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Protein Separation by Preparative Multicompartment Electrolyzer Secluded by Isoelectric Membranes

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ABSTRACT

The performance of a multicompartment electrolyzer using amphoteric isoelectric membranes was studied for preparative isoelectric focusing. The transient behaviors of the electrolyzer were monitored. The current through the electrolyzer and the pH in each chamber were stable during a separation run. Two kinds of protein mixture, bovine serum albumin/myoglobin and bovine serum albumin/β-lactoglobulin, were successfully separated.

Key Words. Electrophoresis; Isoelectric focusing; Immobiline; Preparative; Membrane

INTRODUCTION

Techniques for preparative separation and purification of biochemical substances have received considerable attention with the development of biological industry. Electrophoresis and chromatography are among the most popular technologies for biological separation. Although electrophoresis has been extensively used on the analytical scale, preparative separation and purification methods are still under development. Isoelectric focusing is a promising technique for scaling up to the preparative process among various electrophoresis methods.

Isoelectric focusing involves setting up a pH gradient and allowing proteins to migrate in an electric field to the point where the pH equals their isoelectric point (p*I*) in the system. Each protein is finally concentrated

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at that point after complete migration, because the protein has no charge there. In conventional isoelectric focusing, which is basically liquid-phase electrophoresis, a pH gradient is created by electrophoretic migration of carrier ampholytes to stationary zones in a separation column. Thus, conventional isoelectric focusing has several problems, including unstable pH gradients, lack of even conductivity, insufficient buffering capacity, and limited load capacity.

The Immobiline technique has been developed to overcome these problems (1). To improve the capacity and resolution of conventional isoelectric focusing, isoelectric focusing in immobilized pH gradients without using carrier ampholyte has been introduced. The Immobiline technique is an isoelectric focusing in the gel phase where the pH gradient is copolymerized within a polyacrylamide matrix. This is achieved by using acrylamide derivatives, called Immobilines, which are nonamphoteric weak acids and bases with the general formula CH_2=CH—C(O)—NH—R, where R denotes either a carboxyl or a tertiary amino group. These derivatives are designed to form copolymers with acrylamide and *N*,*N*′-methylenebisacrylamide (Bis). They are efficiently incorporated into the gel matrix. The isoelectric point of the gel matrix is determined by the relative amount of carboxyl and amino groups. In contrast to conventional isoelectric focusing, the Immobilines are covalently fixed to the gel matrix, producing stable pH gradients. The resolving power was reported to be 0.001 pH unit.

From a preparative view point, the Immobiline technique with pH gradients does not have enough load ability, which depends on the supporting matrix. An additional problem is that contamination of protein with unreacted monomers and ungrafted oligomers is inevitable. A multicompartment electrolyzer which contains Immobiline membranes was recently developed (2–4) to overcome these problems. An Immobiline gel with a well-defined isoelectric point was used in the form of a membrane. The separation function is located in membranes, while compartments give space for feeding and recovery.

In this paper, separation of a protein mixture was carried out with an multicompartment electrolyzer containing Immobiline membranes. The principle of the electrolyzer used here is the same as reported previously (2–4). The transient behavior of the separation was studied. Two kinds of protein mixture, bovine serum albumin/myoglobin and bovine serum albumin/β-lactoglobulin, were used as the feed materials.

PRINCIPLE OF MULTICOMPARTMENT ELECTROLYZER

Protein is characterized by an inherent isoelectric point, which is defined as the pH where the net charge of a protein becomes zero. A protein

is positively charged in an environment whose pH is lower than the isoelectric point of the protein, and it is negatively charged at a higher pH environment. As illustrated schematically in Fig. 1, the system is comprised of a stack of chambers secluded by pH-controlling amphoteric isoelectric membranes made of Immobiline gel. Two pH-controlling membranes with different isoelectric points, e.g., p*I* 6 and 7, are shown in the figure. The chambers placed at the two ends are anodic and cathodic electrode compartments, and they are secluded by ultrafiltration membranes to prevent the migration of proteins into the electrode chambers.

Proteins are transported electrophoretically from chamber to chamber in the following manner. Suppose the chambers are filled with a buffer

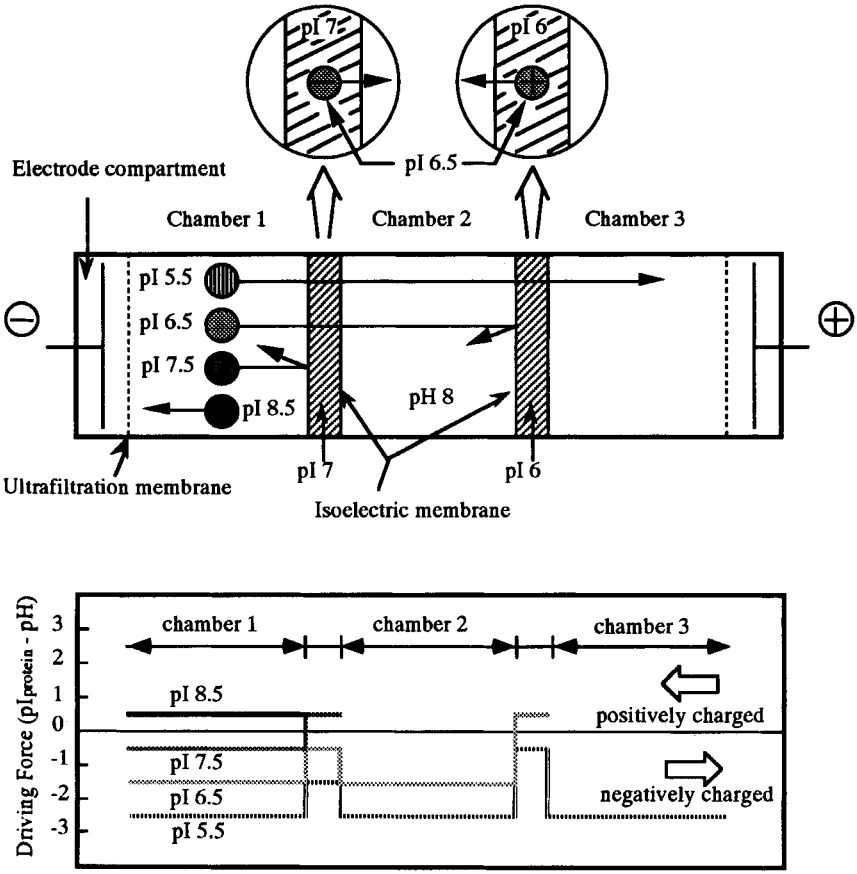

FIG. 1 Principle of multicompartment electrolyzer with isoelectric membranes.

solution of pH 8 and a protein mixture is fed into Chamber 1. Each protein would migrate to the direction determined by the charge of the protein at the environmental pH. Proteins having a smaller p*I* than the pH of the solution migrate toward the anode until they reach the membrane whose p*I* is lower than the p*I* of the protein. For example, the protein of p*I* 6.5 migrates toward the anode in Chamber 1 because it is negatively charged. When it migrates into the membrane of p*I* 7, the protein is still negatively charged, so it moves toward the anode in the membrane. The protein keeps migrating toward the anode in Chamber 2. However, it cannot migrate through the membrane of p*I* 6 because the protein is charged positively in the membrane where the p*I* of the membrane is lower than the p*I* of the protein. Therefore, since the protein cannot pass through the membrane of p*I* 6, it is accumulated in Chamber 2. On the other hand, the protein having p*I* 5.5 can migrate into Chamber 3 because the protein is charged negatively in both membranes. The protein having p*I* 7.5 cannot travel through the membrane of p*I* 7. Consequently, after the migration is completed, only the protein whose p*I* is between the p*I*s of two membranes can be accumulated in Chamber 2. Note that each compartment is flowtight, so that no net liquid bulk flow occurs from a compartment to an adjacent compartment. The transportation of components is caused by electrophoretic migration.

As illustrated in the lower part of Fig. 1, the direction of protein migration in each chamber and membrane is determined by the sign of $pI_{protein} - pI_{membrane}$ or $pI_{protein} - pH_{chamber}$. The protein migrates until it reaches the field where the sign changes.

When the volume of Chamber 2 is smaller than the feed volume in Chamber 1 or a protein mixture is fed continuously into Chamber 1, the protein of interest will be concentrated in Chamber 2. Since different proteins have different p*I*s, each protein will be accumulated in a different chamber. The larger the number of chambers that are stacked, the more protein components that can be separated.

For purification purposes, the membranes must have isoelectric points encompassing the p*I* of the protein of interest. One membrane should have a slightly lower p*I* and another a slightly higher p*I* than the p*I* of the protein. Under ideal conditions, where the p*I*s of membranes are very close to the p*I* of the protein of interest, a homogeneous protein fraction can be isolated in the chamber between two membranes.

The buffer solution in chambers does not directly contribute to separation, but it determines the direction and the migration rate of proteins. When the pH of the solution is distinct from the isoelectric point of the protein, isoelectric denaturation or precipitation can be avoided.

EXPERIMENTS

Figure 2 shows the experimental apparatus. The electrolyzer consists of a stack of three chambers and two electrode compartments at either end. The column is fabricated of acrylic plastic to allow visual observation of the migration. It is 300 mm in total length and of 10 mm i.d. Each chamber is 60 mm in length and 4.3×10^{-6} m^3 in volume. The chambers are attached with flanges. The pH-controlling membranes are housed between the chambers with O-rings to ensure flowtight connections.

Amphoteric isoelectric membranes called Immobiline membranes are prepared from copolymerized acrylamide, Bis, Immobiline, ammonium persulfate and *N,N,N',N'*-tetramethylethylenediamine (TEMED). The Immobiline chemicals (Immobiline II) were purchased from Pharmacia LKB Biotechnology. The Immobiline membranes are supported on both faces by a cellulose acetate coated nonwoven polyester cloth (Advantec, Y100A047A, Toyo Roshi Kaisha Ltd.) to reinforce the mechanical strength. The membrane has a total gel concentration of 10%T and a cross-linking of 3%C. The isoelectric points of the membranes are controlled by the amounts of acidic and basic Immobiline reagent. The thickness of the membrane was 3 mm. The membrane was polymerized in a silicone-rubber ring at 323 K in an oven for 1 hour (5).

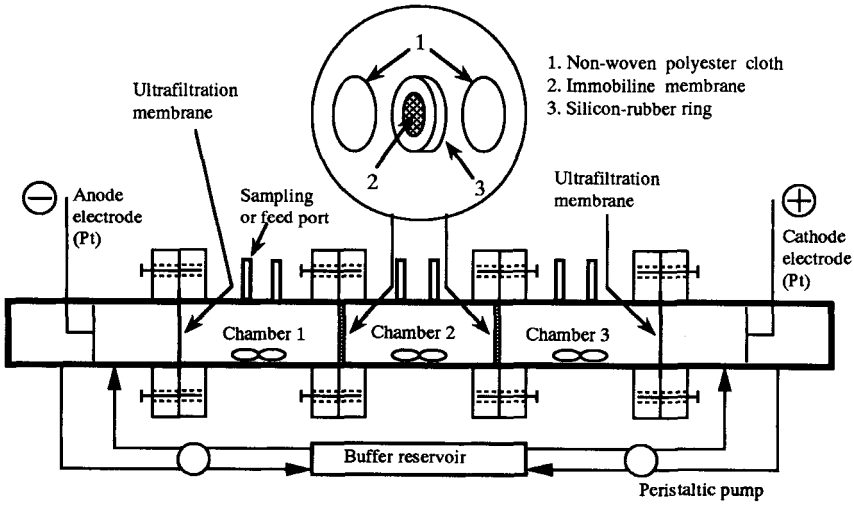

FIG. 2 Experimental apparatus of multicompartment electrolyzer.

The chambers at the ends are electrode compartments containing Pt electrodes. The electrode compartments are secluded by ultrafiltration membranes (Advantec, Q0100, Toyo Roshi Kaisha Ltd.) which prohibit the migration of proteins but allow the migration of buffer ions. The electrode solution is circulated through a buffer reservoir to keep the composition constant. The circulation lines were designed to be electrically disconnected by dropping the liquid. The chambers and the electrode compartments were filled with a buffer solution consisting of an equivalent concentration (10 mol/m^3) mixture of acetic, phosphoric, and boric acid titrated to pH 7.0 with 30 mol/m^3 Bis-Tris.

At the beginning of a run, the protein sample was fed into Chamber 1 and then electrophoretic migration was started by applying an electric field. The concentration in each chamber was measured with HPLC (YMC-Pack, Diol-120, 500 mm × 8 mm i.d., YMC Co.). Myoglobin was monitored with a UV-VIS spectrophotometer at 410 nm, and albumin and β-lactoglobulin were at 280 nm. Before sampling the solution from each chamber, the liquid in the chamber was stirred by a magnetic stirrer. The electrolyzer was held in a cool water bath at 277 K during a run in order to cope with the heat generated. The applied voltage was 300 V.

The proteins used were horse heart myoglobin (p*I* 6.8), bovine serum albumin (p*I* 4.7–4.9), and β-lactoglobulin (p*I* 5.1). These proteins, purchased from Sigma Chemical Co., were used without further purification. The concentration of proteins fed to the chamber was 1.5 kg/m^3.

RESULTS AND DISCUSSION

Current and pH Histories

To check the stability of the system during electrophoresis, changes in current and pH were monitored without feeding any proteins. The histories of the electric current through the electrolyzer during an electrophoretic run are shown in Fig. 3(a). The p*I*s of the membranes were 7.5 between Chambers 1 and 2 and 5.7 between Chambers 2 and 3. The current decreased at the beginning of the run, and reached steady state after 6 hours. The high current at the beginning may be mainly due to the migration of ions already existing in the membranes.

The pH histories in the electrode reservoir and each chamber are shown in Figs. 3(b) and 3(c), respectively. The pHs in the electrode reservoir and the chambers were stable during the run for 12 hours. The pHs in the chambers decreased slightly from Chamber 1 to Chamber 3. At the beginning, the pH in Chamber 1 increased slightly due to the migration of cations, and the pH in Chamber 3 decreased slightly due to the migration of anions.

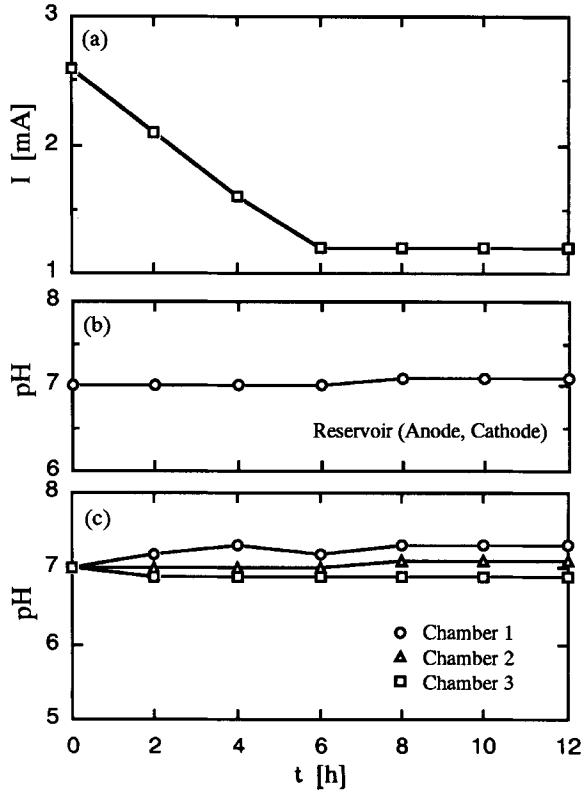

FIG. 3 Histories of (a) electric current, (b) pH in electrode reservoir, and (c) pH in each chamber.

Migration of Myoglobin

We first carried out the electrophoresis of a single component in order to determine the performance of the electrolyzer. The protein used was horse heart myoglobin. Since myoglobin has a visible red color, the migration of the protein can be visually observed. The isoelectric point of the protein is 6.8, which is the value between the p*I*s of two membranes. Thus, the protein is expected to accumulated in Chamber 2 between two membranes.

The histories of the myoglobin concentrations reduced by the feed concentration are shown in Fig. 4 for each chamber. While the concentration in Chamber 1 decreased, the concentration in Chamber 2 increased. A slight increase in Chamber 3 was observed. These results indicate that

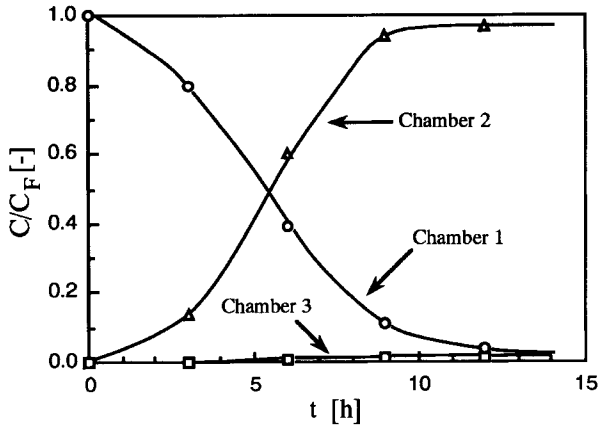

FIG. 4 Concentration histories of myoglobin in each chamber during electrophoresis.

the myoglobin migrated from Chamber 1 to Chamber 2 and was retained in Chamber 2. After 12 hours the migration of myoglobin was almost complete. Although the protein moved from Chamber 1 to Chamber 2, it wasn't transported to Chamber 3. The small increase in Chamber 3 may be due to impurities that existed in the original myoglobin. These phenomena were confirmed by visual observation. The red portion moved from Chamber 1 to Chamber 2. Therefore, myoglobin was successfully accumulated between two membranes.

Separation of Bovine Serum Albumin and Myoglobin

The separation of a mixture of two kinds of protein was carried out. The feed sample was a mixture of horse heart myoglobin and bovine serum albumin. The p*I*s of the membranes were 7.5 between Chambers 1 and 2 and 5.7 between Chambers 2 and 3. The p*I* of myoglobin is a value between the p*I*s of the two membranes, while the p*I* of bovine serum albumin is smaller than the p*I*s of the two membranes. Thus, it is expected that myoglobin will move and be retained in Chamber 2 while bovine serum albumin will move into Chamber 3.

The concentration histories of the proteins in each chamber, which indicate the migration of bovine serum albumin and myoglobin, are shown in Fig. 5. At the beginning, the proteins existed only in Chamber 1, into which the sample was fed. Bovine serum albumin left Chamber 1 in 3 hours and myoglobin left in 12 hours. In Chamber 2, the concentration of bovine serum albumin increased rapidly and then gradually decreased.

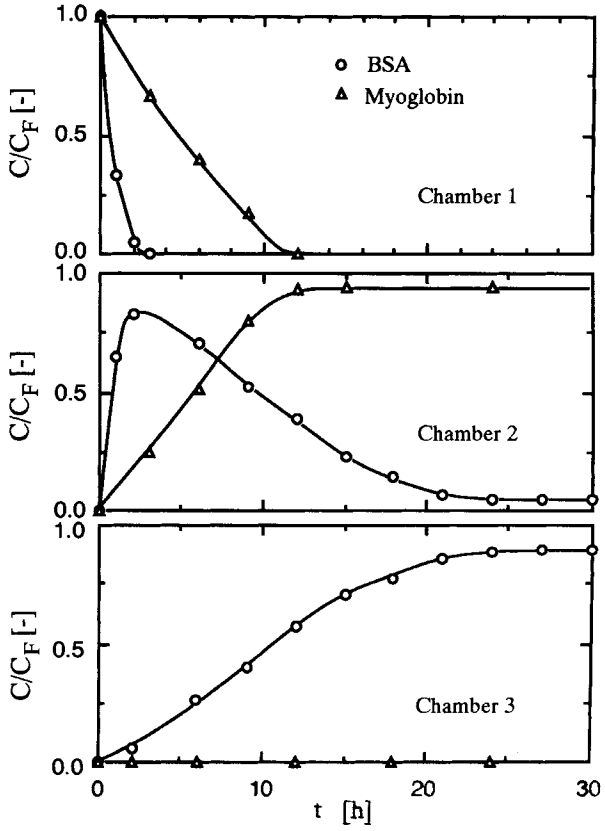

FIG. 5 Separation of a mixture of bovine serum albumin and myoglobin.

On the other hand, the concentration of myoglobin increased and then remained constant after 12 hours. In Chamber 3, only the concentration of bovine serum albumin increased, and it remained constant after 24 hours. Therefore, the majority of bovine serum albumin was transported into Chamber 3 and a very small amount was retained in Chamber 2. The amount retained in Chamber 2 may be due to impurities in the original sample. The migration of myoglobin was completed in 12 hours and that of bovine serum albumin in 24 hours. As a result, bovine serum albumin and myoglobin were separated into Chamber 2 and Chamber 3. The difference in the migration rate between bovine serum albumin and myoglobin may be due to the difference in charge at the pH of the solution.

Separation of Bovine Serum Albumin and β-Lactoglobulin

The separation of a mixture of bovine serum albumin and β-lactoglobulin was studied. These two proteins have closer p*I*s than the proteins examined in the previous section. The difference of their p*I*s is 0.3. The p*I*s of the membranes were 5.3 between Chambers 1 and 2 and 5.0 between Chambers 2 and 3. The chambers and the electrode compartments were filled with a buffer solution of acetic acid (10 mol/m^3) titrated to pH 7.0 with Bis-Tris propane solution (10 mol/m^3). The p*I* of β-lactoglobulin is a value between the p*I*s of the two membranes, while the p*I* of bovine

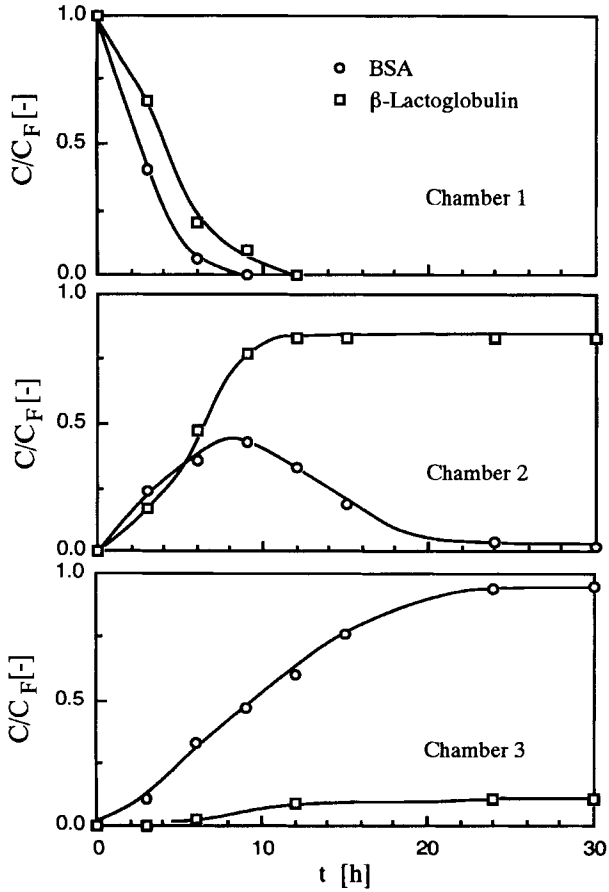

FIG. 6 Separation of a mixture of bovine serum albumin and β-lactoglobulin.

serum albumin is smaller than the p*I*s of the two membranes. Thus, it is expected that these proteins will be separated into Chambers 2 and 3.

The migrations of the proteins are shown in Fig. 6. Both bovine serum albumin and β-lactoglobulin left Chamber 1 in 12 hours. Bovine serum albumin moved from Chamber 1 to Chamber 2 and then migrated further to Chamber 3. On the other hand, β-lactoglobulin was retained in Chamber 2. The small fraction of β-lactoglobulin that migrated to Chamber 3 may be due to impurities in the original sample. As a result of migrations, bovine serum albumin and β-lactoglobulin were successfully separated into Chambers 2 and 3 after 20 hours.

The key requirements for the electrolyzer to succeed are the precise preparation and control of the p*I* of the Immobiline membranes. The separation rate depends on the migration of proteins within the membranes and the liquid in the chambers. The electrophoretical migration rate is proportional to the electrical potential gradient and to the reciprocal of the migration distance. Thus, thinner membranes, a shorter migration path, and a higher voltage will accelerate the separation.

CONCLUSION

1. A preparative multicompartment electrolyzer with isoelectric membranes was investigated for protein separation and/or purification.
2. The pH of the buffer solution in the chambers was stable for a long period, and the electric current through the electrolyzer attained steady state after 6 hours.
3. A mixture of bovine serum albumin and myoglobin was successfully separated. A mixture of proteins having similar p*I*s (bovine serum albumin and β-lactoglobulin) was also separated.
4. Thinner membranes, a shorter migration path, and a higher voltage will accelerate the separation.
5. Accurate control of the p*I*s of the membranes is a crucial factor to achieve high resolution.

SYMBOLS

C	concentration of protein (kg/m^3)
C_F	feed concentration of protein (kg/m^3)
I	current (mA)
pI	isoelectric point (—)
t	time (h)

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