Separation Science and Technology
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713708471

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To cite this Article Liu, Zheng , Huang, Zheng , Chong, Jinyang , Yang, Huan , Ding, Fuxin and Yuan, Naiju(1996) 'Continuous Separation of Proteins by Multichannel Flow Electrophoresis', Separation Science and Technology, 31: 10, 1426 — 1442
To link to this Article: DOI: 10.1080/01496399608001405
URL: http://dx.doi.org/10.1080/01496399608001405

SEPARATION SCIENCE AND TECHNOLOGY, **31**(10), pp. 1427–1442, 1996

Continuous Separation of Proteins by Multichannel Flow Electrophoresis

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ABSTRACT

A new preparative electrophoresis method, multichannel flow electrophoresis (MFE), is proposed in this paper. Mechanisms for different operation modes of MFE are described and a simulation of continuous MFE is proposed. A membrane spaced 5-compartment electrolyzer is developed in which the central compartment and its neighboring compartments are respectively used for sample loading and product discharging. The mass transfer behavior of MFE is investigated by measuring the equivalent migration speed and mobility of bovine serum albumin (BSA) and hemoglobin bovine blood (HBB) at different buffering pH, electric field strength, and sample loading flow rate. The continuous separation of a mixture composed of BSA and HBB, buffered with 0.01 M Tris–HAc at pH 5.9, yields 13.2 mg BSA and 20.0 mg HBB per hour. The application of MFE is exemplified through purification of anti-urokinase mouse IgG. The preliminary results confirm the workability of MFE and point out its promising application potentials in the downstream process of biochemical engineering.

Key Words. Electrophoresis; Multichannel flow electrophoresis; Multicompartment electrolyzer

INTRODUCTION

The excellent performance of electrophoresis techniques used for analytical purposes appears two potential unique advantages of large-scale

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electrophoresis techniques for industrial separation and purification of biomolecules. One is its good versatility because proteins, enzymes and other biomolecules are easily charged by suitable choice of pH. Another is its high productivity since the rate of mass transfer, the decisive factor of productivity of conventional separation processes, can be highly accelerated by electric force. However, the development of large-scale electrophoresis techniques is hindered by the difficulties in Joule heat dissipation, maintaining the steady flow status and pH gradient as well as preventing the mixing of the products. Focused on these problems, recent studies have proposed some new concepts and procedures, such as dividing the outgoing product streams smoothly by a splitter set at the outlet of the thin and ribbon-like separation channel described in *electric split-flow fractionation* (1, 2), recycling the mobile phase to heat exchanger during electrophoresis shown in *recycle isoelectric focusing* (3), combining size elution chromatography with electrophoresis presented in *counteracting chromatographic electrophoresis* (4), as well as generating pH gradient for isoelectric focusing of a mixture by whose components that have different isoelectric points pioneered by *autofocusing* (5). These works have pro-

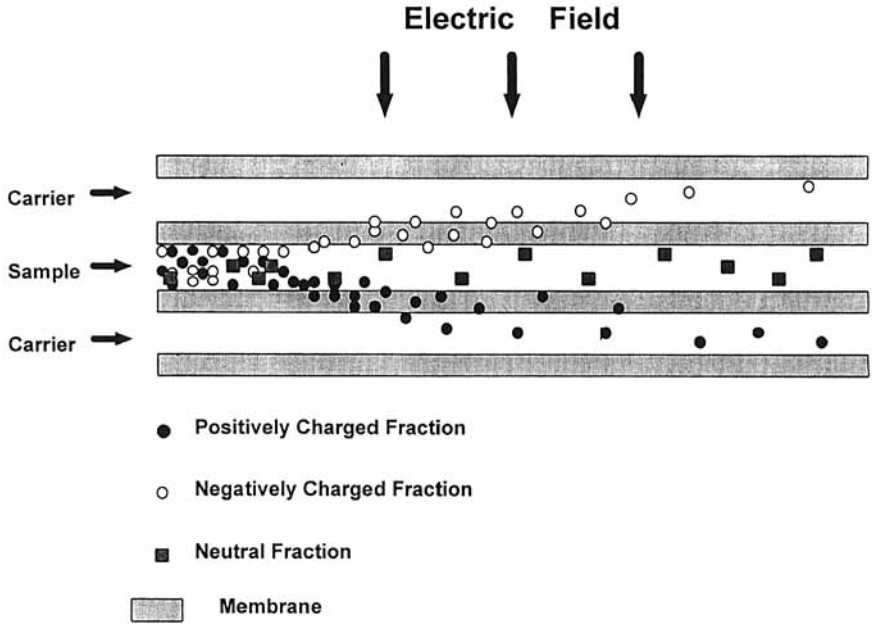

FIG. 1(a) Side view of the separation process of continuous MFE.

vided fresh perspectives of developing large-scale electrophoresis techniques.

In this paper we present a new preparative electrophoresis method, named continuous flow electrophoresis (MFE). MFE is conducted in an adiabatic electrolyzer partitioned into five compartments by gel membranes in which the central and its neighboring compartments are respectively used for sample loading and products discharging. An electric field is applied perpendicular to the flow of protein solution to move charged components from the central compartment into the neighboring compartments. As shown in Fig. 1, separation by MFE can be operated either in a continuous mode or in a dynamic mode.

The continuous mode of MFE is illustrated in Fig. 1(a). During a run the mixture is continuously introduced, each charged component is transmitted to its corresponding elution compartment according to its electric

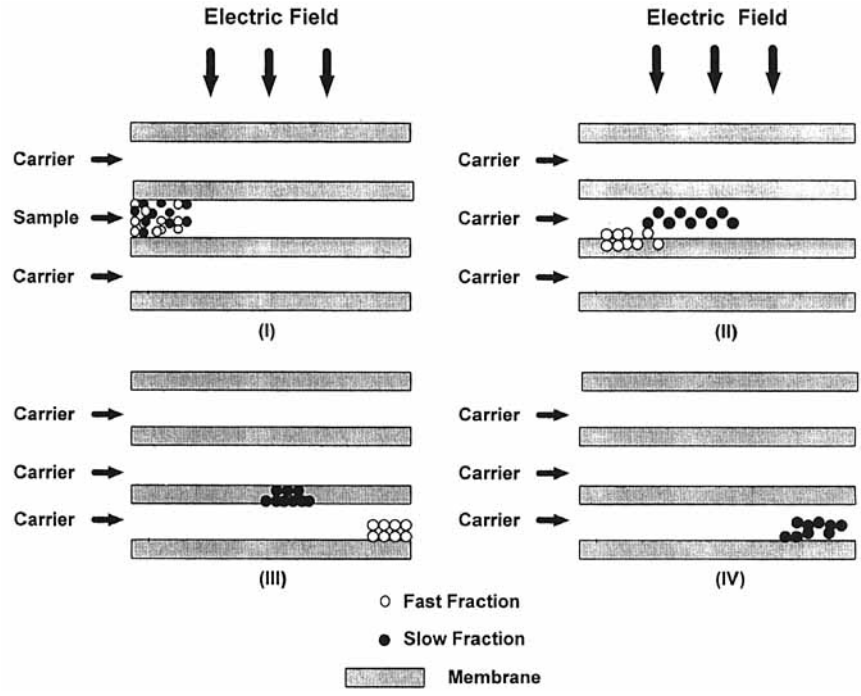

FIG. 1(b) Side view of the separation process of dynamic MFE. (I) Sample injection. (II) Electrophoretic migration. The fast fraction is moving across the membrane. (III) The fast fraction is being washed out of elution compartment while the slow faction is moving across the membrane. (IV) The slow fraction is being carried out of the elution compartment.

charge, and washed out subsequently by carried flow while the neutral component is carried through the central compartment. The separated component is then collected at the outlet of its corresponding compartment.

Various separation strategies are available for MFE through different choices of the buffering pH. For example, MFE could be buffered at a pH that lies between the isoelectric points of two components for recovery of them simultaneously. In this case the separation strategy of MFE is similar to that of *electric split-flow fractionation* proposed by Giddings's group (1, 2). Analogous to *preparative isoelectric point electrophoresis* (6) developed in Yuan's group, MFE could also be carried out at the isoelectric point of the target component to remove the charged impurities out of the central compartment. One unique advantage of MFE is employing an elution compartment for product discharge which makes it more convenient to maintain a steady flow status inside the central compartment.

The dynamic mode of MFE is shown in Fig. 1(b). The sample is injected into the central compartment. Each charged component is transmitted to its respective elution compartment at a specific speed determined by its charge, molecular weight, structure, and electric field strength as well as the gel friction force. Subsequently, different components are washed out by a carrier and collected at the outlet of the elution compartment in an order based on their specific migration speeds. We previously named this separation process *membranous chromatographic electrophoresis* (7), and it is suitable for the separation of a mixture of proteins which have close isoelectric points and distinct molecular weights.

For this paper a small multicompartment electrolyzer for MFE was developed. A one-dimensional simulation model was proposed to describe protein migration in MFE. The equivalent migration speeds and electrophoretic mobilities of BSA and HBB were measured at different buffering pHs, electric field strengths, and sample-introducing flow rates in order to understand the mass transfer characteristics of MFE. In order to demonstrate its workability, the separation of BSA and HBB by MFE was conducted at pH 5.9 to recover BSA and HBB simultaneously. The application of MFE to the separation of complex protein mixtures was exemplified through purification of anti-urokinase mouse IgG from mouse serum.

ONE-DIMENSIONAL SIMULATION OF CONTINUOUS MFE

In the separation process of continuous MFE, the concentration of charged protein changes along the flow axis inside the central compartment due to its removal by electrostatic force. Meanwhile, similar to con-

ventional membrane filtration processes, concentration polarization occurs on the surface of the gel membrane. Consequently, a two-dimensional distribution of protein concentration is generated. In order to simplify the mathematical simulation model while retaining its reliability, we introduce two concepts to describe the migration of protein. One is *equivalent migration speed*, which represents the net transmitting speed of a charged protein from the central compartment to the elution compartment. Another is *equivalent electrophoretic mobility*, defined as the migration speed obtained at a given electric field strength. Here the electric field is regarded as homogeneous because the gel membrane we used has negligible electroconductive resistance when it is placed in the buffer solution. Based on these concepts, a one-dimensional simulation model was developed.

If we assume that the velocity profile is plug flow, a steady-state conservation equation is thereby applied to a thin element of the central compartment taken perpendicular to its flow axis. By considering an arbitrary concentration $C(x)$ of the protein at x, as illustrated in Fig. 2, the mass balance on this element is

$$\frac{dV}{dt}[C(x + dx) - C(x)] = -U \cdot C(x) \cdot H \cdot dx + D \cdot \frac{C(x + dx) - C(x)}{dx} H \cdot W \tag{1}$$

$$dV = dx \cdot H \cdot W \tag{2}$$

$$dt = dx \cdot H \cdot W / \upsilon \tag{3}$$

Here U is the equivalent electrophoretic migration speed of the protein in terms of per unit distance per time. υ is the flow rate of the fluid in

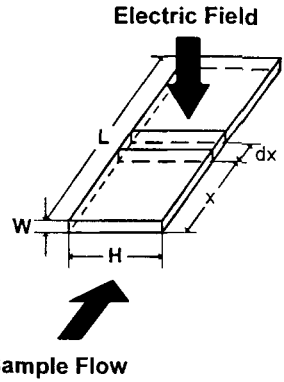

FIG. 2 Mass transfer in the thin element taken in the central compartment.

terms of per volume per time, D is the molecular diffusion coefficient of protein in solution, and L, H, and W stand for the length, height, and width of the central compartment, respectively.

Compared with mass transfer contributed by fluid flow, the mass diffusion item can be neglected. Introducing Eqs. (2) and (3) into Eq. (1) and rearranging Eq. (1) yields

$$\frac{dC(x)}{C(x)} = -\frac{U \cdot H}{\upsilon} \cdot dx \tag{4}$$

Let $C(x)$ be $C(0)$ at $X = 0$, then the final form of $C(x)$ is

$$C(x) = C(0) \exp\left(-\frac{U \cdot H}{\upsilon} x\right) \tag{5}$$

Therefore the protein concentration at the outlet of the central compartment is

$$C(L) = C(0) \exp\left(-\frac{U \cdot H \cdot L}{\upsilon}\right) \tag{6}$$

Introducing $U \cdot H \cdot L/\upsilon = U \cdot \tau / W$ into Eq. (6), where τ stands for the residence time of fluid inside the central compartment, yields

$$C(L) = C(0) \exp\left(-\frac{U \cdot \tau}{W}\right) \tag{7}$$

Recalling $\mu = U/E$ and introducing it into Eq. (7) yield

$$C(L) = C(0) \exp\left(-\frac{\mu \cdot E}{W/\tau}\right) \tag{8}$$

Repeating the separation procedure to the sample solution for n times, the final concentration of charged protein in the sample solution is

$$C(L, n) = C(0) \exp\left[n\left(-\frac{\mu \cdot E}{W/\tau}\right)\right] \tag{9}$$

Equation (9) indicates alternative ways of optimizing the performance of continuous MFE. Among the factors included in Eq. (9), μ is intrinsically responsible for the separation speed and resolution of MFE. Determination of μ and U is thus the first step in optimizating MFE.

By measuring the protein concentration at the inlet and outlet of the central compartment, the equivalent migration speed U and the mobility μ are calculated respectively from

$$U = \ln\left(\frac{C(0)}{C(L)}\right)\cdot\frac{W}{\tau} \tag{10}$$

$$\mu = \frac{1}{E}\ln\left(\frac{C(0)}{C(L)}\right)\cdot\frac{W}{\tau} \tag{11}$$

EXPERIMENTAL

Apparatus of MFE

The separation apparatus of MFE is shown schematically in Fig. 3. The heart of this apparatus is the 5-compartment electrolyzer partitioned by gel membranes, which was synthesized according to Ref. 8. Each compartment is individually connected with a pump (made by Qingyun Machinery Factory, Beijing). The length and width of each compartment is 8 and 0.6 cm, respectively. The depths of the electrode, elution, and central compartment are 0.2, 0.2 and 0.05 cm, respectively. The distance between the two electrode is about 1.10 cm.

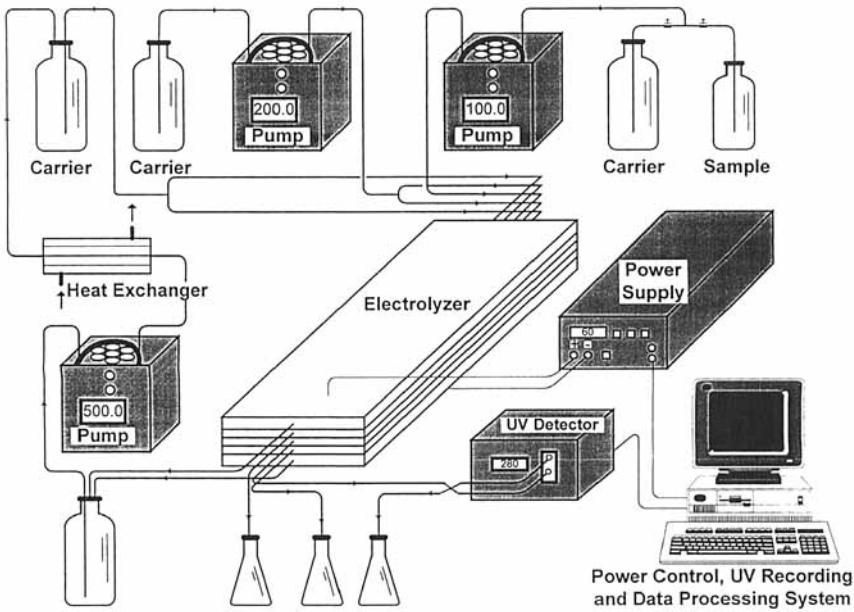

FIG. 3 Experimental system for multichannel flow electrophoresis (MFE).

Materials

The chemicals used in this study were bovine serum albumin (Sigma), hemoglobin bovine blood (Shanghai Dongfeng Bioreagent Company), Tris (Beijing Lanli Fine Chemical Company), and acetate acid (Beijing Chemical Factory). Monoclonal anti-urokinase mouse IgG was produced through a hybridoma cell line described in Ref. 9.

Determination of the Purity and Amount of Products

The purity of the materials and the products obtained after MFE were analyzed by SDS-PAGE assay, in which the concentration of polyacrylamide was 12.5% (w/v) in the case of BSA and HBB or 15% (w/v) in the case of anti-urokinase mouse IgG. The concentrations of BSA and HBB were determined from their own calibration curve established at 280 and 420 nm, respectively. The concentration of the purified anti-urokinase mouse IgG was determined by the Bradford method (10) using BSA to establish the calibration curve.

Procedure

Measuring the Equivalent Migration Speed and Electrophoretic Mobility of Protein

The protein solution was continuously introduced into the central compartment whose outlet was connected with the UV detector operated at 280 nm. Then the electric field was turned on and the absorbance of the effluent started to decrease. Until the absorbance curve of effluent approached a steady value, the elution sample was collected and its protein concentration was measured at 280 nm for BSA or 420 nm for HBB. The equivalent migration speed and mobility were then calculated from Eqs. (10) and (11), respectively. After a run, a counter electric field was applied by altering the cathode and anode to remove the protein from the membrane in order to regenerate the system for a subsequent run.

Separation of Protein Mixture and Anti-Urokinase Mouse IgG

After turning on the electric field, protein mixture solution was continuously introduced into the central compartment. The products were simultaneously collected from the elution compartments in the case of separation of BSA and HBB or from the central compartment in the case of purification of mouse IgG.

RESULTS AND DISCUSSION

Characterization of Electrophoretic Migrations of BSA and HBB

These experiments were carried out at four levels of electric field strength, 20, 40, 60, and 80 V/cm. The sample flow rate was set to 20, 40, 60, 80, and 160 mL/h at each electric field strength while the carrier rates for the elution and electrode compartments were 200 and 400 mL/h, respectively. The concentration of protein in the sample was maintained at 0.5 mg/mL in all experiments. The Tris–HAc buffer (0.01 M) at pH 4.9, 5.4, 5.9, 6.4, and 6.9 was used to prepare sample and carrier solutions.

The Effect of Electric Field Strength on Equivalent Migration Speed and Mobility of BSA

Similar results were obtained in each buffering pH. The equivalent migration speeds and mobilities of BSA measured at pH 6.9 under different electric field strengths are shown in Figs. 4 and 5, respectively.

Figure 4 suggests that a higher electric field strength results in a higher migration speed. However, as illustrated in Fig. 5, the electrophoretic mobility decreases when the electric field strength increases.

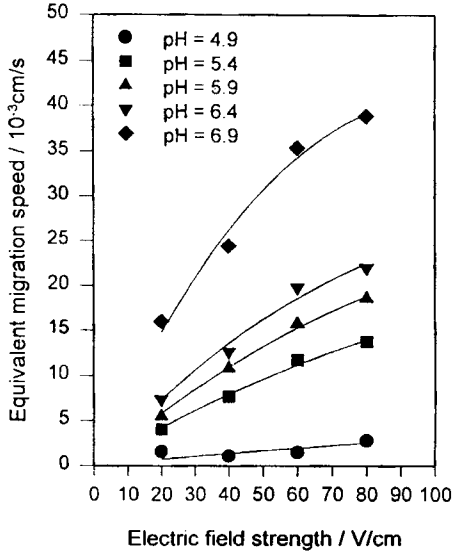

FIG. 4 Equivalent migration speed of BSA obtained at different electric field strengths. The sample loading flow rate was maintained at 80 mL/h in each experiment.

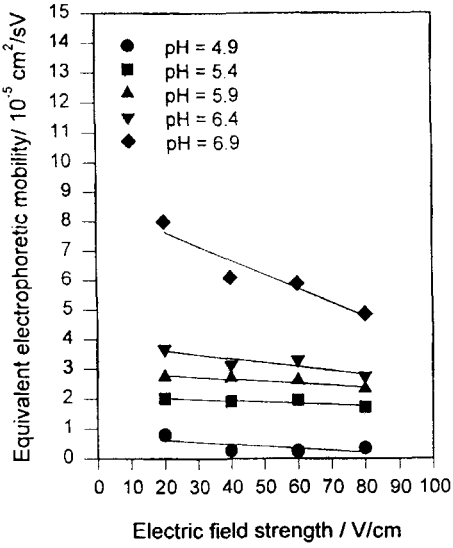

FIG. 5 Equivalent electrophoretic mobility of BSA obtained at different electric field strengths. The sample loading flow rate was maintained at 80 mL/h in each experiment.

As mentioned above, the accumulation of charged protein at the surface of a membrane causes concentration polarization that introduces a mass diffusion whose direction is opposite to electrophoresis. Increasing the electric field strength accelerates both protein migrations in the solution and the membrane and the accumulation of charged protein onto the surface of the membrane, which results in an enhanced mass diffusion that causes the loss of an equivalent amount of migration mobility.

The Effect of Fluid Flow Rate in Central Compartment on Equivalent Migration Speed of BSA

The equivalent migration speeds of BSA obtained for different samples by introducing flow rates for four levels of electric field strength at pH 6.9 are shown in Fig. 6.

As Fig. 6 shows for the experiments described above, the flow rate at which a sample is introduced does not have a visible effect on the migration speed of a protein. This fact suggests that in all the experiments described above the fluid flow state has no effect on reducing the concentration polarization.

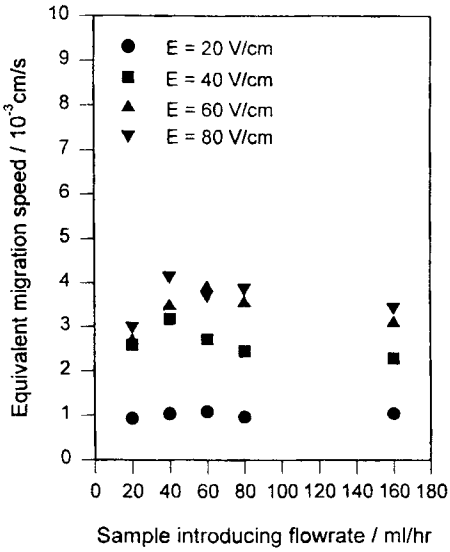

FIG. 6 Equivalent migration speed of BSA obtained at different flow rates of introducing the sample solution. The experiments were conducted using 0.01 M, pH 6.9 Tris–HAc buffer.

The Effect of Buffering pH on Equivalent Migration Speed and Electrophoretic Mobility of BSA and HBB

The equivalent migration speeds and mobilities of BSA and HBB at different buffer pH values are shown in Figs. 7 and 8, respectively. The curves suggest that the migration of BSA and HBB changes significantly in response to the buffering pH.

The driving force for the electrophoresis of a protein in solution is intrinsically decided by its net charge, a function of the difference between its isoelectric point and the buffering pH. It is thus predictable that the larger the difference between the isoelectric point of the proteins, the higher the separation productivity of MFE.

It is concluded from the above results that the equivalent migration speed and electrophoretic mobility of a protein is determined principally by the buffering pH and the electric field strength. An increase of the electric field strength can accelerate the migration speed of a charged protein. When the central compartment is maintained in continuous flow status, the flow rate of introducing the protein solution has no strong effect on the electrophoretic migration of protein in electrophoresis by MFE.

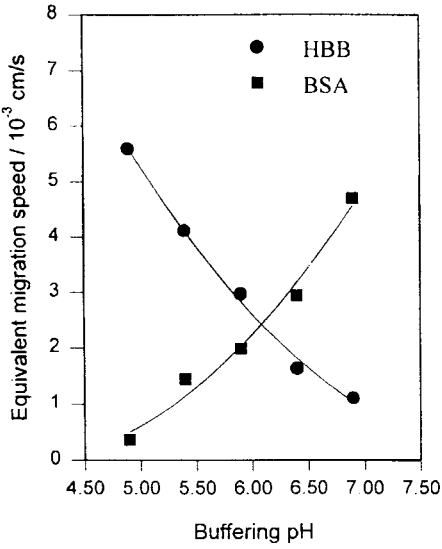

FIG. 7 Equivalent migration speeds of BSA and HBB obtained at different buffering pH values. In each experiment the electric field strength was 80 V/cm and the sample was introduced at a flow rate was 160 mL/h.

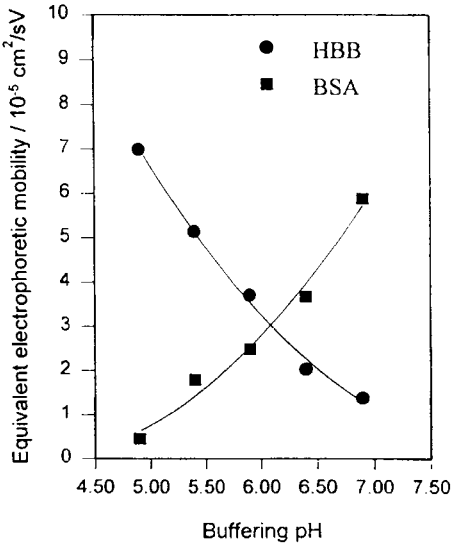

FIG. 8 Equivalent electrophoretic mobility of BSA and HBB obtained at different buffering pH values. In each experiment the electric field strength was 80 V/cm and the sample was introduced at a flow rate was 160 mL/h.

Separation of the Mixture of BSA and HBB

The pure protein was dissolved in 0.01 M, pH 5.9 Tris–HAc buffer, which was also used as the carrier solution for the elution and electrode compartments. The flow rates of the protein solution and the carrier for the elution and electrode compartments were 40, 160, and 320 mL, respectively. The applied electric field strength was maintained at 60 V/cm. In the first run, 0.5 mg/mL BSA solution was first introduced into the central compartment. The protein concentration of the effluent from each compartment was determined from its absorbance at 280 nm. In the second run the 0.5 mg/mL HBB solution was introduced. Analogously, the protein concentration of the product collected from each compartment was interpreted from its absorbance at 420 nm. Finally, the 0.5 mg/mL BSA and HBB mixture was introduced. After checking the purity of the product by SDS-PAGE, the protein content of the product was determined by the absorbance at 280 nm for BSA and 420 nm for HBB. Each separation was conducted for 30 minutes.

Figure 9 shows the results of SDS-PAGE analysis of the products of the third run, which shows that the purities of the products are the same as the corresponding commercial product used for preparing the mixture solution. Listed in Table 1 are the amounts of the materials and products used in each experiment.

The above results demonstrate the workability of this small-scale MFE apparatus in the separation of two oppositely charged proteins. The separation output in the above experiments are about 13.6 mg/h for BSA and 20.0 mg/h for HBB, which means a 100 to 1000 mg protein per day separation output is attainable. Although the yield of one operation run is only about 35 to 55%, the total yield could be easily increased to 90% by repeating the separation of the mixture residue several times with the same procedure. If the buffer pH is changed a little in each run, recycling the mixture into the central compartment is also an easy way to obtain a high yield. One advantage of these two approaches is that the volume of the residue solution does not increase in each run. Consequently, the separation is time-saving.

Purification of Monoclonal Anti-Urokinase Mouse IgG from Mouse Serum

The immunized mouse serum was first precipitated by 50% saturated $(NH_4)_2SO_4$. Then the precipitate was dissolved in 80 mL water and subjected to dialysis with water and with the 0.02 M pH 7.0 Tris–HAc buffer used for continuous MFE. The electrophoresis was carried out under a 60-V applied potential. The volume flow rates for the central, elution, and

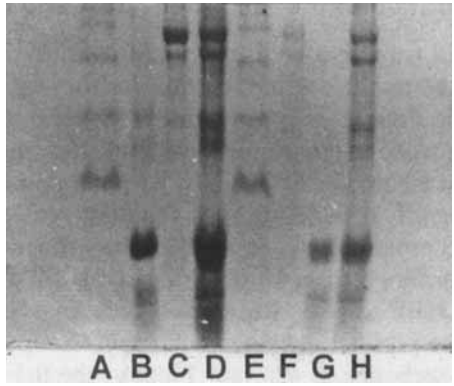

FIG. 9 Diagram of SDS-PAGE analysis of the protein mixture, BSA and HBB products. **Column A**: Standard protein marker (from top to bottom: phosphorylase, MW = 94,000; albumin, MW = 67,000; actin, MW = 43,000; carbonic anhydrase, MW = 30,000; and TMV shell protein, MW = 17,500). **Column B**: HBB used for preparing the protein mixture. Four belts appear in this column. The molecular weights of the two major belts are 12,000 and 6000, respectively. **Column C**: BSA used for preparing the protein mixture. Two belts appear in this column. The molecular weights are 67,000 and 50,000, respectively. **Column D**: Protein mixture composed of HBB and BSA. **Column E**: Standard protein maker, which is the same as column A. **Column F**: BSA recovered from the mixture. Again, two belts appear. The molecular weights are 67,000 and 50,000, respectively. **Column G**: HBB recovered from the mixture. Two belts appear in this column. The molecular weights are 12,000 and 6000, respectively; **Column H**: Residue collected at the outlet of the central compartment.

electrode compartments were 40, 160, and 360 mL/h, respectively. After the first run the product was adjusted to pH 7.0 and then placed in the central compartment for the second run and, analogously, for the third run. After checking the product purity by SDS-PAGE, the concentration of IgG was analyzed by the Bradford method using BSA to establish the calibration curve.

Figure 10 is the SDS-PAGE of mouse serum in solution, the precipitates in $(NH_4)_2SO_4$ precipitation, the soluble fractions in $(NH_4)_2SO_4$ precipita-

TABLE 1

Sample content (mg)	BSA (15.0)	HBB (16.0)	BSA + HBB (18.5) (18.5)
Product from elution compartment next to cathode, mg	0.0	9.0	10.0 (HBB)
Product from elution compartment next to anode, mg	5.5	0.0	6.8 (BSA)

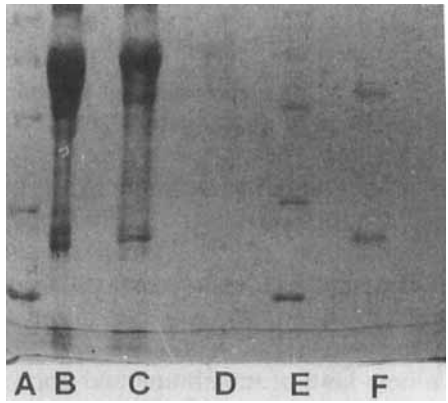

FIG. 10 Diagram of SDS-PAGE analysis of mouse serum and IgG product. **Column A**: Standard protein marker (from top to bottom: phosphorylase, MW = 94,000; albumin, MW = 67,000; actin, MW = 43,000; carbonic anhydrase, MW = 30,000; and TMV shell protein, MW = 17,500). **Column B**: Original immunized mouse serum. **Column C**: Precipitates in $(NH_4)_2SO_4$ precipitation. **Column D**: The soluble fractions in $(NH_4)_2SO_4$ precipitation. The molecular weight of the major belts is 67,000. **Column E**: Standard protein marker. **Column F**: Anti-urokinase mouse IgG product. Two chains appear in this column. The molecular weights are 50,000 and 24,000, respectively. The total molecular weight of anti-urokinase mouse IgG is 98,000.

tion, and the IgG product. The molecular weights of the light and heavy chains of IgG are 24,000 and 50,000, respectively. The concentration test suggests that about 12.6 mg anti-urokinase mouse IgG was recovered from 15 mL mouse serum. The productivity of purifying mouse IgG was about 2.1 mg/h.

The conventional purification scheme of mouse IgG includes ion-exchange chromatography, gel elution chromatography, as well as dialysis procedures between each step, all of which are time- and reagent-consuming and not easy to scale up. Applying MFE to the purification of mouse IgG presents an effective and economic way of approaching high separation output and high resolution.

CONCLUSIONS

The preliminary results presented in this paper have demonstrated the workability of MFE for the separation of simple protein mixtures in which the isoelectric point of one component is different from that of another. The application of MFE in the purification of anti-urokinase mouse IgG

has pointed out its promising application potential in the isolation and purification of complex mixtures. The above practices also exhibit the good versatility and operability of MFE. Our experimental investigation of the electrophoretic transmittance of charged proteins has generated a preliminary view of the mass transfer characteristics of MFE, which are important for further studies.

The development of MFE is in its beginning stage. In coming research focused on scaling-up of this technique, we will direct our attention to a further study of the mechanisms of protein migration in MFE in order to establish analytical or empirical expressions of equivalent migration speeds and mobilities of protein. We will extend our efforts to develop procedures for reducing concentration polarization, such as the synthesis or employment of a new kind of membrane and applying an oscillatory electric field in electrophoresis. Also included in the scope of our further study are the optimization and scaling-up of the geometric structure of the electrolyzer, the durability of the system, and the application of this system to the separation of other real protein mixtures.

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Received by editor August 24, 1995