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Voltage-controlled separation of proteins by electromobility focusing in a dialysis hollow fiber

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Abstract

Electromobility focusing (EMF) is a relatively new protein separation technique that utilizes an electric field gradient and a hydrodynamic flow. Proteins are focused in order of electrophoretic mobility at points where their electrophoretic migration velocities balance the hydrodynamic flow velocity. Steady state bands are formed along the separation channel when equilibrium is reached. Further separation and detection can be easily achieved by changing the electric field profile. In this paper, we describe an EMF system with on-line UV absorption detection in which the electric field gradient was formed using a dialysis hollow fiber. Protein focusing and preconcentration were performed with this system. Voltage-controlled separation was demonstrated using bovine serum albumin and myoglobin as model proteins. The limitations of the current method are discussed, and possible solutions are proposed.

Keywords: Electromobility focusing; Hollow fibers; Proteins; Albumin; Myoglobin

1. Introduction

Separation of complex protein mixtures is a major challenge in modern bioanalytical chemistry. Currently, the most effective method is two-dimensional gel electrophoresis, in which proteins are separated in one dimension on the basis of p*I* value and in a second dimension based on molecular size [1]. There is a growing interest among researchers to develop new methods for the analysis of complex protein mixtures. Recent noteworthy developments include automated two-dimensional liquid phase separation techniques [2,3], high-resolution capillary isoelectric focusing [4], and packed capillary column liquid chromatography coupled to Fourier transform ion cyclotron resonance mass spectrometry [5].

Electromobility focusing (EMF) is a relatively new technique that is promising for protein separation. EMF is a member of a family of equilibrium gradient methods originally described by Giddings [6]. In an equilibrium gradient method, the net force on each analyte species induced by an external field decreases monotonically along the direction of the force, and more importantly, changes direction at some point in the separation channel. Thus, wherever an analyte species is placed inside the channel, the net force will draw it to its unique equilibrium point where the force is zero. Unlike common separation techniques, steady state bands will be formed when equilibrium is reached. Based on how the force gradient is formed, equilibrium gradient methods can be classified into two categories, i.e. constant field

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and field gradient [7]. In the constant field category, a constant external field combined with a gradient in some property, such as pH or density, exerts a force gradient on the analyte species. This category covers most of the known equilibrium gradient methods, such as density gradient sedimentation [8] and isoelectric focusing [9]. In the field gradient category, the force gradient results directly from a gradient in the external field or combination of fields. Since a field gradient is not always easy to form in the first place, methods in this category have not developed as quickly.

In 1985, O'Farrell introduced a new technique for protein separation which he called counteracting chromatographic electrophoresis [10]. In this technique, proteins were focused at the interface between two different gel filtration media packed into upper and lower halves of an electrochromatography column. Since the gradient was discrete rather than continuous, it was not strictly an equilibrium gradient method. However, it did stimulate some new ideas on how to establish an equilibrium gradient method. In the 1990s, Ivory and co-workers introduced a new equilibrium gradient method for protein separation by using an electric field gradient and a hydrodynamic flow, which they called ''field gradient focusing'' [11–15]. Following the idea behind the name ''isoelectric focusing'' in which analytes are focused according to their isoelectric points, we refer to Ivory's technique as ''electromobility focusing'' or EMF, where analytes are focused according to their electrophoretic mobilities. Unlike methods in the constant field category, EMF uses an electric field gradient (as opposed to isoelectric focusing in which the electric field is constant, or in other words, the gradient is zero). In an EMF method, as proteins move along the separation channel from a low electric field to a higher one, their electrophoretic migration velocities gradually increase. The electrophoretic migration of the proteins is countered by an opposing hydrodynamic flow. Thus, each protein reaches its focusing position along the separation channel where its electrophoretic migration velocity just balances the constant opposing flow velocity. The major concern in EMF is how to form the electric field gradient along the separation channel. Several methods have been explored by Ivory et al. The first method involved the formation of a gradient by changing the cross-sectional area of the separation channel. Although this approach successfully produced a continuous electric field intensity gradient, it showed only mediocre performance [11,12]. In the second method, the gradient was formed by using a dialysis membrane to gradually decrease the buffer conductivity in the separation channel [13]. This system was easy to operate and equilibrium could be reached in less than 10 min when run in free solution. In the third method, the gradient was established and maintained using an array of electrodes for which voltages were individually monitored and adjusted by a computer-controlled circuit board [14,15]. The advantages of this approach were that the electronically-generated field could take on various shapes, including nonlinear profiles and step changes. The work by Ivory et al. primarily involved preparative separations. Focusing was demonstrated with colored proteins, and detection was achieved by taking photographs with a camera. As we show below, EMF offers additional unique potential advantages for analytical separations.

The basic theory of EMF has been given elsewhere [7] and, in brief, some important equations are given below. Assuming that the focused band of protein is narrow compared to the change in the electric field gradient, it is a Gaussian distribution with a standard deviation of:

$$\sigma = \left(\frac{D_T}{b\mu}\right)^{1/2} \tag{1}$$

where D_T is a coefficient that represents the sum of all contributions to effective dispersion of the analyte, b is the negative value of the slope of the electric field and μ is the mobility of the protein. For positively charged proteins ($\mu > 0$), b must be positive, which means that the electric field must decrease along the separation channel; for negatively charged proteins ($\mu < 0$), b must be negative, which means that the electric field must increase along the separation channel. In other words, only proteins with the same charge (positive or negative) can be focused in one run; those with opposite charge cannot remain in the channel. The resolution in EMF can be expressed as:

$$R_s = \frac{|u|}{4}\left(\frac{\bar{\mu}}{bD_T}\right)^{1/2}\frac{\Delta\mu}{\bar{\mu}^2} \tag{2}$$

where u is the hydrodynamic flow-rate. It can be seen that resolution is inversely proportional to the square root of the slope of the electric field, which means that a shallow gradient will increase resolution although bands will become broader.

Compared with other protein separation methods, EMF has some unique advantages. Firstly, injection is not as important since band broadening caused by injection has no influence on the final band profile. Secondly, proteins can be simultaneously concentrated as they are separated. Thirdly, EMF is not affected by problems associated with protein aggregation and precipitation at the pI point, which remains a serious problem in isoelectric focusing.

A unique characteristic of EMF is that the shape of the electric field is easy to change, which makes it easy to change the focusing positions of the proteins. As we demonstrate elsewhere [16], the peak capacity can be greatly improved by utilizing an electric field gradient with a nonlinear shape; and based on mobility difference, selective concentration of trace target proteins can be achieved, while interfering components in high concentrations are rejected.

In comparison, the shape of the gradient cannot be easily changed for the methods in the constant field category such as isoelectric focusing, which makes detection difficult. On-line imaging is an alternative [17], but this makes the system more complicated and more expensive. Another possibility is to move the bands out of the channel using another field or flow, and detecting them at the end of the channel [18–20]. Unfortunately, band broadening usually occurs when using this approach. This is not a problem in EMF; after equilibrium has been reached, by simply decreasing the total voltage drop across the channel, the electric field decreases, and proteins lose their focusing positions one by one and move out of the separation channel in order of electrophoretic mobility.

As demonstrated in this paper, separation of charged analytes such as proteins, can be easily achieved by voltage control in EMF. That is, analytes with high electrophoretic mobilities can be focused and retained in the channel while those with low mobilities can be eluted by simply decreasing the voltage drop across the EMF channel. An EMF method with on-line UV absorption detection in which the electric field gradient is formed by using a dialysis hollow fiber is described. Voltage-controlled separation is demonstrated using bovine serum albumin and myoglobin as model proteins. The limitations of the current method are discussed, and possible solutions are proposed.

2. Experimental

2.1. Chemicals and materials

The hollow dialysis fiber was a modified cellulose fiber from Membrana (Wuppertal, Germany) with an internal diameter of 200 μm and a dry wall thickness of 8 μm. The molecular mass cut-off (MWCO) of this fiber was 10,000. Untreated fused-silica capillary tubing (250 μm I.D.×365 μm O.D., and 535 μm I.D.×693 μm O.D.) were purchased from Polymicro Technologies (Phoenix, AZ, USA). Model proteins, bovine serum albumin (BSA) and myoglobin from horse heart (Mb), were obtained from Sigma (St. Louis, MO, USA). The buffer solutions were prepared with deionized water from a Millipore water purifier, filtered through a 0.22 μm filter, and degassed with an ultrasonic vibrator before use.

2.2. Instrumentation

A schematic diagram of the dialysis hollow fiber-based system is shown in Fig. 1. A 6-cm-long dialysis hollow fiber was glued to 15-cm lengths of fused-silica capillary (250 μm I.D.×365 μm O.D.) at each end using epoxy. The fiber was then inserted coaxially inside another fused-silica capillary (535 μm I.D.×693 μm O.D.) tubing, and assembled with other parts of the system using two low pressure crosses and standard low pressure fittings from Upchurch (Oak Harbor, WA, USA). A syringe pump (Model PHD2000, Harvard Apparatus, Holliston, MA, USA), combined with a 250-μl gas tight syringe (Hamilton, Reno, NV, USA) was used to deliver the sample and high concentration buffer into the fiber. Another syringe pump (Model 55-1199, Harvard Apparatus), combined with a 60-ml plastic syringe (Becton Dickinson, Franklin Lakes, NJ, USA), was used to deliver the low concentration buffer through the outside fused-silica capillary tubing. The high voltage source was from Spellman

(Model CZE 1000R, 30 kV, 300 μA, Hauppauge, NY, USA). The distance between the two electrodes was ~12 cm, and the polarity was as shown in Fig. 1. Sample injection was achieved by using a 1-μl volume rotary injection valve from VICI (Houston, TX, USA), and on-line detection was achieved at the end of the fiber using a UV–Vis absorption detector with fiber optics detection accessory from ThermoQuest (Model UV3000, Riviera Beach, FL, USA).

2.3. Focusing procedure

The high concentration buffer inside the dialysis hollow fiber was 100 m*M* Tris–HCl, pH 8.7, and the low concentration buffer was 1 m*M* Tris–HCl, pH 8.7. The inside fiber flow-rate was 0.5 μl/min, which corresponds to a linear velocity of 2.65×10^{-2} cm/s, and the outside flow-rate was 200 μl/min. For the protein focusing and preconcentration experiments described in this study, a 0.1 mg/ml solution of BSA in 100 m*M* Tris–HCl was injected, a voltage of 5 kV was applied across the fiber, and at 61 min, the voltage was decreased to 3 kV. For voltage-controlled separation, a mixture of 0.5 mg/ml Mb and 0.5 mg/ml BSA in 100 m*M* Tris–HCl buffer, pH 8.7, was injected. A voltage of 8 kV was first applied across the fiber. At 61 min, the voltage was decreased to 5 kV, and at 90 min, the voltage was decreased to 3 kV. Detection was performed at 214 nm.

3. Results and discussion

3.1. Formation of the electric field gradient

In the dialysis hollow fiber-based EMF system shown in Fig. 1, a high concentration buffer passes through the inside dialysis fiber, and the same buffer, at a lower concentration, passes through the outside capillary. Since there is a concentration difference between the two buffers, the buffer ions inside the inner fiber diffuse across the membrane to the outside capillary tubing. A concentration and, thus, conductivity gradient is formed along the length of the fiber. When a high voltage is applied, an electric field gradient is formed. Greenlee and Ivory [13] gave an equation to simulate the electric field profile

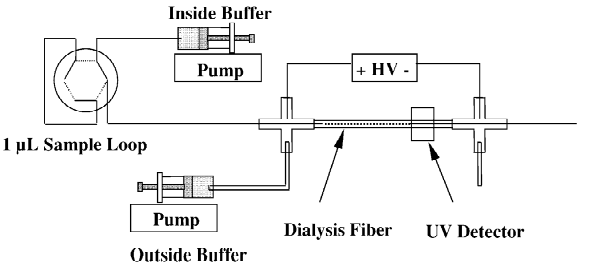

Fig. 1. Schematic diagram of the dialysis hollow fiber-based EFGF system.

for a dialysis membrane-based EMF system. With some modification, this equation can be used to simulate the electric field profile in a dialysis hollow fiber-based EMF system:

$$E = \frac{I_T + \pi r^2 F z_+ (D_+ - D_-)\lambda_1 (c^0_{+,1} - c_{+,2}) \exp(\lambda_1 x)}{\pi r^2 F z_+ (\mu_+ - \mu_-)\left[(c^0_{+,1} - c_{+,2})\exp(\lambda_1 x) + c_{+,2}\right] + \pi\left[R^2 - (r+\delta)^2\right] F z_+ (\mu_+ - \mu_-) c_{+,2}} \quad (3)$$

where I_T is the total current; z_i, D_i, μ_i and c_i are the charge, diffusivity, mobility and concentration of the buffer ions, i, respectively; R and r are the internal radii of the outside capillary and the fiber, respectively; δ is the fiber wall thickness; λ_1 is a parameter describing the concentration gradient inside the fiber, and F is the Faraday constant.

Using the operating parameters given in Section 2 and parameters for the buffer ions from the paper by Greenlee and Ivory [13], a simulated electric field profile along the fiber at a voltage of ~8 kV was determined as shown in Fig. 2. The electric field is very steep in the beginning of the fiber, increasing from 45.56 V/cm at 0 cm (the start of the fiber) to 712.49 V/cm at 0.5 cm, which corresponds to an average slope of 1334 V/cm^2. However, the field along the remainder of the fiber is very shallow, increasing from 712.49 V/cm at 0.5 cm to 712.59 V/cm at 6 cm (the end of the fiber), which corresponds to an average slope of 0.02 V/cm^2. Such an electric field profile is mainly due to the fast diffusion rate of the buffer ions across the wall of the dialysis hollow fiber. The fiber used in this study is a commercially available fiber used for hemodialysis. It has a relatively large inner diameter, a large MWCO and a very thin wall, which leads to a very

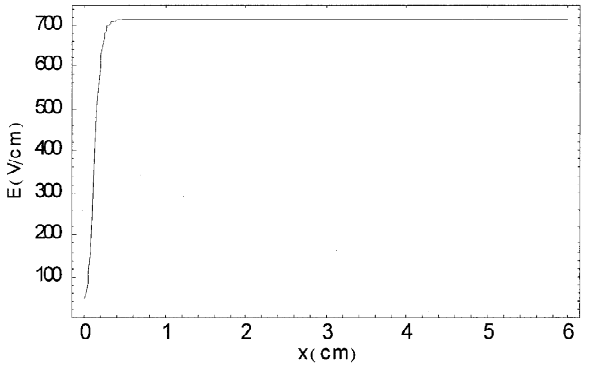

Fig. 2. Simulation results of the electric field profile along the dialysis hollow fiber.

high diffusion rate across the fiber. Further theoretical calculations using Eq. (3) show that the pore size in the wall of the dialysis hollow fiber has a great influence on the diffusion rate across the fiber.

3.2. Protein focusing

Fig. 3 shows how a protein can be focused in the dialysis hollow fiber. Without applying voltage to the fiber, BSA was carried through the fiber by hydrodynamic flow. A broad peak eluted from the fiber at about 35 min after 1 μl of 0.1 mg/ml BSA was injected (bottom trace). When a voltage of 5 kV was applied and all other conditions were kept the same, the protein did not elute from the fiber even after 61 min. The protein was negatively charged at pH 8.7, and the electrophoretic force drew the protein toward the beginning of the fiber while hydrodynamic flow pushed it toward the end of the fiber. In this case, the electrophoretic migration velocity balanced the hydrodynamic flow velocity and the protein was focused inside the fiber. When the voltage was decreased to 3 kV, the electric field decreased and the electrophoretic migration velocity could not balance the hydrodynamic flow velocity. Thus, the protein lost its focusing position and eluted from the fiber. The eluted protein peak had an S/N ratio of over 200, which indicates that a protein sample with much lower concentration can be detected after focusing. Some tailing was observed, which was probably due to adsorption of protein on the untreated fused-silica capillary wall. It should be mentioned that although the peak eluted at about 61 min, it did not take this long to focus the protein. Earlier experiments with colored proteins showed that proteins could be focused in ~10 min after they were introduced into the fiber. The relatively long focusing time in Fig. 3 was chosen to demonstrate that the protein could be retained in the fiber as long as needed before the voltage was decreased.

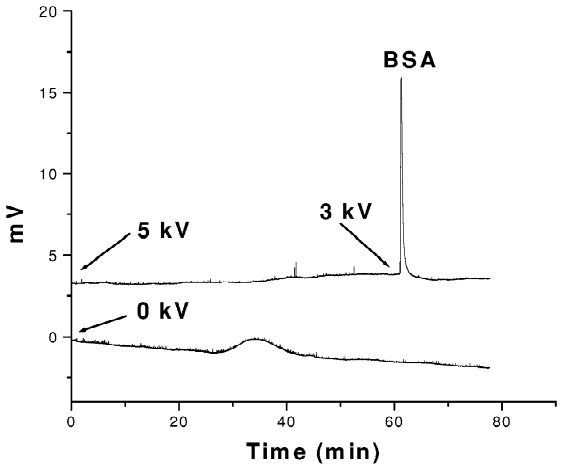

Fig. 3. Protein focusing using the dialysis hollow fiber-based EFGF system. Conditions are listed in Section 2.

3.3. Preconcentration

Simultaneous preconcentration during separation is an advantage of equilibrium gradient methods. The hydrodynamic counter flow in EMF makes injection easy and straightforward. A large volume sample can be injected into the system by either a large volume sample loop or just simply by multiple injections. For methods in the constant field category such as isoelectric focusing, the maximum injection volume is the volume of the separation channel. However, in EMF, the injection volume can be as large as desired. Fig. 4 shows the preconcentrating power of the dialysis hollow-fiber based EMF system. Five consecutive injections of the same sample increased the peak area approximately five times compared with one injection. Five consecutive injections also gave a similar peak width compared with one injection, which indicates that multiple injections did not cause a loss in efficiency. The upper limit of

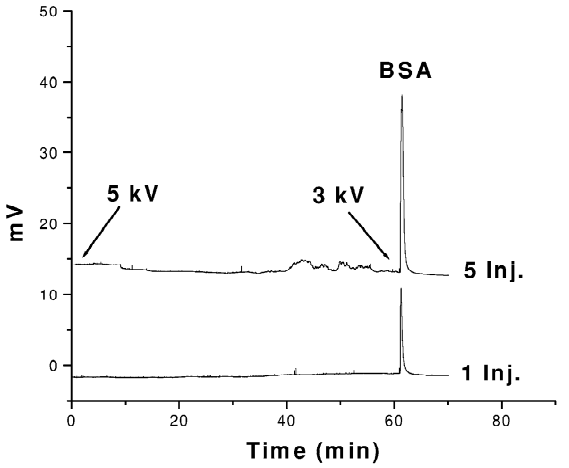

Fig. 4. Preconcentration by multi-injection using the dialysis hollow fiber-based EFGF system. Conditions are listed in Section 2.

preconcentration in EMF is probably only limited by the solubility of the protein.

3.4. Voltage-controlled separation

After equilibrium is reached in an EMF method, proteins stay focused inside the channel in order of electrophoretic mobility. Proteins with low mobilities focus in the high electric field region, while proteins with high mobilities stay in the low electric field region. If the total voltage drop along the separation channel is decreased, the electric field will also decrease, and the equilibrium will be changed. All of the focused proteins will move to new equilibrium points closer to the end of the channel. Therefore, proteins with the lowest mobilities will sequentially move out of the channel for detection, while other proteins will move toward the end of the channel and be refocused. If the voltage continues to decrease, proteins will move out of the channel one by one in order of mobility. If the voltage is kept constant after the first protein elutes, all other proteins will stay focused in the channel without eluting. In this way, separations can be easily controlled by voltage.

Greenlee and Ivory [13] gave an equation to simulate the concentration profile of focused proteins in the dialysis membrane-based EMF system, and the same equation can be used in the dialysis hollow fiber system:

$$c_p(x) = \frac{M_{pT}}{\pi r^2 \int_0^L \exp\left[\int_0^x \frac{u + \mu_p E(x')}{D_T}\,dx'\right] dx \times \exp\left[\int_0^x \frac{(u + \mu_p E(x')}{D_T}\,dx'\right]} \quad (4)$$

Various properties of the model proteins are listed in Table 1. The mobility of Mb was determined by capillary electrophoresis in our laboratory, the mobility and the diffusivity of BSA were taken from the literature [13], and all other parameters were taken from the CRC Handbook of Biochemistry [21].

Using parameters for model proteins listed in Table 1 and the operating parameters given in Section 2, simulation results for voltage-controlled separation are shown in Fig. 5. When the voltage was ~8 kV (Fig. 5A), both BSA and Mb were retained in the first part of the fiber. However, since BSA has a larger electrophoretic mobility than Mb, the focusing position of BSA was closer to the beginning of the fiber than that of Mb. Therefore, BSA and Mb were stored in order of mobility, although they were not totally resolved. When the voltage was decreased to ~5 kV (Fig. 5B), the electrophoretic migration velocity of Mb could not balance the hydrodynamic flow. Therefore Mb lost its focusing position and

Table 1
Properties of the model proteins

	Bovine serum albumin	Myoglobin, horse heart
Molecular mass	67,000	16,890
Isoelectric point	4.9	7.3
Mobility at pH 8.7 ($cm^2\ V^{-1}\ s^{-1}$)	-1.138×10^{-4}	-8.04×10^{-5}
Diffusivity (cm^2/s)	6.1×10^{-7}	1.13×10^{-6}

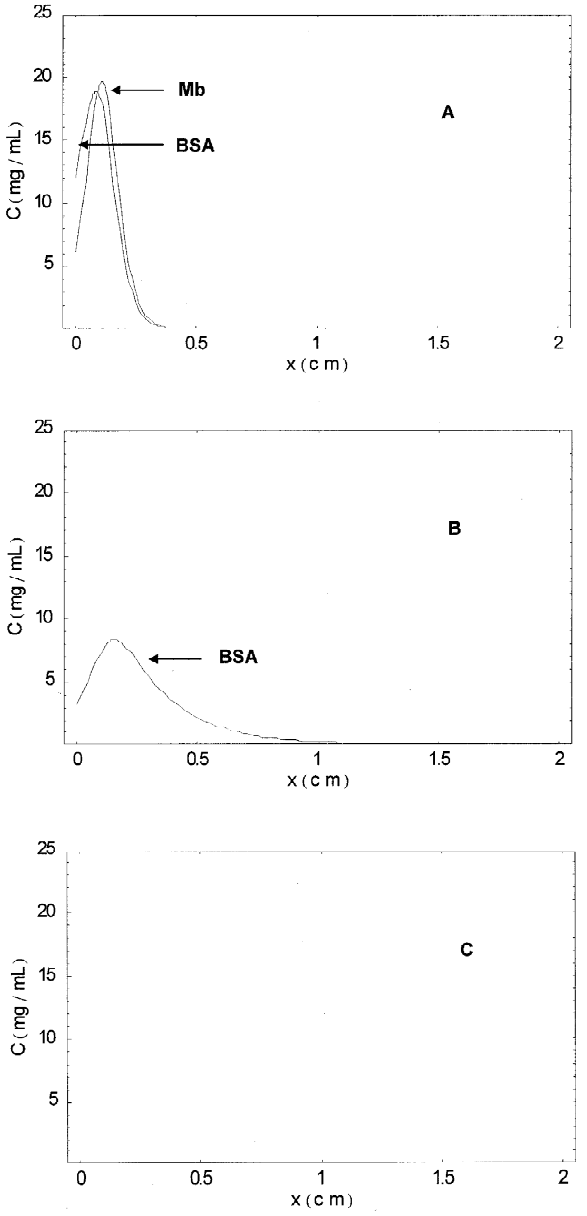

Fig. 5. Simulation results of voltage-controlled separation of BSA and Mb. (A) 8 kV, (B) 5 kV, and (C) 3 kV.

eluted from the fiber. At the same time, BSA remained focused inside the fiber, however, its focusing position shifted slightly toward the end of the fiber, and the band became broader due to a reduced electric field. It can also be seen that the BSA band became asymmetric because the band shifted partly into the shallow electric field region. Finally, when the voltage was decreased to about 3 kV (Fig. 5C), BSA also lost its focusing position and eluted from the fiber.

Fig. 6 shows experimental results of voltage-controlled separation of BSA and Mb. Initially, 8 kV was applied along the fiber. At this voltage, no bands eluted, which indicated that both BSA and Mb were retained in the fiber. When the voltage was decreased to 5 kV, Mb could no longer be focused in the fiber due to its low mobility, and it was detected by the UV absorption detector. However, at this voltage, BSA was still focused inside the fiber. Finally, when the voltage was decreased to 3 kV, BSA also lost its focusing position and eluted. These results clearly show that protein separation can be easily achieved by voltage control in a dialysis hollow fiber-based EMF system.

Compared with other equilibrium gradient methods such as capillary isoelectric focusing, relatively broad peaks were observed in the dialysis hollow fiber-based EMF experiments described in this paper. As is known from Eq. (1), the standard deviation of the peak width in EMF is proportional to the square root of the total dispersion, D_T, and inversely proportional to the square root of the slope of the electric field. As we showed earlier, the slope of the electric field went almost to zero at the end of the fiber, which led to a relaxation of the self-focusing

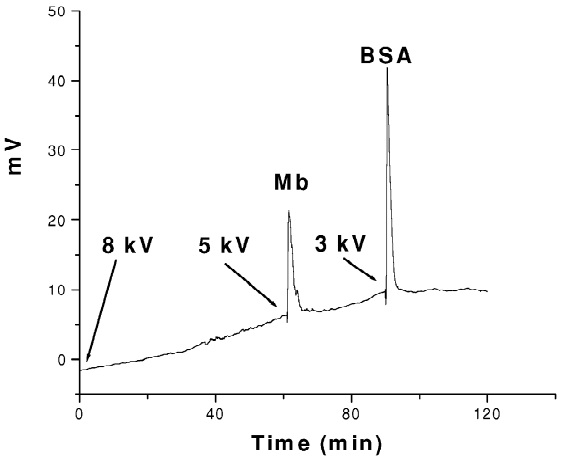

Fig. 6. Voltage-controlled elution of BSA and Mb. Conditions are listed in Section 2.

forces as the proteins moved to the detector. Furthermore, the dispersion caused by hydrodynamic flow also increased the total band width beyond what would be expected from molecular diffusion alone. In the dialysis hollow fiber system, the total dispersion can be expressed as [22]:

$$D_T = D + \frac{u^2 r^2}{48D} \quad (5)$$

It can be seen from Eq. (5) that the total dispersion increases at a rate proportional to the product of the hydrodynamic flow and the fiber radius. Under the operating conditions listed in Section 2, the total dispersion for BSA is almost 4000 times larger than the diffusivity of BSA. The very shallow electric field gradient and flow-generated dispersion limited the performance of the current dialysis hollow fiber-based EMF method. However, there are several possibilities for improving the performance of the current system. A ''poor'' dialysis fiber with smaller pore size and thicker wall should improve the shape of the electric field profile for protein focusing. The flow-generated dispersion can be reduced by using a smaller diameter fiber and by packing the fiber with chromatographic particles or hydrophilic monolithic material.

4. Conclusions

EMF is a relatively new technique for protein separation that belongs to the field gradient category of equilibrium gradient methods. Steady state bands are formed along the separation channel in the order of electrophoretic mobility. Simultaneous preconcentration can be achieved during separation. After equilibrium is reached, further separation and detection can be easily achieved by changing the profile of the electric field gradient.

An EMF system with on-line UV absorption detection was constructed in which the electric field gradient was formed by diffusion of buffer ions across the wall of a dialysis hollow fiber. Due to the properties of the fiber, a steep electric field gradient was formed only in a narrow part of the beginning of the fiber, while the gradient in the rest of the fiber was almost flat. Protein focusing was performed with the fiber EMF system, and preconcentration after multiple injections was demonstrated. In an EMF method, proteins can be separated simply by changing the total voltage drop, and this was demonstrated for the first time using a dialysis hollow fiber-based EMF system with BSA and Mb as model proteins. Due to the very shallow gradient in most of the fiber and the large dispersion from hydrodynamic flow, relatively broad peaks were observed compared with other equilibrium gradient methods such as isoelectric focusing. These problems hopefully can be solved by packing an optimized dialysis hollow fiber with some chromatographic particles or hydrophilic monolithic material.

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