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Journal of Chromatography A, 229 (1996) 229–236

JOURNAL OF CHROMATOGRAPHY A

Focusing proteins in an electric field gradient

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Received 13 June 1995; revised 12 September 1995; accepted 21 September 1995

Abstract

Gradient techniques are a class of separation methods that combine the steps of isolation and concentration. This class of techniques uses one or more gradients of counteracting forces to create an 'equilibrium point' at which a protein can focus. Equilibrium gradient techniques can be adapted to a specific separation by choosing appropriate counteracting forces based on differences in the physical characteristics of proteins. The method introduced here, field gradient focusing, is an addition to this class of separation techniques which employs a gradient in the electric field to simultaneously separate and concentrate molecules.

Keywords: Field gradient focusing; Proteins

1. Introduction

Advances in genetic engineering and cell culture have greatly increased the variety of proteins available for use as diagnostic and therapeutic agents [1,2]. This has challenged the biotechnology industry to find separation techniques that can achieve high purity, yet be gentle enough to retain the protein's biological activity. While much work has been done to adapt conventional systems to biological product recovery and to scale-up existing laboratory techniques, the unique properties of biological products suggest a need for alternative techniques [3].

O'Farrell [4] brought renewed interest to the field of electrochromatography as a preparative-scale separation medium with his paper on "Counteracting chromatographic electrophoresis", alias, CACE. Perhaps more importantly, he identified CACE as a member of a larger class of methods that create a stable equilibrium point about which a protein will focus. These techniques have been classified as equilibrium gradient methods [5,6]. They work by applying a net force that changes direction at some point in a column so that this force acts everywhere to push constituents towards the 'equilibrium' point (see Fig. 1). Because the applied force depends on the properties of the molecule, e.g., surface charge, solutes with different properties will migrate to unique equilibrium positions and remain there. Existing equilibrium gradient techniques include density gradient sedimentation and isoelectric focusing.

A net force that can change direction at a fixed point is generated by applying two or more coun-

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0021-9673/96/$15.00
SSDI 0021-9673(95)01069-6

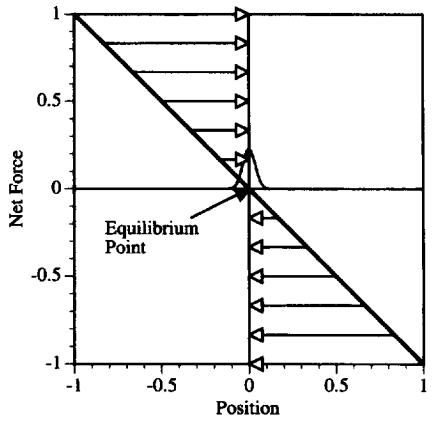

Fig. 1. Generic equilibrium gradient. Solute focuses at the equilibrium point where the net force is zero. Upstream of this point, the net force is positive and pushes the solute in the positive direction, towards the equilibrium point. Solute located downstream is pushed in the negative direction by a negative force, again towards the equilibrium point.

teracting forces[2], usually with a gradient in one while all others are held constant. Various methods for doing this have been suggested [6] and include using combinations of gravitational, magnetic and electrophoretic forces as the constant forces. Most electrochromatographic research has been limited to using velocity and pH gradients to vary the net force.

This paper examines a new equilibrium gradient method where variation in the net force is created by varying the electric field strength throughout the column. The fundamental behavior of the technique is first described by a simple linear model and then illustrated using an electrochromatography column designed for this purpose.

2. Theory

In field gradient focusing (FGF) a constant solvent velocity is opposed by a gradient in a solute's electrophoretic velocity which has been created by spatially varying the electric field. The basic requirements to achieve focusing with this method (or any other equilibrium gradient method) can be estimated by using a linear model to predict the steady-state concentration profile. A charged particle in solution will migrate in an electric field with a velocity, v_i, that is proportional to the charge of that particle, z_i, and the electric field, E, i.e.:

$$v_i = z_i \omega_i E \tag{1}$$

where ω_i, is defined as the mobility of the particle and depends on the size of the particle, its density, and the strength of interactions with the solvent.

At very low particle concentrations the one dimensional flux of a charged particle can be described by the flux equation:

$$J_i(x) = -D_i \frac{\mathrm{d}}{\mathrm{d}x} c_i + [u + z_i \omega_i E(x)] c_i \tag{2}$$

where u is the convective velocity, c_i is the concentration of the ion, and D_i is a dispersion coefficient which includes the effects of diffusion and mechanical mixing. Note that u and v_i must have opposite signs if they are to be 'counter-acting'. This requirement is met if z_i is negative or if the polarity of the field is switched.

The geometry of the system is a chamber of length $2L$ with its origin at the chamber's center, a linear electric field gradient can be defined as:

$$E(x) = E_0 + E_1 x \tag{3}$$

where E_0 is the average field in the chamber and E_1 is the increase in field strength per unit length. Because the electric field cannot switch polarity at any point in the chamber, E must be greater than zero everywhere and E_0 must be greater than or equal to $E_1 L$. At steady-state the net flux is zero and the flux equation can be integrated to find the concentration distribution:

$$c_i(x) = k \exp\left\{ \frac{1}{D_i} \left[(u + z_i \omega_i E_0) x + \frac{1}{2} z_i \omega_i E_1 x^2 \right] \right\} \tag{4}$$

The constant of integration, k, can be found by specifying the total mass of i. If A is the area of the chamber, then the total moles of i, M_i, can be found by integrating over the length of the column. If the chamber is long enough so that the concentration

[2] This is not the case for isoelectric focusing. Because the charge on the protein can change sign depending on the pH, the electrophoretic force can change direction and a counter-acting partner is not needed.

approaches zero at the boundaries, the limits on the integration can be replaced by plus and minus infinity to simplify the integration, i.e.:

$$M_i = A\int_{-L}^{L} c_i(x)\mathrm{d}x \approx A\int_{-\infty}^{\infty} c_i(x)\mathrm{d}x \tag{5}$$

Using Eq. 5 to solve for k in Eq. 4, the concentration profile is now given by:

$$c_i(x) = \frac{M_i}{A}\sqrt{-\frac{z_i\omega_i E_1}{2\pi D_i}} \times \exp\left[\frac{z_i\omega_i E_1}{2D_i}\left(x + \frac{u + z_i\omega_i E_0}{z_i\omega_i E_1}\right)^2\right] \tag{6}$$

which yields a Gaussian distribution about the focal point:

$$x_f = -\frac{u + z_i\omega_i E_0}{z_i\omega_i E_1} \tag{7}$$

with variance:

$$\chi^2 = \frac{D_i}{|z_i\omega_i E_1|} \tag{8}$$

Note that z_i and E_1 must have opposite signs or else the solution would be imaginary due to the quantity under the square root in Eq. 6. This implies that, if the electric field gradient slopes in the wrong direction, the net force will act to push everything away from the equilibrium point, dispersing the solute rather than focusing it. The variance (Eq. 8), χ^2, is a measure of the width of the focused protein peak and suggests that the band will be tighter if the dispersion coefficient is decreased or if the slope of the electric field is increased. Eq. 7 for the focal point shows that the flow-rate and the average electric field determine the location of the protein peak.

The linear model derived above applies only at low protein concentrations and assumes that both the buffer ion concentrations and the electric field gradient are unaffected by the presence of focused protein. In reality, the local conductivity changes where the protein concentration exceeds about 0.5%. This alters the local electric field and changes the shape of the protein peak in a manner which is not predicted by the linear model.

3. Electric field 'shaping'

The electric field gradient can be created by varying the area through which the total current flows. At constant electrolyte concentration, the electric field is approximately related to the total current passing through a chamber by the expression:

$$E(x) = \frac{I}{\sigma A(x)} \tag{9}$$

where σ is the conductivity of the buffer and $A(x)$ is the cross-sectional area of the chamber normal to the flow. Simply varying the area of the column would cause the opposed flow to change at precisely the same rate as the field and would not yield a unique focal point in the chamber. That is, for a fixed volumetric flow, $Q = uA(x)$, and current, $I = \sigma EA(x)$, Eq. 4 becomes:

$$c_i(x) = k\exp\left\{\frac{1}{D_i}\left[\int\left(\frac{Q}{A(x)} + \frac{z_i\omega_i I}{\sigma A(x)}\right)\mathrm{d}x\right]\right\} = k\exp\left\{\left(\frac{Q}{D_i} + \frac{z_i\omega_i I}{\sigma D_i}\right)\left[\int\frac{1}{A(x)}\mathrm{d}x\right]\right\} \tag{4*}$$

where the integral on the far right cannot be equal to zero since the cross-sectional area must always be positive. The premultiplicative term in this expression is not a function of position and so no unique focal point can exist in this case, i.e., when the electric field and the velocity vary at the same rate.

This limitation is removed by dividing the chamber into two parallel regions separated by a membrane which is permeable to the current but not to bulk fluid flow (see Fig. 2). The cross-sectional area on the 'working' side of the membrane is constant so that the flow-rate in this region will also be constant. However, the current can pass through the dialysis membrane (via the buffer ions) and so the electric field is shaped according to the cross-sectional area of the chamber as determined by Eq. 9.

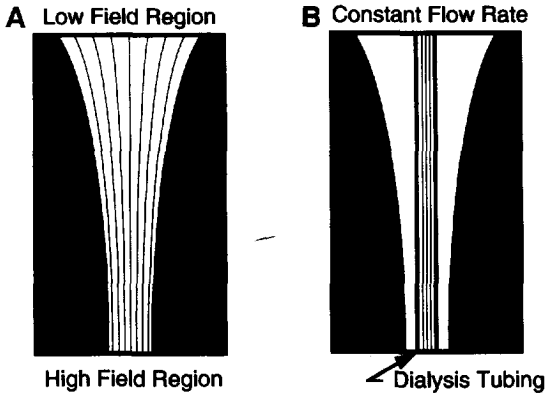

Fig. 2. Current (A) and flow (B) streamlines. The electric field is shaped by varying the area through which the current flows. As the area decreases (A), the current density increases, as does the field; small ions will carry the current across the membrane so that the total area shapes the field. However (B), the flow-rate inside the dialysis tubing remains constant because there is no bulk fluid flow across the membrane.

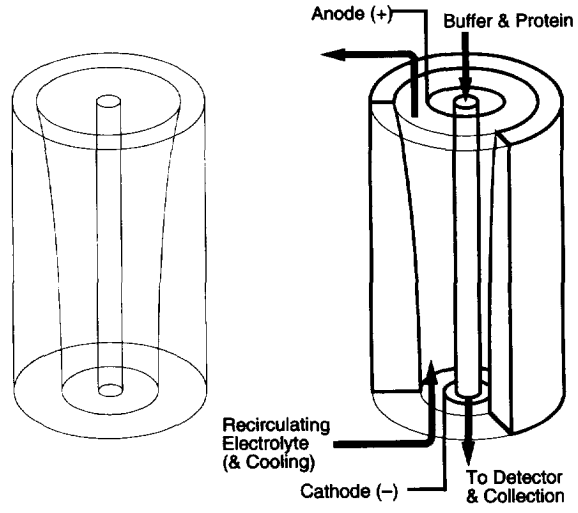

Fig. 3. Simplified diagram of apparatus. In both figures, the outer shell is the shaped Plexiglas cylinder and the inner tubing is the dialysis tubing. The figure on the right shows the placement of electrodes and identifies the flows.

An expression for the spatial variation in the area needed to create a linear electric field can be found by substituting Eq. 3 into Eq. 9 to get:

$$A(x) = \frac{I}{\sigma(E_0 + xE_1)} \tag{10}$$

Once the equipment is fabricated, the variation in the area is fixed but the field strength can be changed by changing the current or the conductivity. It is more useful to describe the area with the equation:

$$A(x) = \frac{2A_T A_B}{\frac{x}{L}(A_T - A_B) + (A_T + A_B)} \tag{11}$$

where A_T and A_B are the total cross-sectional areas at the top and bottom of the column, respectively.

4. Experimental

The focusing apparatus consisted of a 6.4-mm wet diameter dialysis tube (BioDesign) mounted in the center of a shaped Plexiglas cylinder (see Fig. 3). Two platinum electrodes (one at the top and one at the bottom) were located in the annular space through which the externally cooled electrolyte buffer flowed. This recirculating buffer was cooled in a glass heat exchanger in countercurrent flow against de-ionized water cooled to 4°C. The dialysis tubing was packed with ToyoPearl HW040F size-exclusion resin with a nominal particle size of 45 μm and an exclusion limit of 10 000 amu. The bed was supported at the bottom by a cotton plug but the top surface of the bed was free. The working buffer inside the dialysis tube flowed downward to prevent fluidization. The column was mounted in an ABI Model 230A HPEC system to use the unit's UV spectrophotometer which was located 20 cm from the bottom of the column. A back-pressure regulator (Rainin) was installed downstream of the detector to prevent outgassing in the detector and to pressurize the dialysis tubing.[3]

Although more than a few experiments have been performed reproducibly, only two representative examples are included here. The first experiment is the focusing of hemoglobin from a dilute solution to a band of more concentrated protein to demonstrate that FGF is an equilibrium gradient technique. The second experiment demonstrates the purification

[3] Dehydrated dialysis tubing has permanent creases that create corner effects. By expanding the tube slightly, these effects can be mitigated somewhat.

potential of this new method by separating two different oxidation states of myoglobin.

4.1. Hemoglobin

Focusing of bovine hemoglobin (Sigma) was accomplished in a 5 m*M* Tris–13.3 m*M* glycine buffer at pH 8.7 with a flow-rate of $2.54 \cdot 10^{-4}$ ml/s and a voltage of 1000 V. The initial current was 12 mA, but this increased over the course of the experiment, indicating that the bulk conductivity of the recirculating buffer was increasing. The final current was 22 mA, seven hours after the electric field was turned on.

The experiment began by loading approximately 1 ml of a 0.1% (w/v) solution (approx. 1 mg) of the hemoglobin onto the column. After the leading edge of the sample reached the bottom of the column, the field was turned on. Fig. 4 is a series of pictures taken at 3-h intervals showing the progressive focusing of hemoglobin from the 0.1% (w/v) solution. In the last picture in this series, there is a second band above the major band which could be an isoform of hemoglobin, or perhaps a different oxidation state. The final band does not resemble the Gaussian distribution predicted by the theory; the downstream edge is very sharp while the upstream edge is diffuse and shows considerable skew.

In addition to the observed skewing, the edges of the focused bands are rarely flat around the perimeter of the dialysis tube and in Fig. 4c and Fig. 6 they show considerable deformation. This maldistribution of the solute is a result of a slight mismatch between the hydrodynamic flow and electrophoretic migration in the opposite direction. It might be due to slight temperature variations around the perimeter caused by uneven cooling, or edge effects where the column packing contacts the dialysis tubing, or even an instability in the current flow due to dielectric or conductivity gradients. However, on the basis of our observations, it seems most likely that minor imperfections in the shape of the dialysis tube, e.g., a crease or seam along one side of the tube, are responsible for this maldistribution. This problem could perhaps be solved by placing the dialysis tube inside a rigid, porous support with a precision-machined inner surface.

After the protein focused, the field was turned off and the protein was eluted through a spectrophotometer. A second pulse of approximately 1 ml of 0.1% hemoglobin was then run through the column without focusing for comparison. Fig. 5

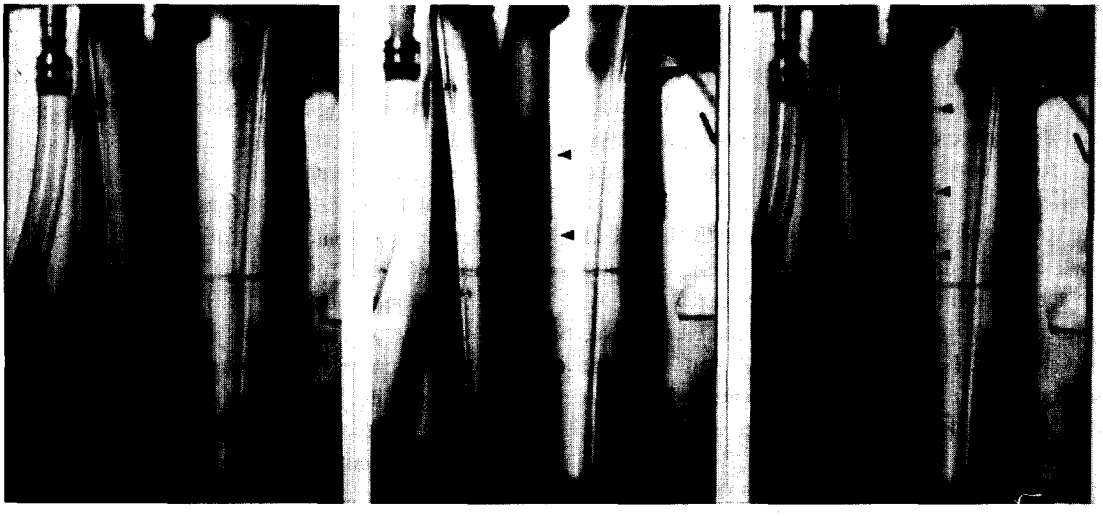

Fig. 4. Focusing of hemoglobin. The gray cylinder in the center of each picture is the packed bed. (left) The bed is completely filled with 0.1% (w/v) hemoglobin solution, $t=0$. (middle) Progression to a focused band at 3 h; the arrowheads mark the edges of the partially focused band(s). (right) The major band is focused between the arrowheads just above the center mark. There is also a secondary band near the top arrowhead, focused on the right-hand side of the bed.

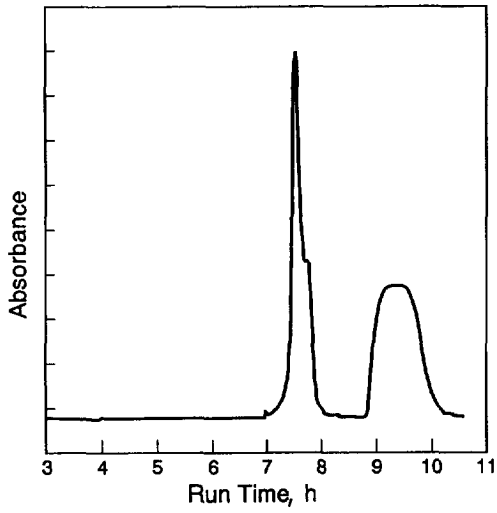

Fig. 5. Absorbance trace of hemoglobin-focusing experiment. The first peak is the focused hemoglobin band. The cusp on this peak is the secondary band. The second peak is the unfocused hemoglobin at 0.1% (w/v) concentration, shown for comparison.

shows the absorbance trace at 300 nm of the run. Assuming a linear correlation between concentration and absorbance, a two- to three-fold increase in protein concentration is estimated from the relative heights of the two peaks.

4.2. Myoglobin

Separation of myoglobins with different oxidation states was accomplished using the same buffer and a potential difference of 1000 V. The current increased from 12 to 20 mA during the seven hours the field was applied.

The experiment began by loading about 0.3 ml of a 0.5 wt.% horse-heart myoglobin (Sigma) solution into the top half on the chamber (approx. 1.5 mg). At this point all of the protein was in the brown, oxidized form. The field was turned on and the protein focused at the top of the column[4] with a flow-rate of $0.37 \cdot 10^{-4}$ ml/min. After about 1 h, a second band of reddish myoglobin began to form above the other band. When this second band was well established (about 2 h), the flow-rate was increased to $1.83 \cdot 10^{-4}$ ml/s and the bands were refocused near the bottom. The bands were held here for about two more hours until they were of about equal concentration.

The iron atom in the heme group of blood proteins can be found in the ferrous (2+) or the ferric (3+) oxidation state. Ferromyoglobin (2+) can bind oxygen and will become oxygenated even at low oxygen partial pressures. However, prolonged exposure to oxygen will oxidize the molecule to ferrimyoglobin (3+) which can no longer bind oxygen. With the loss of an associated oxygen molecule, myoglobin turns from red to brown in color [7]. Purification of the oxidized form of myoglobin (3+) often results in the formation of two bands as has been reported for capillary zone electrophoresis [8] and isoelectric focusing [9]. It has been proposed that the mechanism of the formation of the second band is the reduction of oxidized myoglobin back to its native state.

A picture of these focused myoglobin bands is shown in Fig. 6. The bands were clearly separated by about 2.5 cm although there is a murky area between the bands, probably consisting of reduced myoglobin moving from one band to the other. A small but distinct doublet of myoglobin bands whose nature is unknown also formed above the major bands, very close to the anode. Only the lower one of these is visible in the figure. These may be isoforms of myoglobin, or contaminants.

After steady-state was reached, the electric field was turned off and the bands were then eluted to the detector. A second pulse of the original myoglobin sample was run through the column for comparison. The absorbance trace at 300 nm is shown in Fig. 7. The degree of separation seen on the absorbance trace is much less than that observed in the chamber at steady-state. These are probably due to extra-column effects resulting from a poor outlet design since there was a lot of peak dispersion near the bottom of this column.

The idea of using a membrane as the boundary between phases was an elegant solution to the field-shaping problem, but proved difficult to implement. Also, the performance of the system was sensitive to

[4] Reduction of the 3+ iron occurs faster when the myoglobin is focused at the top of the chamber (anode) than when it is focused at the bottom of the chamber. This may be due to the higher concentration of Tris at this point, or a slightly higher temperature (the chilled recirculating buffer flows in at the bottom and out the top).

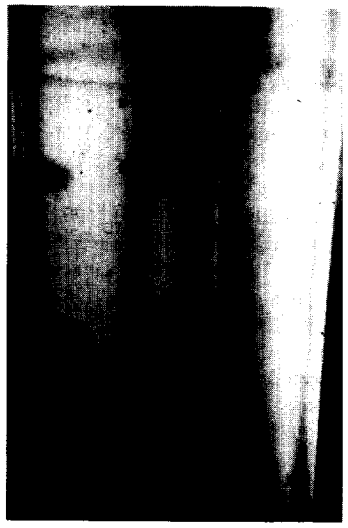

Fig. 6. Separation of myoglobins. The picture shows a close-up of the final steady-state distribution of the separated myoglobin bands. The band between the lower set of arrowheads is the oxidized form of myoglobin (3+). The band between the upper set of arrowheads is the reduced form (2+) which was redder in color. There were two more minor bands above these.

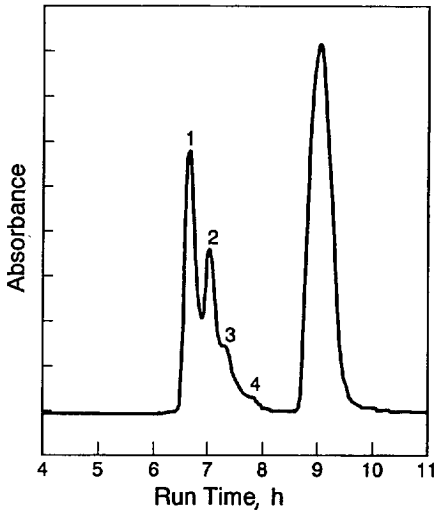

Fig. 7. Absorbance trace of myoglobin separation. The first set of peaks shows the four different components resolved during focusing. Peak 1 is the original ferrimyoglobin (3+) and peak 2 is the reduced ferromyoglobin (2+). The last peak is the unfocused sample at 0.5% (w/v) concentration, shown for comparison.

how well the membrane was aligned along the axis of the chamber. If either the top or bottom of the tubing was 'kinked' during assembly, or if the piece of tubing itself was not at least moderately straight, the protein would focus against one side of the bed and form a long smear on the side of the column. Because the field lines are bent due to the varying area of the chamber (see Fig. 2), there is a radial component to the electric field that pushes the protein away from the center of the chamber.

An additional hemoglobin-focusing experiment was performed to check the solute distribution inside the bed. Instead of eluting to the detector after the hemoglobin focused, the apparatus was disassembled and the bed frozen. Cutting the bed longitudinally revealed that, although the hemoglobin band was distributed evenly around the bed, it did not penetrate more than 2 mm into the interior of the bed. If the bed is not aligned properly, the radial component of the electric field will act unevenly and the protein will be pushed to one side of the bed.

5. Conclusion

Equilibrium gradient methods hold promise in bioprocessing because they can combine the steps of isolation and concentration. They can also be adapted to a separation as needed by choosing appropriate counteracting forces based on physical differences in the specific proteins involved. Field gradient focusing is a new addition to this class of separation techniques with the unique property of using a gradient in the electric field instead of using a gradient in the flow or in column pH.

The experiments presented above verify that FGF is an equilibrium gradient method. Hemoglobin was focused from a dilute solution to a concentrated band and different oxidation states of myoglobin were focused and separated. These experiments, carried out in a first-generation apparatus, demonstrate that FGF has the power to separate proteins that differ by only one charge on a milligram scale, and can do so in a moderate amount of time, with minimal power consumption, no special buffer requirements, and without having to add chemical modifiers such as ampholytes.

One question that still needs to be addressed is the

scale-up of this separation method. While electrochromatography appears to be limited by heat transfer in the bed, field gradient focusing can be implemented in free solution and may therefore be adaptable to larger scale, albeit, in a non-chromatographic system.

Acknowledgments

W.S. Koegler was supported by a National Science Foundation Graduate Research Fellowship during her MS research.

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