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# Isoelectric Split-Flow Thin (SPLITT) Fractionation of Proteins

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## ABSTRACT

Electric split-flow thin (SPLITT) fractionation permits the continuous separation of charged species, particularly proteins, at gram and subgram levels. We characterized this system and separated protein mixtures based on the difference between protein isoelectric points (pI). For characterization, we examined seven variables. Buffer stability was determined by measuring pH changes per hour and the UV spectrum before and after an electrical potential of 50 V was applied. The electrical field across the channel was determined by measuring the buffer conductivity and the current passed through it. The experimental and theoretical (calculated) fractional retrieval of proteins was determined by the relative magnitude of field-induced and outlet flow rates. The protein response at various electrical fields (0, 10, 20, 30 V) and solution pHs (4.85, 5.60, 6.87, and 7.80) was examined, as were the effects of the ionic strength of the buffer, protein recovery, and protein separation with pulsed sample injection. To separate protein mixtures after the system was characterized, we ran continuous SPLITT fractionation of five protein mixtures for more than 8 hours. Characterization results show that 1) buffer stability was good for acetate and phosphate buffers, 2) the electrical field across the channel was about 60% of that predicted by a geometric estimation, 3) experimental retrieval of four proteins (ferritin, BSA, hemoglobin, and cytochrome c) agreed well with calculated retrieval, 4) protein response at the four electrical fields and four solution pHs corresponded to the difference between protein pI and solution pH, 5) lower buffer ionic strength was better for protein

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separation, 6) protein sample recovery was reasonable from 78 to 90% (mean 85%) for six proteins, and 7) pulsed sample injection led to successful separation of five protein mixtures. In the second part of the study, three protein mixtures were successfully separated using continuous separation over 8 hours. The collected fractions showed clean separation as confirmed by flow field-flow fractionation and spectrophotometer analysis. The throughput was around 15 mg/h and the minimum difference between protein pIs that permitted separation was about two units. We conclude that isoelectric SPLITT fractionation has potential for use in protein purification.

## INTRODUCTION

The split-flow thin (SPLITT) cell is designed for continuous separation of various molecular species. The common type of SPLITT cell has both inlet and outlet flow splitters. The inlet flow splitter allows two inlet flows to be smoothly joined and the resolution of two fractionated products to be controlled. The outlet flow splitter divides differently populated samples in two fractions that emerge at separate outlets. Various forces can be used to drive samples selectively and transversely into different distributions across the channel based on the sample properties (1).

In electric SPLITT fractionation, an electrical potential is applied across the cell thickness, thus making this technique applicable to charged species. Proteins are natural choices for the application of electric SPLITT fractionation because of their charged nature and importance to human and animal life. The overall charge of a protein depends on the difference between its isoelectric point (pI) and the solution pH. The protein has a net positive charge when the pI is greater than the pH and a net negative charge when the pI is less than the pH.

Electric SPLITT fractionation (SF) can be performed in either a transport mode or an equilibrium mode (1–3). In this study we will focus only on the latter, in which the various components are driven into different equilibrium positions and are separated into different fractions by the outlet flow splitter. Two proteins will tend to carry opposite charges if the solution pH lies between their isoelectric points. Upon application of an electrical field, the two proteins thus will migrate in opposite directions. The protein with a net negative charge will move toward the positive electrode and the protein with a net positive charge will move toward the negative electrode (Fig. 1). Consequently, proteins with opposite net charges will be driven to different equilibrium positions and will be concentrated on opposite sides of the channel. These differently distributed proteins can then be separated by the outlet flow splitter and eluted at separate outlets. In this equilibrium operation the inlet flow splitter is

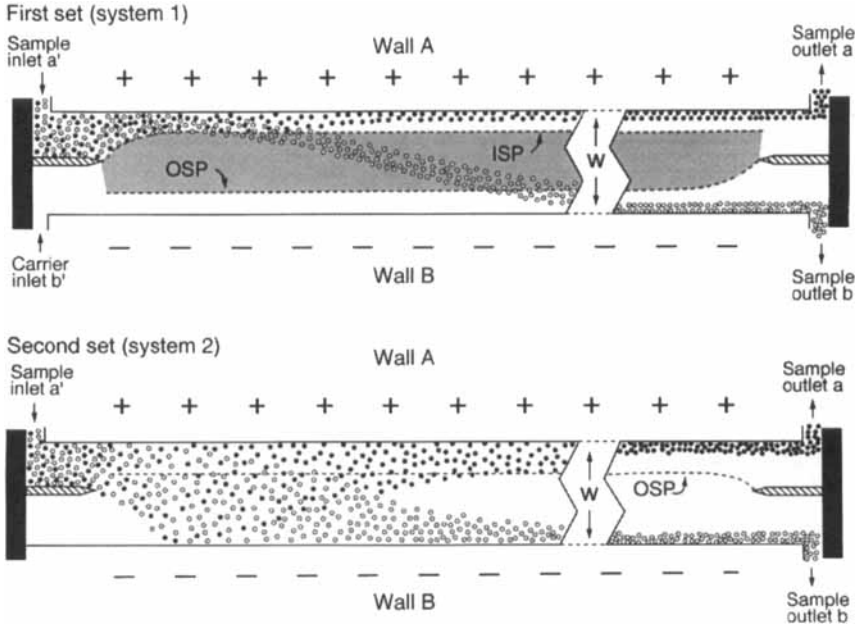


FIG. 1 The two experimental systems used in isoelectric SPLITT fractionation.



optional because each component will eventually be driven to its equilibrium position regardless of its initial position. This equilibrium operation will be illustrated by two sets of experiments that compare the experimental and theoretical retrieval of proteins.

Like all other SPLITT systems, the separation speed is very high because the separation takes place over a short distance, about 100 μm. Sample injection can be pulsed or continuous. The former is normally used for analytical or characterization purposes and is termed *analytical SPLITT fractionation* (4, 5). The latter is used for preparative purposes and is called *continuous SPLITT fractionation* (CSF) when operated with continuous sample introduction (3). Isoelectric SF was used in this study because the SF separation mechanism is based on the difference between the isoelectric points of the samples. This technique has good potential in the preparative separation and purification of proteins, both of which are under extensive study (6–8).

Several experimental variables must be considered if fractionation is to be successful. Buffer stability is important when using isoelectric SPLITT

systems. Upon application of the electrical field, redox reactions occurring at the anode and cathode can cause drifts in baseline measurements and changes in solution pH. Severe baseline drifts are undesirable as are changes in the solution pH, which may adversely affect biological samples, e.g., denature proteins. The voltage drop across the channel is used to calculate an accurate value for the electric field that is applied to the sample species. The ionic strength of the buffer regulates buffer conductivity and the sample response to the electrical field. To determine the ability of isoelectric SPLITT fractionation to separate and purify proteins, we conducted two sets of experiments. We characterized the isoelectric SF system in the first part of this study in regard to the following variables: 1) buffer stability, 2) the electrical field across the channel, 3) experimental and theoretical retrieval of proteins, 4) protein response under applied electrical fields of various magnitudes and solution pH, 5) effect of ionic strength of the buffer on protein retrieval, and 6) separation of protein mixtures with pulsed sample injection. In the second part of this study we used continuous SPLITT fractionation to show whether the system can successfully separate proteins for preparative purposes.



## THEORY

Experiments were done using the two system setups shown in Fig. 1, with the positive charge assigned to wall $A$ and the negative charge assigned to wall $B$ for representative illustration. In system 1, the outlet flow rate, $\dot{V}(a)$, was larger than the inlet flow rate of sample $\dot{V}(a')$, and the sample-free inlet flow rate, $\dot{V}(b')$, was greater than zero. In system 2, $\dot{V}(a) < \dot{V}(a')$ and $\dot{V}(b') = 0$. Both systems were used as part of the study on system characterization. The theory of the operation of system 1 has been discussed previously (2), so the theory for system 1 will first be briefly summarized here and then the theory for system 2 will be deduced.

For a species to be transported across a lamina of flow rate $\dot{V}_i$ with an electrical driving force, the conditions of the following equation must be met (2):

$$bL\mu E \geq \dot{V}_i \tag{1}$$

where $b$ is the channel breadth, $L$ is the channel length, $E$ is the electrical field, and $\mu$ is the electrophoretic mobility ($\mu$ equals $\mu_+$ for positively charged species and equals $\mu_-$ for negatively charged species).

In the first set of experiments (system 1), $\dot{V}(a) > \dot{V}(a')$, $\dot{V}(b') > 0$, and it is clear that the positive species must traverse the outlet splitting plane (OSP) to be fully recovered at outlet $b$. Thus, the following inequality must apply:

$$bL\mu_+E \geq \dot{V}(a) \tag{2}$$

The negative species require no transport across the OSP for total recovery at outlet $a$.

For the case of a negative charge on wall $A$ and a positive charge on wall $B$, the following equation must apply for the negative species to be recovered fully at outlet $b$:

$$bL\mu_-E \geq \dot{V}(a) \tag{3}$$

No transport is required for the positive species to be recovered fully at outlet $a$.

Using the same principles, it is possible to derive the criteria that must be met for a second set of experiments (system 2). In this second set of experiments, $\dot{V}(a) < \dot{V}(a')$ and $\dot{V}(b') = 0$. As Fig. 1 shows, in system 2 both positive and negative species clearly must transverse the OSP to be recovered completely at different outlets. The following two equations must apply if positive and negative species are to be fully recovered at outlets $b$ and $a$, respectively:

$$bL\mu_+E \geq \dot{V}(a) \tag{4}$$

$$bL\mu_-E \geq \dot{V}(b) \tag{5}$$

For the case of a negatively charged wall $A$ and a positively charged wall $B$ (not shown in Fig. 1), the following two equations must apply for positive and negative species to be fully recovered at outlets $a$ and $b$, respectively:

$$bL\mu_+E \geq \dot{V}(b) \tag{6}$$

$$bL\mu_-E \geq \dot{V}(a) \tag{7}$$

To characterize the system, four proteins with different pI values were used with different electrode polarities. Results obtained from both sets of experiments were compared with those calculated from theory. The results of the characterization studies were then used in the second part of the study to determine optimal conditions. The second set of experiments was used in the CSF study involving the increase of sample throughput and concentration of the fractionated proteins.

## EXPERIMENTAL

### System Description and Equipment and Reagents Used

The channel length, breadth, and thickness were 28.8 cm, 2.0 cm, and 0.0635 cm, respectively. The calculated void volume of the cell was 3.66 mL. The cell construction has been discussed previously (2). The distance

between the two electrodes was 2.5 cm. The multilayered structure was designed in large part to prevent perturbations caused by electrolytic gases. To eliminate the gases and joule heat from electrode compartments, buffer was circulated at high flow rates and a cooling loop was used. Fresh buffer was passed through frit compartments to ensure this elimination. The cell was cleaned after each run by pulsing the electrode polarity (+30 V and −30 V) with the flow rate set at approximately 1 mL/min.

Figure 2 shows a schematic of the entire system. Two peristaltic pumps (Minipuls 2, Gilson, Middleton, WI), designated as pumps 1 and 2, provide the flow to feed inlets *a'* and *b'*, respectively. To degas the carrier, another Minipuls 2 pump (pump 3) was used to pump the carrier through the frit and out to waste containers. A Masterflex peristaltic pump (Cole-Parmer, Chicago, IL), designated as pump 4, circulated the buffer solution through three 4-L reservoirs at a flow rate high enough to degas and cool the electrode chamber (about 110 mL/min). Outlet flows were controlled by using various lengths of capillary tubing as pressure restrictors. Variable



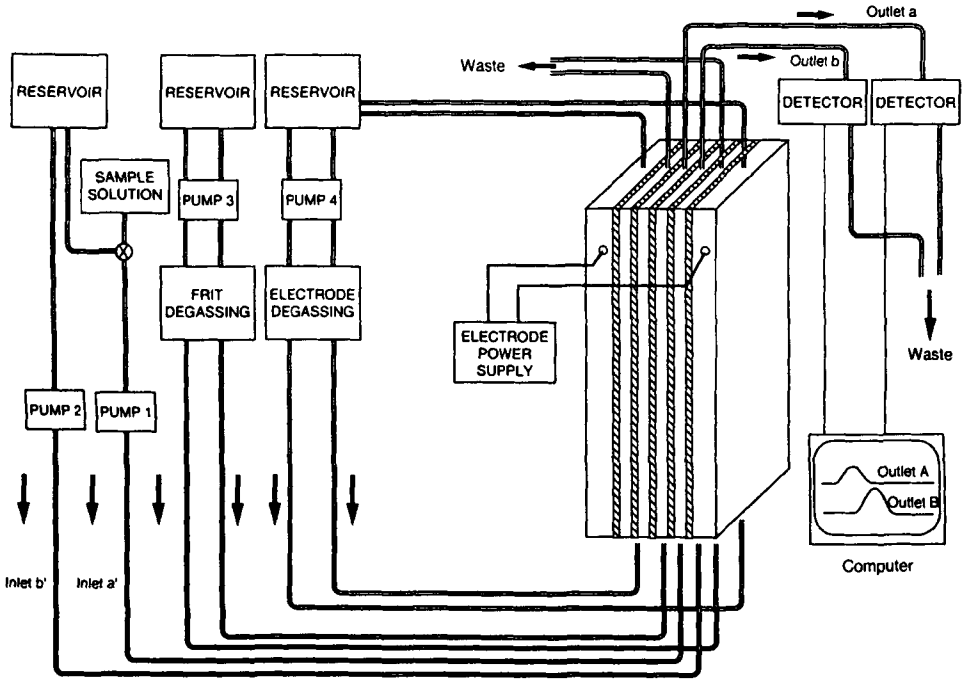


FIG. 2 Experimental system for isoelectric SPLITT operation.

wavelength detectors (Shimazu SPD-6A, Kyoto, Japan, and Spectroflow SF 769, Applied Biosystems, Ramsey, NJ) were connected to each outlet in all experiments. The wavelength was set at 280 nm. Data acquisition and analysis were done with a PC-compatible computer and software developed at the FFF Research Center.

Trizma base [tris-(hydroxymethyl)aminomethane], MES (2-[*N*-morpholino]ethanesulfonic acid), HEPES (*N*-[2-hydroxyethyl]piperazine-*N'*-[2-ethanesulfonic acid]), and sodium azide were purchased from Sigma Chemical (St. Louis, MO). Sodium acetate, sodium hydroxide, and sodium phosphate (monobasic and dibasic) were purchased from Mallinckrodt (Paris, KY). Glacial acetic acid was purchased from J. T. Baker (Phillipsburg, NJ). All proteins were purchased from Sigma Chemical (St. Louis, MO): hemoglobin (human), IgG (human), cytochrome c (horse heart), ferritin (horse spleen), bovine serum albumin (BSA), albumin (human), and α-chymotrypsinogen A (bovine pancreas). All sample solutions were prepared by dissolving the proteins in the selected buffer and then filtering with a 25-mm disk filter (Alltech, Los Altos, CA) with a pore size of 0.22 μm before use.



## Characterization of the Isoelectric SF System

### *Buffer Stability*

The tested buffers were Tris-acetate, MES, HEPES, acetate, and phosphate. They were evaluated according to three categories: 1) color change, 2) pH change per hour, and 3) changes in UV spectra of the buffers collected from different parts of the system after an electrical voltage of 50 V had been applied for 8 hours. Only the buffer running through the electrode compartment was circulated, not those running through the frit and channel. The buffer solution flowing through the channel, frit outlet, and electrode compartment was collected at various times during the 8 hours for UV spectra analysis. A digital pH meter (Model 609, Extech, Boston, MA), calibrated frequently with standard pH solutions, was used to monitor the pH of the buffer solutions used in this study.

### *Electrical Field across the Channel*

The voltage drop across the channel in this particular study was calculated from the product of the resistance inside the channel and the current through the channel. The electrical field was adjusted using a laboratory-built dc power supply operated at different voltages. The resistance inside the channel was calculated from the specific conductivities of electrolytes and known channel geometry; the specific conductivities were obtained

from conductivity measurements. Constant conductivity is assumed in this voltage calculation. The electrical field was then calculated from the voltage drop across the channel divided by the channel thickness. The specific conductivities of two well-known standards, $NaH_2PO_4$ and $Na_2HPO_4$, each with a concentration of 5 g/L (9), were used to estimate the voltage drop across the channel by measuring the current flowing across the channel.

Specific conductivities of the buffers were measured using a Leeds Northrup conductivity cell (Model LN 4920, Philadelphia, PA). The conductivity cell was calibrated with 0.01 and 0.02 M potassium chloride solutions before use.

### *Experimental and Theoretical Protein Retrieval*

The fractional retrieval (retrieval) at outlet $b(F_b)$ was calculated using the following equation:

$$F_b = \frac{\dot{V}(b)A(b)}{\dot{V}(a)A(a) + \dot{V}(b)A(b)} \tag{8}$$

where $\dot{V}(a)$ and $\dot{V}(b)$ are the volumetric flow rates at outlets $a$ and $b$, respectively, and $A(a)$ and $A(b)$ are the peak areas of the fractograms at outlets $a$ and $b$, respectively.

The system configurations shown in Fig. 1 were used for comparison of experimental and theoretical retrievals of proteins. In system 1, $\dot{V}(a) > \dot{V}(a')$ and $\dot{V}(b') > 0$, and in system 2, $\dot{V}(a) < \dot{V}(a')$ and $\dot{V}(b') = 0$. Different polarities in each set of experiments were also tested. The electrical field was calculated as discussed in the last section.

### *Buffer Ionic Strength*

Most buffers were prepared as 0.01 M. In the study of ionic strength effect of buffer, 0.01, 0.05, and 0.1 M acetate buffers were used. Phosphate buffer with a concentration of 0.005 M was used in the comparison of protein retrievals and separation of protein mixtures.

### *Separation of Protein Mixtures*

A spectrophotometer (Model Spectronic 20, Bausch and Lomb, Rochester, NY) operated at 415 nm was used to measure the amount of hemoglobin eluted and collected in different fractions. The spectrophotometer was calibrated with a series of hemoglobin concentration standards before use.

Flow field-flow fractionation (FFF) was used to check the purity of the fractionated mixture of IgG and albumin. The channel length, breadth,

and thickness were 28.5 cm, 2.0 cm, and 0.0178 cm respectively. The frit inlet, channel, and cross flow rates were 1.0, 2.1, and 10.6 mL/min respectively.

Pulsed sample injections were made with a metal-free type 50 Teflon rotary valve 5025 (Rheodyne, Cotati, CA) equipped with a 90-μL loop. Continuous sample introduction was achieved by pumping sample solution into inlet *a'*.

In the recovery experiment, known amounts of protein were first injected into the system (but bypassing the channel) and through the two detectors that were connected in series. Fractograms were obtained; flow rate and peak area of the fractograms were used to calculate the total amount of protein injected and the sensitivity ratio of the two detectors. Subsequently, the same amount of protein was injected through the channel with each outlet connected to one detector. The peak area and flow rates obtained at the two outlet detectors then were used to calculate the amount of protein that flowed through each detector. The two amounts were added to yield the total amount of protein recovered. This calculation was done at applied voltages of 0, 10, 20, and 30 V.



## RESULTS AND DISCUSSION

The first part of this section covers the characterization of the isoelectric SPLITT system. The second part is devoted to preparative applications of proteins.

### System Characterization

#### *Buffer Stability*

This study examined the stability of potential buffers for use in this system. The results are shown in Table 1. The baseline stability of a buffer is related to the pH change per hour; using this criterion, we found that acetate and phosphate buffers were more stable than the other buffers tested. The chemical structure of the buffers tested suggests that the baseline instability may be the result of the redox reaction of the hydroxyl functional group or six-member-ring structure at the electrodes. Thus, buffers containing such structures should be avoided when using this system.

#### *Electrical Field across the Channel*

The purpose of this study was to measure accurately the electrical field across the channel and to determine the relationship between the current and the applied voltage. Accurate knowledge of the voltage drop across

TABLE I
Stability of Four Buffers Tested to Characterize the Isoelectric SPLITT Fractionation System[a]

| Buffers | Color change | pH change per hour (8 hours) | Baseline stability[b] (280 nm) |
|---|---|---|---|
| Tris-acetate | Not obvious | 0.040 | No |
| MES | Yellow green (after 4 hours)<br>Golden yellow (after 8 hours) | 0.020 | No |
| HEPES | Light green (after 4 hours)<br>Light yellow green (after 8 hours) | 0.016 | No |
| Phosphates | Not obvious | 0.009 | Yes |
| Acetates | Not obvious | 0.002 | Yes |

[a] Tris: Tris-(hydroxymethyl)aminomethane. MES: 2-(*N*-Morpholino) ethane sulfonic acid. HEPES: *N*-(2-Hydroxyethyl)piperazine-*N'*-(2-ethanesulfonic acid). The applied voltage was 50 V; the system was run without frit circulation.

[b] To determine baseline stability of each buffer, UV spectra analysis was done at 280 nm. A buffer having absorption at a wavelength of 280 nm is indicated by "No."



the channel is crucial in calculating the amount of driving force applied to the samples. The relationship between the current and the applied voltage can reveal the main source of voltage consumption in the system. Using geometric estimates as references, the voltage drop across the channel was found to be approximately 60% of the geometric estimate (Fig. 3). The geometric estimate was obtained from the product of the applied voltage and the fraction of channel thickness divided by the distance between the two electrodes. Overall, the voltage drop across the channel when different buffers were used varied between 50 and 65% of the geometric estimates. This finding suggests that about 40% of the applied voltage was lost across the frit, membrane, and electrodes.

Current versus applied voltage for the different buffers is plotted in Fig. 4. A linear plot was obtained for each buffer, but each has a different slope, which suggests that the *iR* voltage drop is the main source of voltage loss. This information on voltage loss shows that more voltage is lost in the frit and membrane than in the electrode compartment. The applied voltage across the channel is related to the resistance and distance between the two electrodes. For the same buffer, the closer the two electrodes were, the higher the efficiency of the applied voltage across the channel. However, the electrodes must be far enough apart to prevent disturbances caused by electrolysis products.

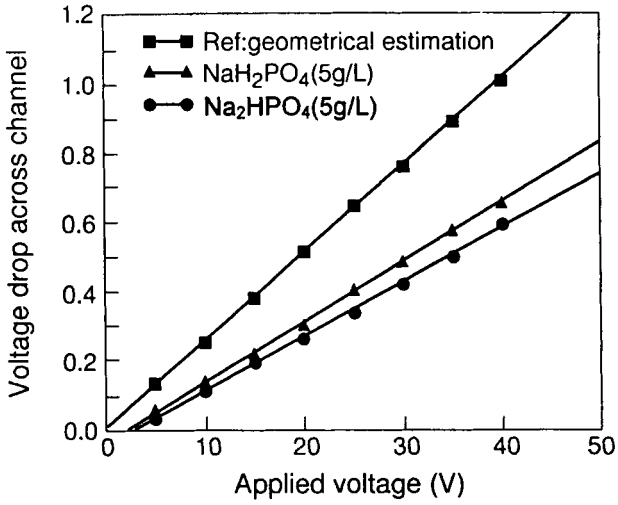


FIG. 3 Voltage drop across the channel as estimated using the specific conductivities of two standards, $NaH_2PO_4$ and $Na_2HPO_4$.

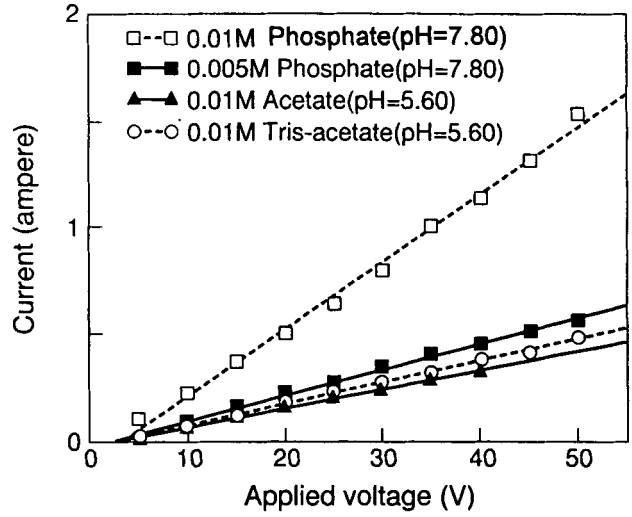


FIG. 4 Current vs applied voltage of buffers: 0.005 M and 0.01 M phosphate (pH 7.8), 0.01 M acetate (pH 5.6), 0.01 M Tris-acetate (pH 5.6).

### Comparison of Experimental and Theoretical Retrieval of Proteins

Experimental and theoretical retrievals at outlet $b$ ($F_b$) of four proteins with different pI values were compared. The effect of different solution pHs on $F_b$ was also studied. To determine theoretical retrieval values, we had to collect reliable mobility ($\mu$) data from the literature (10, 11). Because such data were scarce, to roughly estimate $\mu$ we assumed that $\mu$ was proportional to the absolute value of the difference between the experimental pH and the protein pI. This assumption has been shown to be a good approximation according to the literature (12–14). The literature pI (15) and the estimated $\mu$ of proteins are shown in Tables 2 and 3. Table 2 shows the results of the set of experiments using system 1. The agreement between experiment and theory is fairly good, indicating that the approximations for $\mu$ are reasonable. Overall, experimental and theoretical values of $F_b$ differ by 6%. Table 3 show the results using system 2. Again, the agreement between experiment and theory is good, within 6% difference.



### Protein Response at Various Electrical Fields and Solution pHs

The retrieval $F_b$ for four proteins at three different solution pHs (4.75, 5.60, and 6.87) in system 2 were plotted against applied voltage as shown in Fig. 5. These plots reflect the relative magnitude of $\mu$ that corresponds to the values shown in Table 3 and indicate that the protein mobility increases as the difference between the solution pH and protein pI increases for a specified protein. The $F_b$ response of BSA and hemoglobin at pH 4.75 and 6.87 illustrates this point very clearly in Figs. 5(A) and 5(C), respectively. These two proteins were in solutions that had pHs very close to their pI values. Therefore, their net charges were very small and responded very little to the increase in applied voltage.

### Effect of Buffer Ionic Strength on Protein Retrieval

Differences in buffer ionic strength could change the net charge of a protein or change the screening efficiency on the protein from the electrical field. Such a change would alter the protein response when an electrical field is applied. A higher ionic strength could also cause more thermal instability. Figure 6 shows the fractional retrieval of hemoglobin plotted against applied voltage at three different ionic strengths. The lowest ionic strength (0.01 M) solution shows a larger response (indicating a larger change in retrieval) than those at higher ionic strengths (0.05 and 0.10 M). The retrieval of hemoglobin at 0.05 and 0.10 M also increases as voltage

TABLE 2
Comparison of Experimental and Theoretical Protein Retrieval at Outlet b ($F_b$) in Tris-acetate Buffer (first set) with $\dot{V}(a') < \dot{V}(a)$, $\dot{V}(b') > 0$ for Four Proteins at Three Different pHs[a]

| Protein studied | pI (ref) | $\mu \times 10^5$ cm$^2$/V·s | $bL\mu E$ (mL/min), calculated | $bL\mu E$ (mL/min), required | | $F_b(A+B-)$ | | $F_b(A-B+)$ | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | $(A+B-)$ | $(A-B+)$ | Theory | Exp | Theory | Exp |
| pH 4.75: | | | | | | | | | |
| Ferritin | 4.4 | −0.94 | 0.23 | 0.00 | 0.40 | 0.00 | 0.05 | 0.58 | 0.52 |
| BSA | 4.9 | 0.63 | 0.16 | 0.40 | 0.00 | 0.40 | 0.34 | 0.00 | 0.03 |
| Hemoglobin | 6.9 | 2.74 | 0.68 | 0.40 | 0.00 | 1.00 | 1.00 | 0.00 | 0.00 |
| Cytochrome c | 10.6 | 0.93 | 0.23 | 0.40 | 0.00 | 0.58 | 0.56 | 0.00 | 0.00 |
| pH 5.60: | | | | | | | | | |
| Ferritin | 4.4 | −3.96 | 0.98 | 0.00 | 0.40 | 0.00 | 0.00 | 1.00 | 0.99 |
| BSA | 4.9 | −2.30 | 0.57 | 0.00 | 0.40 | 0.00 | 0.00 | 1.00 | 0.98 |
| Hemoglobin | 6.9 | 1.70 | 0.42 | 0.40 | 0.00 | 1.00 | 0.95 | 0.00 | 0.00 |
| Cytochrome c | 10.6 | 0.83 | 0.21 | 0.40 | 0.00 | 0.53 | 0.50 | 0.00 | 0.00 |
| pH 7.80: | | | | | | | | | |
| Ferritin | 4.4 | −11.22 | 2.79 | 0.00 | 0.40 | 0.00 | 0.00 | 1.00 | 1.00 |
| BSA | 4.9 | −9.53 | 2.37 | 0.00 | 0.40 | 0.00 | 0.00 | 1.00 | 1.00 |
| Hemoglobin | 6.9 | −1.18 | 0.29 | 0.00 | 0.40 | 1.00 | 0.98 | 0.73 | 0.70 |
| Cytochrome c | 10.6 | 0.46 | 0.11 | 0.40 | 0.00 | 0.28 | 0.25 | 0.00 | 0.00 |

[a] $E$ = 30 V; $A+B-$: wall $A$ positively charged and wall $B$ negatively charged; $A-B+$: wall $A$ negatively charged and wall $B$ positively charged. Ferritin = 0.03 mg/mL, BSA, hemoglobin, cytochrome c = 0.125 mg/mL. $\dot{V}(a')$ = 0.07, $\dot{V}(b')$ = 0.53, $\dot{V}(a)$ = 0.40, $\dot{V}(b)$ = 0.20 mL/min. Detector wavelength = 280 nm.

TABLE 3
Comparison of Experimental and Theoretical Protein Retrieval at Outlet $b$ ($F_b$) in Acetate and Phosphate Buffers for Four Proteins with $\dot{V}(a') > \dot{V}(a)$, $\dot{V}(b') = 0$ (second set)[a]

| Protein studied | pI (ref) | $\mu \times 10^5$ $cm^2/V \cdot s$ | $bL\mu E$ (mL/min), calculated | $bL\mu E$ (mL/min), required | | $F_b(A+B-)$ | | $F_b(A-B+)$ | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | $(A+B-)$ | $(A-B+)$ | Theory | Exp | Theory | Exp |
| pH 4.75:[b] | | | | | | | | | |
| Ferritin | 4.4 | −0.94 | 0.25 | 0.46 | 0.13 | 0.54 | 0.57 | 1.00 | 0.94 |
| BSA | 4.9 | 0.63 | 0.16 | 0.13 | 0.46 | 1.00 | 0.92 | 0.65 | 0.61 |
| Hemoglobin | 6.9 | 2.74 | 0.68 | 0.13 | 0.46 | 1.00 | 0.98 | 0.00 | 0.06 |
| Cytochrome c | 10.6 | 0.93 | 0.24 | 0.13 | 0.46 | 1.00 | 0.99 | 0.48 | 0.45 |
| pH 5.60:[c] | | | | | | | | | |
| Ferritin | 4.4 | −3.96 | 1.03 | 0.37 | 0.21 | 0.00 | 0.00 | 1.00 | 1.00 |
| BSA | 4.9 | −2.30 | 0.60 | 0.37 | 0.21 | 0.00 | 0.00 | 1.00 | 1.00 |
| Hemoglobin | 6.9 | 1.70 | 0.44 | 0.21 | 0.37 | 1.00 | 1.00 | 0.00 | 0.04 |
| Cytochrome c | 10.6 | 0.83 | 0.22 | 0.21 | 0.37 | 1.00 | 1.00 | 0.41 | 0.36 |
| pH 7.80:[d] | | | | | | | | | |
| Ferritin | 4.4 | −11.22 | 2.19 | 0.37 | 0.21 | 0.00 | 0.00 | 1.00 | 1.00 |
| BSA | 4.9 | −9.53 | 1.86 | 0.37 | 0.21 | 0.00 | 0.00 | 1.00 | 0.98 |
| Hemoglobin | 6.9 | −1.18 | 0.23 | 0.37 | 0.21 | 0.62 | 0.58 | 1.00 | 0.96 |
| Cytochrome c | 10.6 | 0.46 | 0.09 | 0.21 | 0.37 | 0.43 | 0.40 | 0.76 | 0.70 |

[a] $E$ = 30 V; $A+B-$: wall $A$ positively charged and wall $B$ negatively charged; $A-B+$: wall $A$ negatively charged and wall $B$ positively charged detector wavelength = 280 nm; ferritin = 0.03 mg/mL; BSA, hemoglobin, cytochrome c = 0.125 mg/mL.
[b] $\dot{V}(a') = 0.59$, $\dot{V}(b') = 0.0$, $\dot{V}(a) = 0.13$, $\dot{V}(b) = 0.46$ mL/min for pH 4.75, 0.01 M acetate buffer.
[c] $\dot{V}(a') = 0.58$, $\dot{V}(b') = 0.0$, $\dot{V}(a) = 0.21$, $\dot{V}(b) = 0.37$ mL/min for pH 5.60, 0.01 M acetate buffer.
[d] $\dot{V}(a') = 0.58$, $\dot{V}(b') = 0.0$, $\dot{V}(a) = 0.21$, $\dot{V}(b) = 0.37$ mL/min for pH 7.80, 0.005 M phosphate buffer.

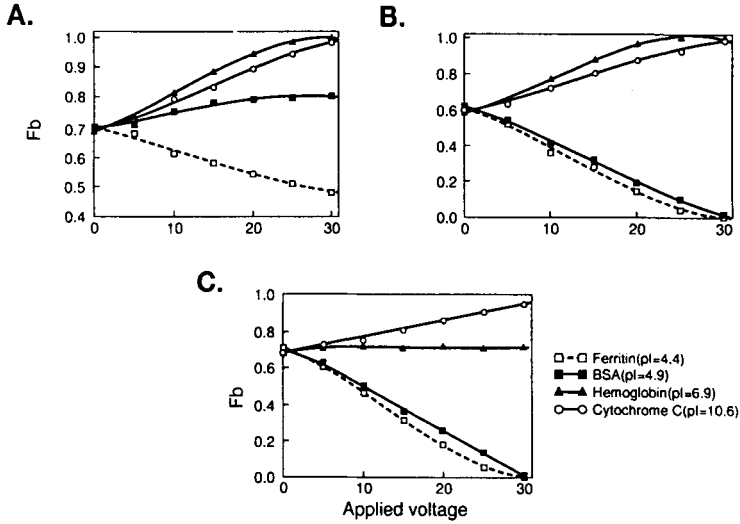


FIG. 5 $F_b$ response of proteins at various electrical fields and solution pHs. (A) pH 4.75, 0.01 M acetate, $\dot{V}$ (mL/min): $a' = 0.59$, $b' = 0$, $a = 0.13$, $b = 0.46$. (B) pH 5.60, 0.01 M acetate, $\dot{V}$ (mL/min): $a' = 0.55$, $b' = 0$, $a = 0.18$, $b = 0.37$. (C) pH 6.87, 0.01 M phosphate, $\dot{V}$ (mL/min): $a' = 0.56$, $b' = 0$, $a = 0.15$, $b = 0.41$.



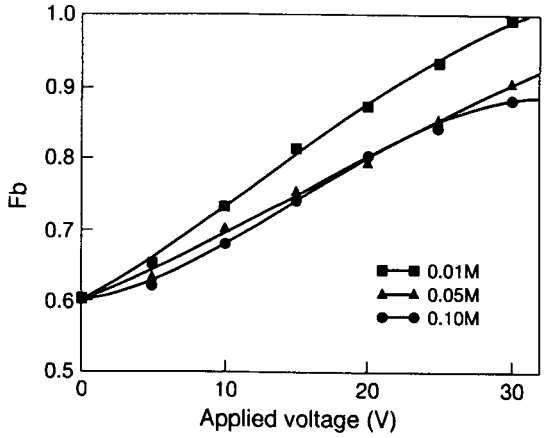


FIG. 6 Effect of ionic strength of acetate buffer on retrieval $F_b$ response of hemoglobin at pH 4.75, $\dot{V}$ (mL/min): $a' = 0.63$, $b' = 0$, $a = 0.24$, $b = 0.39$.

increases but to a lesser degree than that at 0.01 M. Cytochrome c retrieval showed the same trends as hemoglobin. The 0.01 M buffer was used for the rest of the experiments. The effect of buffer ionic strength on the $F_b$ value was clear in the case of BSA, where the values of solution pH and protein pI were nearly identical. The $F_b$ of BSA was constantly low at different applied voltages and at buffer ionic strengths of 0.05 and 0.10 M. However, the $F_b$ of BSA in 0.01 M buffer increased slowly with the applied voltage. This finding shows that different ionic strengths can affect the pI value of protein and thus the mobility, as reported elsewhere in the literature (16).

### *Protein Recovery*

The acetate and phosphate buffers used in the recovery study yielded similar results. Results for the acetate buffer at pH 5.6 are shown in Table 4. Recoveries of the six different proteins studied here ranged from 78 to 90%, with cytochrome c having the lowest recovery. This may have occurred because cytochrome c has a hydrodynamic size close to the pore size of the membrane. Thus, a membrane with a lower molecular weight cutoff (and uniform pore size) would improve the recovery of cytochrome c and other proteins with comparable hydrodynamic diameters. Increasing



TABLE 4
Mass Balance Study of Proteins Using 0.01 M Acetate Buffer, pH 5.6, in an Isoelectric SPLITT System[a]

| Protein[b] | MW[c] (g/mole) | Through the channel 30 V[d] | | |
|---|---|---|---|---|
| | | Eluted[e] | Late released[f] | Loss[g] |
| Cytochrome c | 13,400 | 0.78 | 0.05 | 0.17 |
| α-Chymotrypsinogen A | 24,000 | 0.85 | 0.06 | 0.09 |
| BSA | 68,000 | 0.88 | 0.05 | 0.07 |
| Hemoglobin | 76,000 | 0.90 | 0.05 | 0.05 |
| IgG | 150,000 | 0.86 | 0.09 | 0.05 |
| Ferritin | 450,000 | 0.84 | 0.09 | 0.06 |

[a] $\dot{V}(a') = 0.60$, $\dot{V}(b') = 0.0$, $\dot{V}(a) = 0.34$, $\dot{V}(b) = 0.26$ mL/min.
[b] Initial sample concentration (mg/mL): Hemoglobin, BSA, cytochrome c, IgG, α-chymotrypsinogen A: 0.50; ferritin: 0.25.
[c] MW cutoff of the membrane is 8000.
[d] Sample bypass of the channel was taken as reference (1.0).
[e] Eluted: Detected at both detectors at 30 V.
[f] Late released: Detected at both detectors when the electrical field is turned off.
[g] Loss: Total amount bypass the channel - come out - absorption.

the flow rate from 0.6 to 1.2 mL/min improved recovery by only a few percent.

### *Separation of Protein Mixtures with Pulsed Sample Injection*

All the separations in this experiment were run using system 2 of Fig. 1 to show how isoelectric SPLITT fractionation can increase the throughput of the separation and concentrate the fractionated proteins. Pulsed sample injection was used to test the applicabilty of the isoelectric SPLITT system for protein separation and to optimize experimental conditions for subsequent work involving CSF. Protein mixtures containing various combinations of cytochrome c and BSA, hemoglobin and ferritin, IgG and albumin, hemoglobin and BSA, and hemoglobin and α-chymotrypsinogen A were successfully separated. The resulting fractograms are shown in Figs. 7 through 9. Experiments were done by first injecting each protein separately as shown in the right-hand portion of each fractogram. The detector signals show that the two individual proteins were fully recovered at their respective outlets. Then a sample mixture was injected that contained the two proteins present in the same amounts as in the individual injections. The results are shown in the left-hand part of each fractogram. The separation of a protein mixture into the individual components was



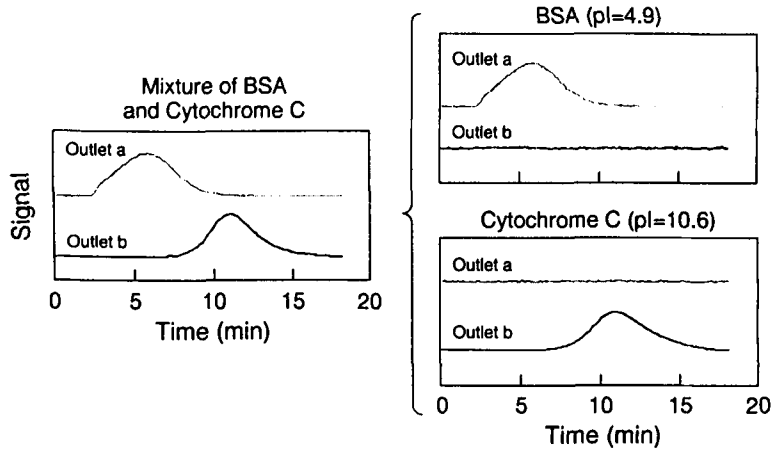


FIG. 7 Separation of protein mixture: cytochrome c and BSA, pH 5.60, 0.01 M acetate buffer, $\dot{V}$ (mL/min): $a' = 0.46$, $b' = 0$, $a = 0.31$, $b = 0.15$, 30 V, with a positively charged wall $A$ and a negatively charged wall $B$.

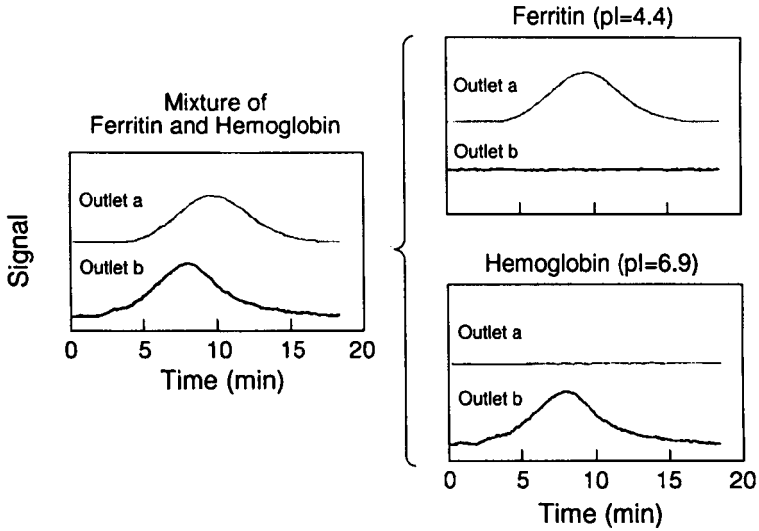


FIG. 8 Separation of protein mixture: hemoglobin and ferritin, pH 5.60, 0.01 M acetate buffer, $\dot{V}$ (mL/min): $a' = 0.44$, $b' = 0$, $a = 0.22$, $b = 0.22$, 30 V, with a positively charged wall $A$ and a negatively charged wall $B$.

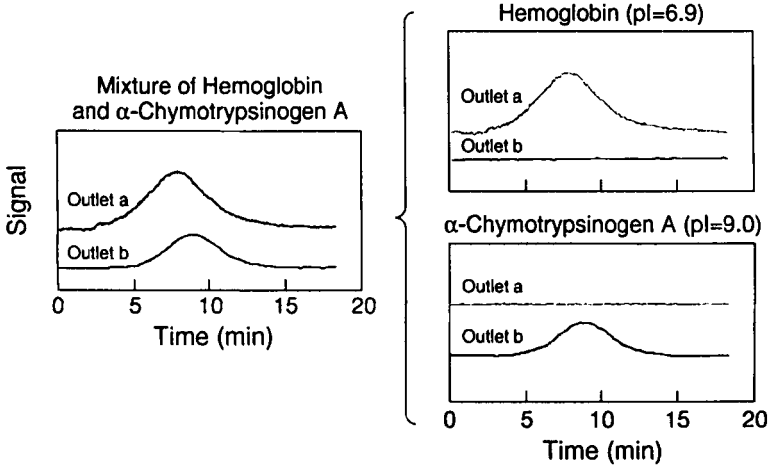


FIG. 9 Separation of protein mixture: hemoglobin and α-chymotrypsinogen A, pH 7.8, 0.01 M phosphate buffer, $\dot{V}$ (mL/min): $a' = 0.41$, $b' = 0$, $a = 0.22$, $b = 0.19$, 30 V, with a positively charged wall $A$ and a negatively charged wall $B$.

complete as indicated by the same peak areas for the proteins in a mixture and those injected individually. The concentration shown in the figures was the highest concentration that could be run without contaminating the other outlet. Ferritin could be separated at a lower concentration than the other proteins. The minimum pI difference between proteins that could be separated was approximately two pI units. All these examples show that the isoelectric SPLITT system can successfully separate the proteins.

## Continuous SPLITT Fractionation of Protein Mixtures

The separation of protein mixtures was further studied with continuous sample introduction. Protein mixtures (IgG and albumin, hemoglobin and BSA, and hemoglobin and α-chymotrypsinogen A) were continuously introduced into the channel for fractionation over 8 hours. The fractionated proteins were collected at different times for further purity analysis. Flow FFF was used to check the purity of the separation involving IgG and albumin. The fractions were collected at the end of each 8-hour run. The reference fractograms of human albumin and IgG were run with pure samples, respectively. The data in Fig. 10 clearly show a very clean separation. The recovery of IgG was 89%. Spectrophotometric analysis at 415 nm was used to check the purity of the separation of hemoglobin from



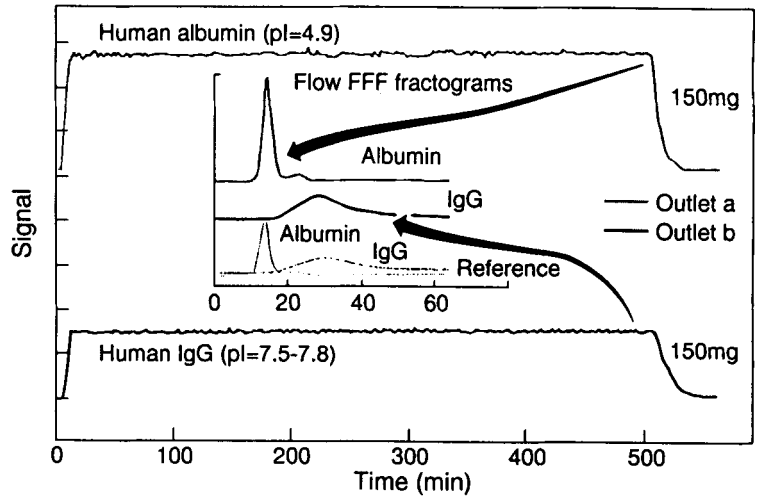


FIG. 10 Continuous SPLITT fractionation of human IgG and albumin, pH 5.6, 0.01 M acetate buffer, $\dot{V}$ (mL/min): $a' = 0.45$, $b' = 0$, $a = 0.29$, $b = 0.16$, 30 V, with a positively charged wall $A$ and a negatively charged wall $B$. Purity was confirmed with flow FFF.

BSA and hemoglobin from α-chymotrypsinogen A. The CSF fractograms for these two mixtures are shown in Figs. 11 and 12, respectively. In Fig. 11, outlet *b* shows that the concentration of hemoglobin was 1.36 mg/mL based on previously established calibration curves described in the Experimental Section. The original sample concentration of hemoglobin was 0.7 mg/mL. Thus, the recovery of hemoglobin was 88% after correcting for the effect of flow rate. Outlet *a* shows no detectable amount of hemoglobin, confirming complete separation of hemoglobin and BSA. In Fig. 12, the concentration of hemoglobin eluted from outlet *a* was 0.8 mg/mL, a recovery of 86%. Outlet *b* did not show any detectable amount of hemoglobin, verifying the successful separation of hemoglobin and α-chymotrypsinogen A. Acetate buffer yielded a slightly higher throughput than phosphate buffer.

Isoelectric CSF has been demonstrated to be capable of the preparative separation of proteins with pI differences equal to or larger than two units. Also, the fractionated proteins were more concentrated than that of the original sample. The throughput was approximately 15 mg/h. The throughput of this system is proportional to 1) sample concentration, 2) sample inlet flow rate, 3) applied electrical field, and 4) cross-sectional area of



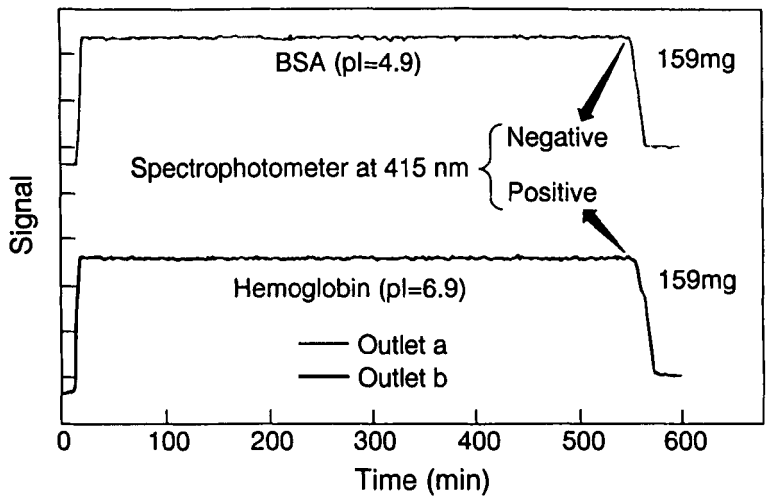


FIG. 11 Continuous SPLITT fractionation of hemoglobin and BSA mixture, pH 5.6, 0.01 M acetate buffer, $\dot{V}$ (mL/min): $a' = 0.42$, $b' = 0$, $a = 0.23$, $b = 0.19$, 30 V, with a positively charged wall *A* and a negatively charged wall *B*. Purity was confirmed with spectrophotometric analysis at 415 nm.

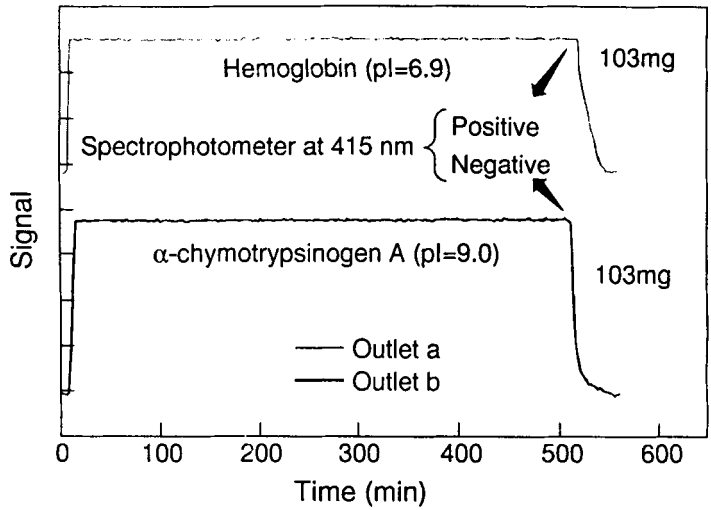


FIG. 12 Continuous SPLITT fractionation of hemoglobin and α-chymotrypsinogen A, pH 7.80, 0.005 M phosphate buffer, $\dot{V}$ (mL/min): $a' = 0.41$, $b' = 0$, $a = 0.22$, $b = 0.19$, 30 V, with a positively charged wall *A* and a negatively charged wall *B*. Purity was confirmed with spectrophotometric analysis at 415 nm.



channel (channel length multiplied by channel breadth). A sample concentration of 0.7 mg/mL was used in this experiment with clean and successful separation. The highest sample concentration that can be used for clean and successful separation under highest voltage need further study. The sample interaction under very high concentration (overloadings) could cause deviations from theory in separation. The present setup can apply voltage up to 45 V for clean separation of the protein mixtures tested in this study. To increase the throughput with a higher applied voltage requires further study. Free-flow electrophoresis studies of stability might be useful as a reference for future study of stability (17). The throughput could also be increased to some extent by using a longer channel length, channel breadth, or both than in the present setup.

## Comparison with Other Techniques for Protein Separation

Isoelectric focusing has been used previously for preparative separation of proteins (18). However, a pH gradient is needed. In contrast, isoelectric SF can concentrate the fractionated samples without a pH gradient, which can cause adverse effects (e.g., precipitation). The extent of the concen-

trated outlet fraction is proportional to the flow ratio $\dot{V}(a')/\dot{V}(a)$ or $\dot{V}(a')/\dot{V}(b)$ for outlet $a$ and $b$, respectively. HPLC has been useful for microscale separation (19). Preparative HPLC has also been used for preparative separation of protein, but the possibility of denaturing caused by the packing material is still a concern (17). Isoelectric SF has some similarities with the free-zone operation mode of free-flow electrophoresis (17). Both use thin cell geometry for separation, and the applied field is perpendicular to the flow. The separation axis for SF is the thin dimension, but for free-flow electrophoresis it is the wide dimension. Therefore, the separation distance for SF is much shorter than that for free-flow electrophoresis. Also, because the separation distance is narrow for isoelectric SPLITT fractionation, only two outlets are required, whereas free-flow electrophoresis has multiple outlets. Isoelectric SF has a higher separation speed and free-flow electrophoresis has a higher resolution between these two techniques due to the different separation distances.



## CONCLUSIONS

The separation achieved with isoelectric SF is based on the difference in isoelectric points of the samples. An isoelectric SF system was characterized and shown to be able to separate protein mixtures. Continuous SF has also been run stably under unattended conditions for 8 hours with clean separation. The fractionated proteins were more concentrated than the feeding proteins. The scheme to separate a mixture of three or more proteins would depend on the pI distribution of the protein mixture. The minimal pI difference needed between proteins to be separated was about 2 units, and the throughput was about 15 mg/h in this study. Higher throughput may be achieved by increasing the applied electrical field, using a larger channel cross section (channel breadth $\times$ channel length), or both. In conclusion, our findings indicate that isoelectric SF has promise as a step for the preparative purification of proteins in biotechnology and molecular biology.

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Special thanks to a great scientist, Prof. J. Calvin Giddings, who invented SPLITT fractionation technique and gave me lots of inspiration during my graduate and postdoc work in Utah. Professor Giddings died on October 24, 1996. His passing is a great loss to the separations community.

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