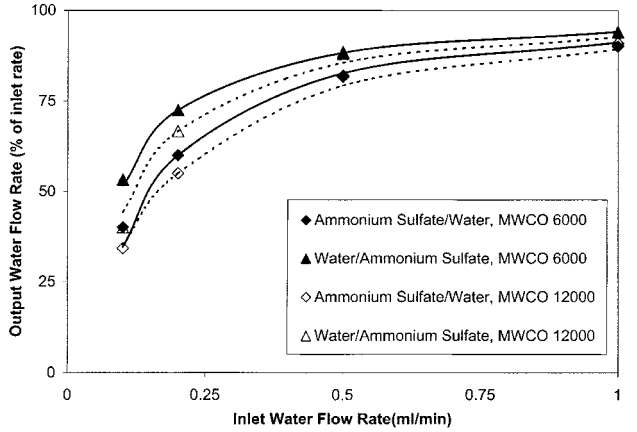

Figure 4. Experimental (symbols) and calculated (lines) output flow-rate percentage of the water channel, defined as the percentage of the flow rate at the outlet to that at the inlet of the water channel as a function of flow rates in the water channel.

Ninety-five % saturation ammonium sulfate solution was fed into the salt channel. No rotation was applied. The length of the channels (L) is 1.7 m. The legend indicates the solution fed in the upper/solution fed in the lower channel and membrane MWCO. The solid lines represent the numerical results of membrane with 6,000 MWCO, and the dashed lines represent the numerical result of membrane with 12,000 MWCO. Experimental data were obtained from Ito (1999).

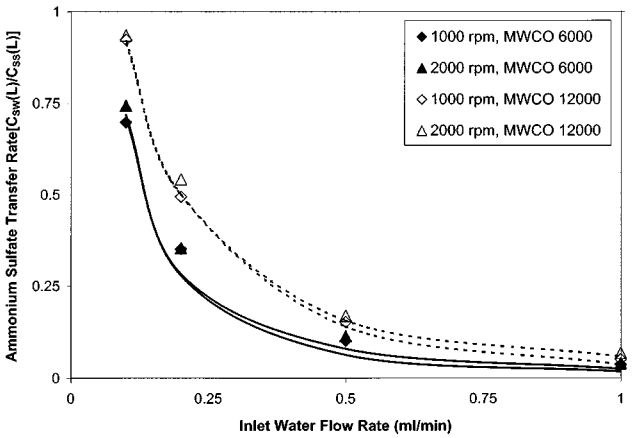

Figure 5. Experimental (symbols) and calculated (lines) ammonium sulfate transfer rate through the membrane, defined as the ratio of the outlet concentration of ammonium sulfate in the water channel to the feed concentration of ammonium sulfate in the salt channel as a function of feed flow rates in the water channel and the revolution of a membrane unit.

Ninety-five % saturation ammonium sulfate solution was fed into the upper channel and water was fed into the lower channel. The length of the channels (L) is 1.7 m. The legend indicates the revolution speed (rpm) of the membrane unit and membrane MWCO. The solid lines represent the numerical results of membrane with 6,000 MWCO, and the dashed lines represent the numerical result of membrane with 12,000 MWCO. Experimental data were obtained from Ito (1999).

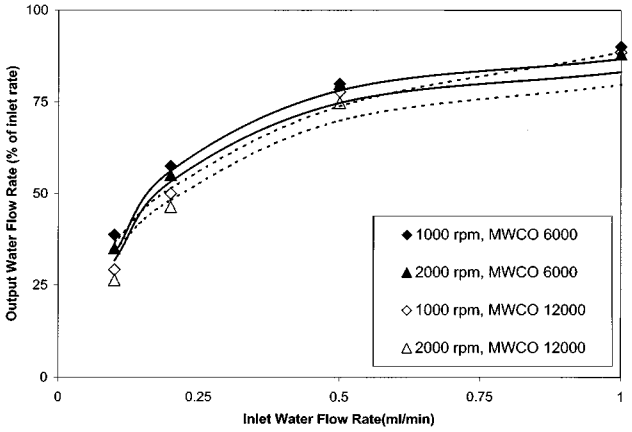

Figure 6. Experimental (symbols) and calculated (lines) output flow-rate percentage of the water channel, defined as the percentage of the flow rate at the outlet to that at the inlet of the water channel as a function of flow rates in the water channel and the revolution of a membrane unit.

Ninety-five % saturation ammonium sulfate solution was fed into the upper channel and water was fed into the lower channel. The length of the channels (L) is 1.7 m. The legend indicates the revolution speed (rpm) of the membrane unit and membrane MWCO. The solid lines represent the numerical results of membrane with 6,000 MWCO, and the dashed lines represent the numerical result of membrane with 12,000 MWCO. Experimental data were obtained from Ito (1999).

hanced by the centrifugal force. Based on the investigations of fluid flowing in a rotating pipe in which it is concluded that centrifugal force would induce the secondary flow in the pipe (Ito and Nanbu, 1971; Launder and Tselepidakis, 1994), it is believed that the centrifugal force also induces a similar secondary flow in the channels of centrifugal precipitation chromatography. As a result, this secondary flow will likely influence both mass and momentum transfers in the channels of centrifugal precipitation chromatography. For a moderately fast rotating pipe and low Reynolds number, as in the case of fluid flowing in both channels of centrifugal precipitation chromatography, Ito and Nanbu (1971) suggested that the frictional losses would depend more strongly on the revolution than the Reynolds number. Therefore, we proposed that the overall mass-transfer coefficient and the frictional losses inside the channels could be modified and expressed as

$$\frac{1}{K_0} = \frac{1}{\alpha(\text{rpm}, \nu_S)/k_S + 1/k_m + \beta(\text{rpm}, \nu_W)/k_W} \quad (6a)$$

$$\text{For salt channel: } \tau_{wS,\text{rotation}} = \lambda_S(\text{rpm}, \nu_S) \times \tau_{wS,\text{no rotation}} \quad (6b)$$

$$\text{For water channel: } \tau_{wW,\text{rotation}} = \lambda_W(\text{rpm}, \nu_W) \times \tau_{wW,\text{no rotation}}. \quad (6c)$$

Equations 6a to 6c imply that the centrifugal force enhances the mass transfer and increases the frictional losses in the channels in ways similar to those generally observed in the fluid flowing in the rotating pipe.

With these assumptions, we investigated the values of β, α, λ_S, and λ_W by fitting the numerical results with the experimental data at revolutions of 1,000 rpm and 2,000 rpm, an inlet water flow rate of 0.2 mL/min, and an inlet ammonium sulfate flow rate of 1.0 mL/min. From the computation, β/k_W was found to be very small, on the order of 10^{-6}, while α was found to be equal to 1 in both revolutions. Therefore, the term β/k_W could be deleted from the expression for the overall mass-transfer coefficient across a membrane. This finding, in turn, implied that the centrifugal force dramatically enhanced the mass transfer in the water channel, as discussed before. For the frictional losses we found from the analysis that while λ_S was equal to 1 at all revolutions, λ_W depended on the revolution. The dependence of λ_W on the revolution is shown in Figure 7. From Figure 7, we saw that the values of λ_W were strongly dependent on the revolution; λ_W was higher when the revolution (rpm) increased. It is interesting to note that while the centrifugal force effect on the transport processes in the water channel is quite strong, the centrifugal force effect on the transport processes in the salt channel is insignificant. Perhaps, these wide differences are caused by the different directions the fluid flows in the water and salt channels with reference to the centrifugal force.

In order to evaluate the proposed correlation for the centrifugal membrane unit, the numerical analysis at different flow rates was made and compared with the experimental results. As seen in Figures 5 and 6, the numerical results agreed well with the experimental data. Some deviations at the high

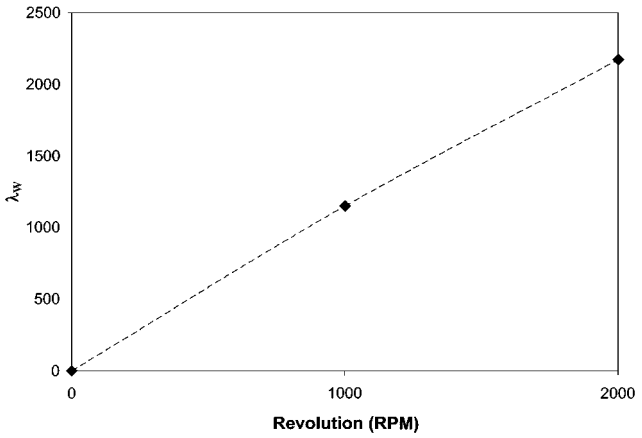

Figure 7. λ_W dependence on the revolution (rpm) of a membrane unit.

inlet water flow rate probably indicate the weak dependence of ν_W on β and λ_W, which were neglected in the correlation.

Steady-state gradient formation in a centrifugal membrane unit

In order to obtain an insight into what happened in both the water and salt channels under the actual operating conditions, the numerical analysis of the steady-state gradient formation in the countercurrent membrane (MWCO 6,000–8,000) unit was evaluated using the model discussed earlier and the parameters listed in Tables 1 and 2. The numerical results were shown in Figure 8. Note that in the numerical analysis, ammonium sulfate was pumped into the upper channel and water was fed into the lower channel, since this countercurrent flow configuration enhanced the transfer of ammonium sulfate across the membrane.

From Figure 8a, it can be seen that the ammonium sulfate concentration in the upper channel does not change much with respect to the distance from the inlet, except when the higher degree of saturated ammonium sulfate solution is fed into the upper channel. With the more concentrated ammonium sulfate solution fed into the upper channel, the mass transfer across the membrane is generally greater, and so a larger amount of ammonium sulfate is permeated out of the salt channel.

On the other hand, the salt concentration in the water channel is a strong function of the distance from the inlet. As shown in Figure 8b, the concentration of ammonium sulfate in the water channel increases rapidly, especially when highly concentrated ammonium sulfate is fed into the salt channel. When highly concentrated ammonium sulfate is fed into the salt channel, the reflection point in the early portion of the curves can be seen, possibly reflecting the changes in the transport mechanism. Perhaps, the greater convective flux from the water channel to the salt channel, due to the greater differences in osmotic pressure, is the main cause of the reflection point.

An analysis of the velocity and pressure profiles was also performed (data not shown). Generally, we found that large convective flux across the membrane from the water channel

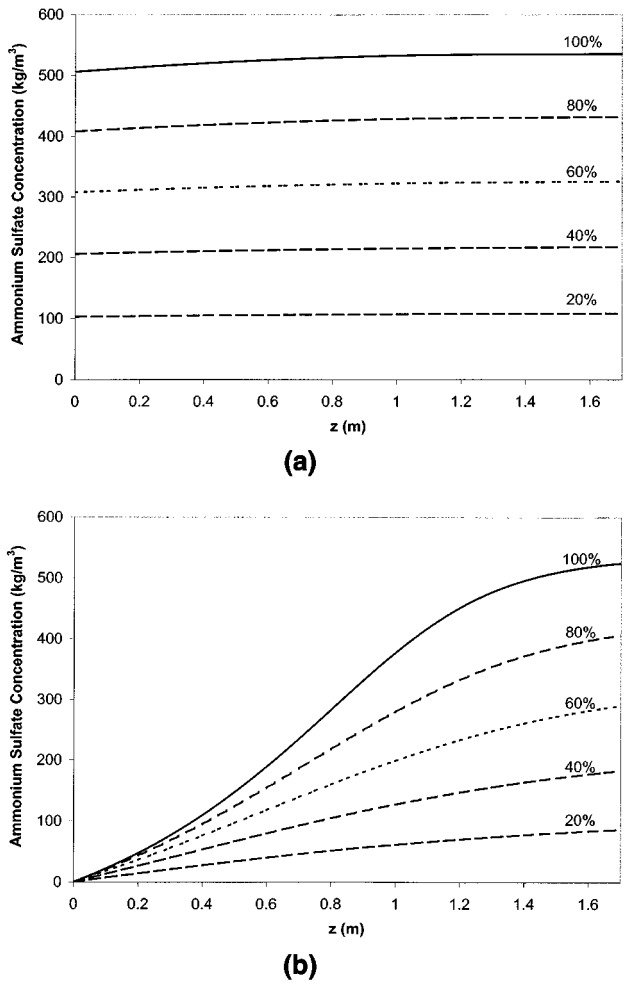

Figure 8. Steady-state concentration profile of ammonium sulfate concentration in the salt channel (a) and in the water channel (b) of a countercurrent unit with rotation of 2,000 rpm.

The different ammonium sulfate solutions were fed into the upper channel as indicated in the legend (% saturation). Empirical correlation discussed in the text was used in the numerical analysis. Ammonium sulfate flow rate was 1.0 mL/min and water flow rate was 0.06 mL/min.

to the salt channel decreased the velocity in the water channel and increased the velocity in the salt channel. This effect was higher when more concentrated ammonium sulfate was fed into the salt channel, since the convective flux from the water channel to the salt channel was higher in that case. As for the pressure profiles (data not shown), there generally were no significant changes when a different ammonium sulfate concentration solution was fed into the channels, especially the salt channel. For the pressure in the water channel, the greatest change was less than 5% for the geometry of the unit considered here. Note that if the channels were longer or if the process was scaled up, the pressure could play an important role.

Similar numerical results were also obtained for the cocurrent membrane unit (data not shown). The important difference between these two different flow directions was that less

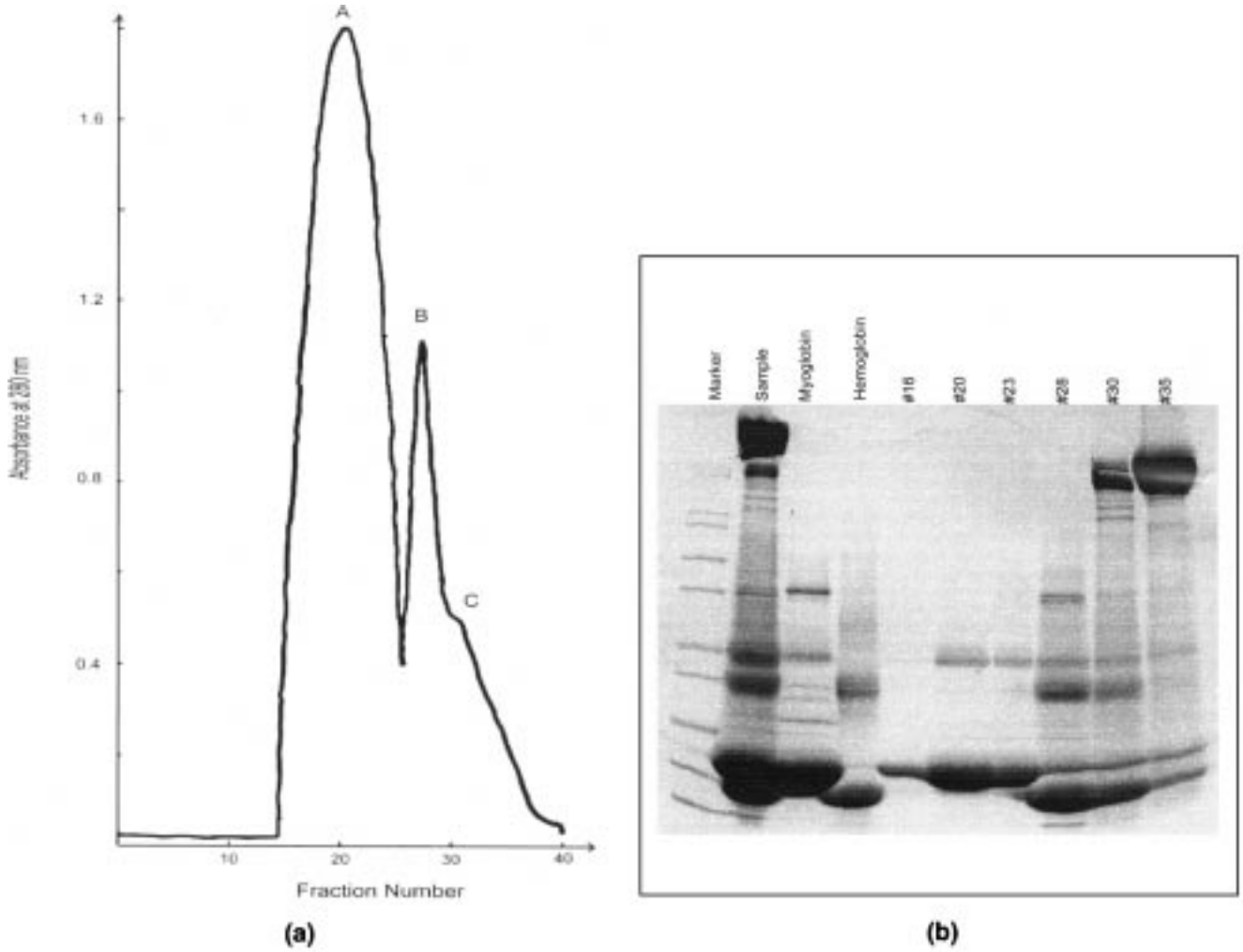

Figure 9. (a) Chromatogram of myoglobin (A), hemoglobin (B), and fibrinogen (C): 10 mg of each protein was dissolved in 1 mL phosphate buffer and injected into the water channel; (b) SDS-PAGE of the fractionated samples.

80% ammonium sulfate solution was fed in the salt channel for 4 h, after which the precipitates were eluted out by feeding water into the salt channel and the centrifugation was stopped.

ammonium sulfate was generally permeated with the cocurrent membrane unit.

As shown in Figure 8b, the ammonium sulfate gradient profiles were formed inside the water channel. These gradients could be used to facilitate the fractionation of proteins by precipitation and dissolution. In fact, the gradient of ammonium sulfate inside the water channel was one of the important factors that determined the retention time and the resolution of proteins in this technique. However, in order to predict the resolution of protein mixtures and the retention time of proteins, the solubility of proteins in ammonium sulfate solution also has to be considered.

Separation of protein mixtures by centrifugal precipitation chromatography

To demonstrate the ability of the technique to separate proteins, a mixture of myoglobin (horse heart), hemoglobin (human), and fibrinogen (bovine plasma) and a mixture of myoglobin (horse heart), human serum albumin (HSA), and bovine γ-globulin were injected into the water channel, while an appropriate degree of saturated ammonium sulfate solution was continuously fed into the salt channel for 4 h to facilitate the precipitation of proteins. After that, the protein precipitates were flushed out. The chromatograms of these separations are shown in Figures 9 and 10. To analyze the fractionated sample qualitatively, the fractionated samples were desalted and subjected to SDS-PAGE. The results of SDS-PAGE are also shown in Figures 9 and 10.

After injecting the mixture of myoglobin, hemoglobin, and fibrinogen into the centrifugal precipitation chromatography, myoglobin was the first protein to elute out, followed by hemoglobin and fibrinogen, as indicated in Figure 9a. The elution sequence of these three proteins from centrifugal precipitation chromatography are in agreement with the solubility properties of these proteins reported by Cohn and Edsall (1943), in which myoglobin is the most soluble protein in an ammonium sulfate solution, while fibrinogen is the least soluble protein in that solution. The same agreement is also

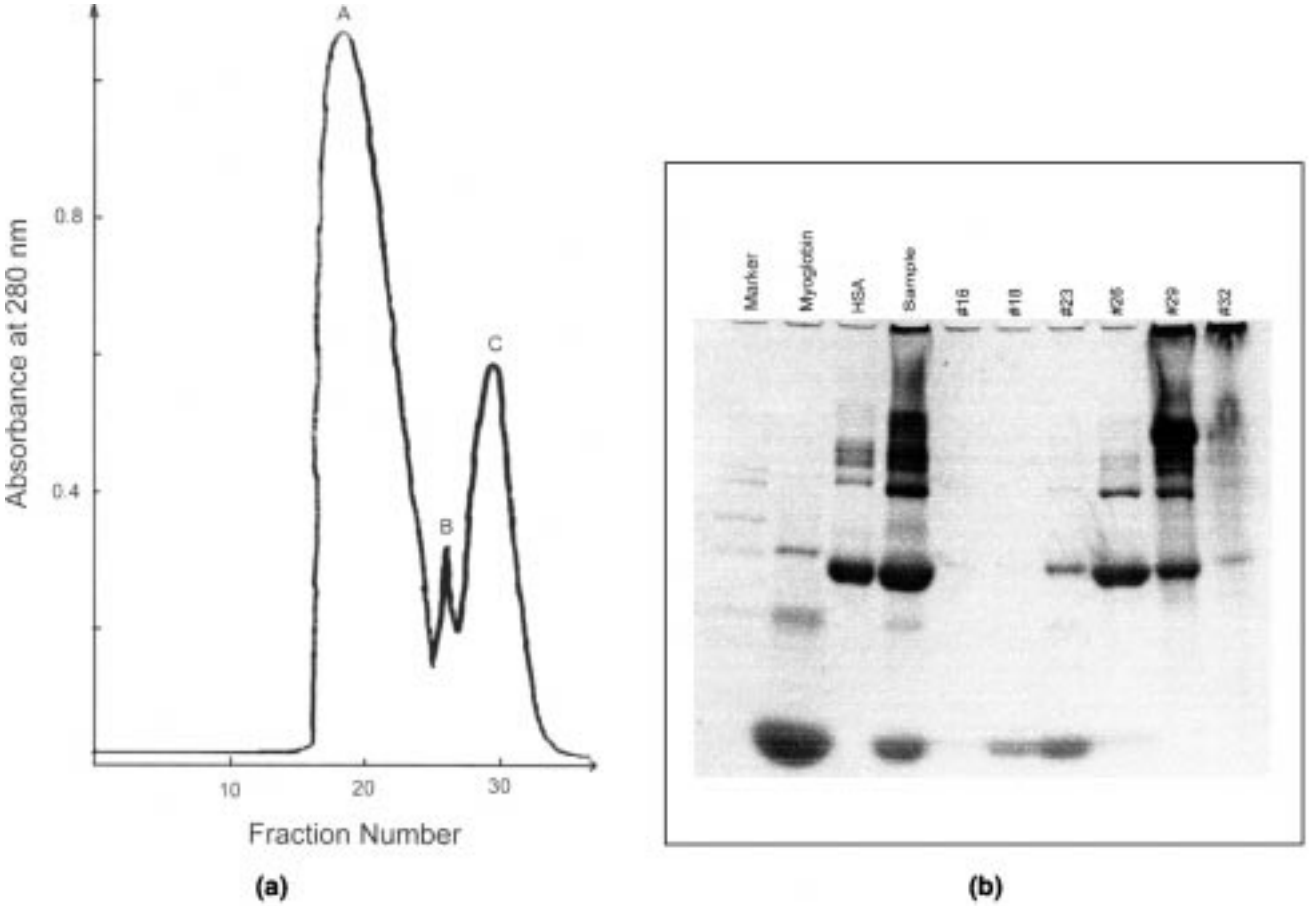

Figure 10. (a) Chromatogram of myoglobin (A), human serum albumin (B), and γ-globulin (C); (b) SDS-PAGE of the fractionated samples.

5 mg of each protein was dissolved in 1 mL phosphate buffer and injected into the water channel; 75% ammonium sulfate solution was fed in the salt channel for 4 h, after which the precipitates were eluted out by feeding water into the salt channel and stopping the centrifugation.

held for the mixture of myoglobin, hemoglobin, and γ-globulin.

As seen in Figures 9 and 10, the partial resolution of proteins was generally achieved. The partial resolution of proteins indicated that the ammonium sulfate gradient existed inside the water channel. Moreover, we observed that the resolution of human serum albumin (HSA) and γ-globulin depended on the ammonium sulfate gradient in the water channel. Consequently, by utilizing the optimum ammonium sulfate gradient inside the water channel, a better resolution of these mixtures of protein should be possible. For example, in order to obtain a better resolution of the myoglobin, human serum albumin, and γ-globulin mixture, a gradual reduction of the ammonium sulfate solution at the inlet of the salt channel should be pursued, instead of a step concentration switch of the inlet ammonium sulfate solution.

Conclusions

In this article, a new technique utilizing the mass transfer of ammonium sulfate across a membrane to establish the gradient profile of ammonium sulfate in the water channel and to facilitate the separation of protein by ammonium sulfate precipitation as proposed by Ito (1999) was discussed. We have solved the mathematical model to explain the ammonium sulfate gradient formation, which is one of the important factors of the technique. From the numerical analysis, the technique should operate well for the fractionation of a mixture of proteins whose solubility in ammonium sulfate solution differs from each other. This article offers the platform for the mathematical model to predict the resolution and the retention of proteins by this technique. A model that can be used to predict the retention time of protein by incorporating the solubility of proteins with the ammonium sulfate gradient profile in the water channel will be presented in the subsequent paper.

To demonstrate the ability of the technique to purify proteins, mixtures of proteins were injected into the water channel. Partial resolutions were obtained when a step concentration switch of inlet ammonium sulfate in the salt channel was applied. From the experimental results, the resolution was a function of the degree of saturation of ammonium sulfate solution fed into the salt channel. Therefore, the resolution could be improved significantly if the gradual decrease of ammonium sulfate concentration at the inlet of the salt channel was applied.

Although most of the discussions in the article were about ammonium sulfate precipitation, the technique should be feasible for differing precipitating agents, such as polyethylene glycol and acid, assuming the precipitating agent could permeate across the membrane. As a result, the separation of proteins using this technique can be applied to the fractionation of other biomolecules by differential precipitation.

Acknowledgment

One of the authors (T. S.) is grateful to Suranaree University of Technology, Thailand, and the Ministry of University Affairs, Royal Thai Government, for providing financial support under the Faculty Development Program during his study at Lehigh University.

Notation

A = parameter correlating Sherwood number with Graetz number
A_{xS}, A_{xW} = cross-sectional flow of the salt and water channel, m^2
D_{sw} = diffusivity of salt in water, $m^2 \cdot s^{-1}$
f = frictional factor
Gz = Graetz number
h_S, h_W = depth of the salt and water channels, m
L = total length of membrane, m
n = parameter correlating Sherwood number to Graetz number
P_f = wet perimeter, m
P_S, P_W = pressure in the salt and water channel, Pa
t = time, s
v_S, v_W = fluid velocity in the salt and water channel, $m \cdot s^{-1}$
w_m = membrane width, m
z = distance from the inlet, m
μ_S, μ_W = viscosity of fluid in the salt and water channels, $kg \cdot m^{-1} \cdot s^{-1}$
ρ_m = average density of fluid inside the membrane, $kg \cdot m^{-3}$
ρ_S, ρ_W = density of fluid in the salt and water channels, $kg \cdot m^{-3}$
Π_S, Π_W = osmotic pressure in the salt and water channel, Pa

Literature Cited

Bailey, J. E., and D. F. Ollis, *Biochemical Engineering Fundamental*, McGraw-Hill, Singapore (1986).

Bell, D. J., M. Hoare, and P. Dunnill, ''The Formation of Protein Precipitates and Their Centrifugal Recovery,'' *Advances in Biochemical Engineering/Biotechnology*, Vol. 26, A. Fiechter, ed., Springer-Verlag, New York (1983).

Bird, R., W. E. Stewart, and E. N. Lightfoot, *Transport Phenomena*, Wiley, Singapore (1960).

Bruin, H. R., ''Coefficients of Diffusion in Liquids,'' *International Critical Tables*, E. W. Washburn, C. J. West, and C. Hull, eds., National Research Council, New York, p. 63 (1929).

Cohn, E. J., and J. T. Edsall, *Proteins, Amino Acids and Peptides*, Reinhold, New York (1943).

Englard, S., and S. Seifter, ''Precipitation Techniques,'' *Methods in Enzymology*, Vol. 182, M. P. Deutscher, ed., Academic Press, San Diego (1990).

Foster, P. R., P. Dunnill, and M. D. Lilly, ''The Kinetics of Protein Salting-Out Precipitation of Yeast Enzymes by Ammonium Sulfate,'' *Biotech. Bioeng.*, **18**, 545 (1976).

Ito, H., and K. Nanbu, ''Flow in Rotating Straight Pipes of Circular Cross Section,'' *J. Basic Eng.*, **93**, 383 (1971).

Ito, Y., ''Centrifugal Precipitation Chromatography Applied to Fractionation of Proteins with Ammonium Sulfate,'' *J. Liq. Chromatog.* **22**, 2825 (1999).

Ito, Y., J. Saudeau, and R. L. Bowman, ''New Flow-Through Centrifuge without Rotating Seals Applied to Plasmapheresis,'' *Science*, **189**, 999 (1970).

Jaffrin, M. Y., L. Ding, and J. M. Laurent, ''Simultaneous Convective and Diffusive Mass Transfers in a Hemodialyser,'' *J. Biomech. Eng.*, **103**, 261 (1990).

Jakob, M., *Heat Transfer*, Vol. 1, Wiley, New York (1949).

Kessler, S. B., and E. Klein, ''Dialysis,'' *Membrane Handbook*, W. S. W. Ho and K. K. Sirkar, eds., Van Nostrand Reinhold, New York, p. 161 (1992).

King, T. P., ''Separation of Proteins by Ammonium Sulfate Gradient Solubilization,'' *Biochemistry*, **11**, 367 (1972).

Launder, B. E., and D. P. Tselepidakis, ''Application of a New Second-Moment Closure to Turbulent Channel Flow Rotating in Orthogonal Mode,'' *Int. J. Heat Fluid Flow*, **15**, 2 (1994).

Perry, R. H., and D. Green, *Perry's Chemical Engineers' Handbook*, 5th ed., McGraw-Hill, New York (1973).

Porath, J., ''Zone Precipitation,'' *Nature*, **196**, 47 (1962).

Rothstein, F., ''Differential Precipitation of Proteins,'' *Protein Purification Process Engineering*, R. G. Harrison, ed., Dekker, New York, p. 115 (1994).

Scopes, R. K., *Protein Purification: Principle and Practice*, 3rd ed., Springer-Verlag, New York (1994).

Virkar, P. D., M. Hoare, M. Y. Y. Chan, and P. Dunnill, ''Kinetics of the Acid Precipitation of Soya Protein in a Continuous-Flow Tubular Reactor,'' *Biotech. Bioeng.*, **24**, 871 (1982).

Manuscript received June 28, 1999, and revision received Dec. 28, 1999.