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OPEN-CHANNEL ISOELECTRIC FOCUSING IN THERMALLY ENGENDERED pH GRADIENTS

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SUMMARY

The use of thermally formed pH gradients permits macromolecular separations in open-channel isoelectric focusing (IEF), simplifying product recovery, shortening run times and eliminating costly carrier ampholytes. Because of these unique advantages and other limitations this IEF method may effect purifications on a preparative scale more effectively than on an analytical scale where other IEF methods may cover a larger pH range. Heat exchangers at both ends of a 3.18 mm $\times$ 5.0 mm $\times$ 28.0 mm channel generated the temperature gradient (4°C cm^{-1}). Owing to the large temperature dependence of the pH ($dpH/dT = -0.028\ K^{-1}$), the Tris–HCl buffer formed the pH gradient in the free solution. In a 25 V cm^{-1} electric field, a dilute mixture of hemoglobin variants A and S (*ca.* 0.8 mg ml^{-1}, isoelectric points 6.98 and 7.2, respectively) were separated and concentrated by a factor of 20.

INTRODUCTION

The separation of proteins in their native states has long been an important research goal. Many methods exist that are reasonably satisfactory under laboratory-scale conditions, including isoelectric focusing (IEF). The need for larger-scale separation methods is becoming an increasingly important part of biotechnology as more commercial or near-commercial products are produced through biochemical means. The value of a "natural" derivative as a useful product is frequently dependent on the purity and concentration with which it may be isolated; many products produced through genetic recombinant methods are too dilute and too impure to be marketed directly. IEF could be a valuable technique if the electro-mechanical generation of pH gradients in gels could be replaced by an open-channel approach in a free solution.

With ampholytes, a class of compounds whose extent and sign of ionization are dependent on the pH of the local environment, IEF is unmatched in its ability to resolve complex mixtures into extremely pure fractions. In this technique, the analyte is placed in a medium, usually an agarose or polyacrylamide gel, containing a monotonic pH gradient that causes an analyte to protonate or deprotonate,

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depending on the pH in the immediate vicinity[1–5]. If an anode is located near the end of the gradient with a lower pH, an electric field applied along the gradient will cause the analyte to migrate toward the point in the gradient where the local pH equals the isoelectric point (p*I*) of the molecule. The isoelectric point of an ampholyte is the pH at which there is no net ionization of the species, thus the mobility is zero. In this way ampholytes of various molecular weights, with p*I* values differing by only 0.001 pH unit, have been separated[6,7].

IEF is usually conducted in pH gradients formed by carrier ampholytes, which are aliphatic polyaminopolycarboxylic acids (a synthesized version is Ampholine, LKB). These carrier ampholytes are pre-focused in the channel, forming the pH gradient owing to their different pK_a values. Generating the gradient in this manner has several disadvantages over thermally engendered pH gradients in which the expensive carrier ampholytes are absent, together with several of the deleterious effects associated with them. With the latter method, the cost of a separation is decreased, the long pre-focusing time is eliminated and purification of the analyte of the carrier ampholytes after focusing is unnecessary. This last advantage is especially beneficial when analyzing peptides that have similar net charge and mass characteristics to carrier ampholytes, as a result of which purification by chromatographic separation methods is ineffective[8]. On focusing cells in the presence of carrier ampholytes, viability can be decreased, leading some researchers to theorize that they are cytotoxic. Interference of carrier ampholytes with cells has been indicated by observations of their adsorption on red blood cells[9].

Other methods of forming the pH gradient have been utilized, such as buffer isoelectric focusing. Stable pH gradients were formed with a weak base and weak acid in a free solution with preformed pH gradients that were not generated by the electric current[10]. This method has the advantage of operating in an open channel, but the disadvantage of imperfect migrational stability[11] and is limited to pH ranges around neutrality[12]. A similar method is rheoelectrolysis in which the internal electric diffusion is balanced by the external flow of buffer to the electrode solutions[13]. Recently, Immobiline, a set of buffers copolymerized in a polyacrylamide matrix, has been used in IEF[14]. All of these have advantages over IEF with carrier ampholytes. IEF with thermally engendered pH gradients has the some of these advantages plus simplicity and the ability to manipulate the pH gradient during the separation run. In addition to the buffer Tris, several other buffers exist with a high temperature dependence of the pH, *e.g.*, ethylenediamineimidazole with $\mathrm{dpH/d}T = -0.027\ \mathrm{K}^{-1}$, p$K_a$ 6.85, which may be used in lower pH regions where its buffer capacity is higher than that of Tris.

In developing an alternative approach to conventional IEF, we have considered the limitations of the technique as it is conventionally performed in terms of where the difficulties lie in larger scale applications in a production or process environment. This technique is not intended to compete with IEF as is normally carried out with carrier ampholytes, in buffer IEF or with Immobiline. This method may have applications in larger scale separations where the species of interest are not separated on the basis of large p*I* differences. Currently only 1 pH unit is spanned in thermally engendered pH gradients with Tris buffer. This is more than adequate for such applications as separations of hemoglobin variants and cells. It is often unnecessary to span a large pH difference when only one species is to be concentrated and separated from a mixture of

unwanted species. The limitations of applications based on 1 pH unit is not often a problem when analyzing for minute differences in isoelectric points. For example, there is no need to operate between pH 2 and 12, as with carrier ampholytes, in order to analyze hemoglobin variants A and S, the p*I* values of which differ by only 0.22 pH unit.

Forming the pH gradient thermally can also allow *in situ* manipulation of the pH gradient, which is not easily accomplished in most of the other isoelectric techniques. The pH gradient can be controlled outside the separation cell without changing the buffer pH at the electrodes. This property of IEF in thermally engendered pH gradients may have application in preparative work where the run may be continuous for extended periods. In this instance the gradient may fluctuate, which can be corrected for by changing the temperature gradient, the temperature or the absolute temperature difference between electrodes.

We consider the following to be important limitations to the development of practical preparative IEF with carrier ampholytes: (1) the cost of the pH gradient-forming ampholytes is high; (2) the gel medium in which separation occurs frequently contributes to the difficulties of the isolation and detection of the species of interest; (3) perhaps the most serious is the long time required to generate the pH gradient, that is, prefocusing of the ampholytes usually adds several hours of preparation time to the already relatively lengthy separations; (4) purifying the analytes of the carrier ampholytes after focusing is a difficult, often impossible, process. IEF in a free-solution approach could reduce or eliminate these limitations.

Previous work with thermally engendered pH gradients

There have been previous attempts to develop free-solution IEF separators including the use of thermally generated pH gradients. Luner and Kolin[15] attempted separation in an apparatus in which open-tubular IEF could be performed in glass tubing located between two water-baths of widely different temperatures (0 and 50°C). The buffer used, Tris–HCl, has a large temperature coefficient of pH ($dpH/dT = -0.028\ K^{-1}$, pK_a 8.2 at 25°C), so the temperature gradient along the long axis of the open tube gave rise to a corresponding pH gradient. Although Luner and Kolin showed that such gradients, formed in a few seconds in Tris–HCl buffers, had promise and could be used for focusing proteins, the approach has never gained favor over conventional gradients formed in gels. In their experiments, the design of the gradient generators, the choice of polystyrene and polyacrylamide materials and the geometry of the channels may exaggerate the difficulties associated with the generation and maintenance of the stability of free-solution gradients. These design problems, coupled with the relative ease of gel methods in laboratory-scale separations, have probably contributed to the dominance of the conventional approaches[16].

Free-solution methodology becomes more attractive in separation problems where time and operating difficulties with the conventional gel approaches become significant concerns. Larger scale separation problems in process and production environments are reduced by the added simplicity and reduction of the total separation time.

EXPERIMENTAL DESIGN AND METHODS

The design of the open-channel IEF separator incorporates approaches directed at overcoming the problems associated with other designs and previous attempts. Experiments were carried out with a device constructed as shown in Fig. 1. The separation channel (dimensions 3.1 mm × 5 mm × 28 mm) and electrode compartments were machined from a copper plate (oxygen-free, 1/8-in. copper plate). A 14000-dalton cut-off dialysis membrane (Spectrapor® 1-cm membrane tubing; Spectrum Medical Industries, Los Angeles, CA, U.S.A.) is placed between the separation channel and the electrode compartments (dimensions 3.1 mm × 5 mm × 7 mm), where a 0.01-in. platinum-wire electrode applies a voltage gradient along the long axis of the separator (MicroPro 3000; Haake Buchler Instruments, Saddle Brook, NJ, U.S.A.). These 14000-dalton cut-off dialysis membranes act as current-passing barriers permeable to small ions and buffer molecules but not to larger protein molecules. The buffer streams flowing through the electrode compartments constantly refresh the electrolyte, removing the products of buffer electrolysis and other unwanted species which may pass through the membranes under the electric field. In this way, the concentration of buffer in the cell itself is held constant despite any inequality in the flows of counter ions.

All copper surfaces in contact with the fluid are electrically insulated with an

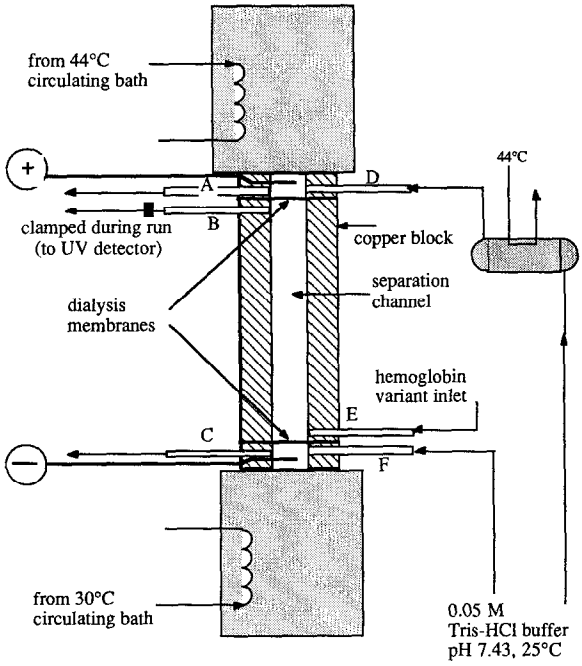

Fig. 1. Schematic diagram of isoelectric focusing prototype. During operation, tube B, which accesses the separation channel, is clamped. After focusing has occurred, tubes A, C, D and F are clamped as buffer flows through tube E and elutes the focused hemoglobin variants through tube B to the UV detector. The channel is Teflon coated. A plate (not shown) is bolted to the cell to enclose the separation channel. The cell is held horizontally so that the temperature gradient is perpendicular to the gravitational vector.

adhesive Teflon® film. Constant-temperature reservoirs at the electrode ends serve both as the caps of the cell and as thermal reservoirs necessary to generate the pH gradient. The pH gradient necessary for the IEF effect is generated indirectly by these constant-temperature reservoirs in contact with the prototype. When held at different temperatures, the reservoirs establish a temperature gradient along the copper walls of the cell and, hence, through the solution within. The Teflon film on the copper walls of the channel prevents protein adsorption and electrically insulates the cell. Natural convection is limited by holding the cell horizontally so that a density gradient, as a result of the temperature gradient, does not develop parallel to the gravity vector. Stability is much greater with a horizontally held cell than a vertical cell, as will be explained later.

Stock 0.05 *M* Tris–HCl buffer (pH 7.34 at 25°C) had a buffer capacity of 0.011 *M*. Ultra-pure Tris was purchased from Schwarz/Mann Biotech (Cleveland, OH, U.S.A.). Syringe pumps (Model 355; Sage Instruments, Cambridge, MA, U.S.A.) passed the buffer solution through polyethylene tubing (Intramedic, 0.062 in. I.D.; Clay Adams, Parsippany, NJ, U.S.A.) to the electrode compartments. The buffer to the anode was preheated to 44°C by passing it through polyethylene tubing immersed in the 44°C circulating bath (Model F; Haake Buchler Instruments), decreasing the apparent pH to 6.8. The cathode buffer was heated to 30°C (pH 7.2) at the cathode reservoir by the low-temperature circulating bath (Model 2095; Forma Scientific, Marietta, OH, U.S.A.).

Hemolysate was prepared by the Duke Medical Center Hematology Laboratory (Durham, NC, U.S.A.) from freshly drawn blood of sickle cell anemia patients. The red blood cells, collected with EDTA as an anticoagulant, were washed with three volumes of 0.9 g/l saline solution, then with a 0.05 g l^{-1} KCN–2.5 g l^{-1} EDTA lysate reagent. The ratio of hemoglobin A to S was 40:60 in a total concentration of aproximately 110 g l^{-1}. The stock solution was diluted 140-fold in the buffer solution to 0.8 mg ml^{-1}.

After focusing, the bands were eluted by a syringe pump (Model 355; Sage Instrument) to a UV detector equipped with a flow cell (TriDet detector; Perkin-Elmer, Norwalk, CT, U.S.A.).

If the pH gradient were linear through the solution, hemoglobin variant A should have focused 0.5 cm from the anode, where the pH of 6.98 was equal to its p*I*. The isoelectric point of hemoglobin variant S lay at 7.2 in the cathode reservoir, so it accumulated against the membrane. By focusing hemoglobin A in the channel and hemoglobin S against the membrane, the distance between the two was greater than if both were focused in the channel. Hemoglobin S formed a more concentrated band because it was farther from its isoelectric point.

RESULTS

The temperature gradient used in this experiment was 5.0°C cm^{-1}, which formed a -0.14 units cm^{-1} pH gradient. A 40:60 mixture of hemoglobin variants S and A (p*I* 7.2 and 6.98, respectively[17], total hemoglobin 0.8 mg ml^{-1}) were focused in 2 h at 1 mA (see Fig. 2) and 0.2 mA (see Fig. 3). The current was held constant in these experiments while the voltage was allowed to vary. At the end of the run, buffer was pumped through the separation cell, sweeping out the hemogobin bands to a UV detector flow cell.

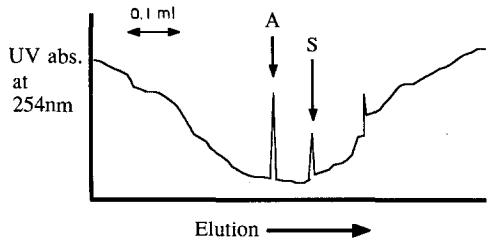

Fig. 2. Isoelectric separation of hemoglobin variants A and S at 25 V cm^{-1} and 1.0 mA showing the UV absorbance of the eluent after passing through a flow-cell detector.

The concentration of the protein bands, as evident from the decreased band width, was increased by a factor of *ca.* 20 in the run at 70 V and *ca.* 5 at 14 V. This may not be the maximum concentration in the separation channel, as the concentration was measured in the UV flow-cell. The process of flowing to the detector introduces dispersion effects, decreasing the concentration. A short length (7 cm) of polyethylene tubing was fitted between the detector and the separation chamber and a parabolic, laminar flow profile would explain the tailing leg of the S peak in Fig. 3. The hemoglobin variant S was focused against the dialysis membrane at the cathode reservoir end. At the steady state, this peak would not have the same symmetrical Gaussian shape as with hemoglobin variant A, which was focused in the middle of the chamber. This could also explain the difference between peaks A and S in Fig. 3.

DISCUSSION AND CONCLUSION

We recognize that there are several problems that could exist in isoelectric focusing in a free solution which would diminish the separation and resolution of the protein bands, including convection due to thermal gradients and build-up of heat in the channel. We tried to take these into account in the designs as follows.

The problems caused by convection and electromigration of the pH gradient were minimized by keeping dimensions *small* [the solution compartment is approximately 3.18 mm × 5 mm × 28.0 mm (0.44 cm^3, 0.158 cm^2 cross-sectional area)] and by operating the separator *horizontally*. As a result, there should not be

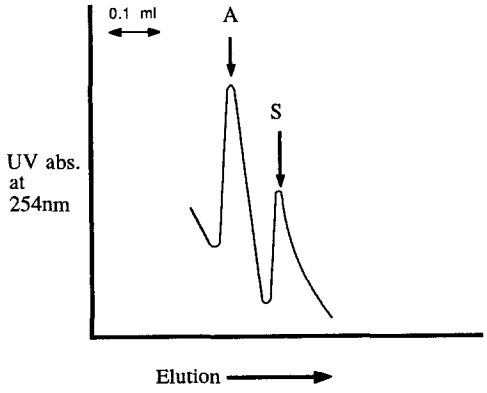

Fig. 3. Isoelectric separation of hemoglobin variants A and S at 5 V cm^{-1} and 0.2 mA.

a significant temperature gradient parallel to the gravity vector and mixing due to buoyant effects should be minimized. Keeping the temperature gradient perpendicular to the force of gravity reduces density gradients, the driving force for free convection.

There is concern over Joulean heating in free-solution electrophoretic separators as it causes mixing owing to convection. Heat generation in the center of the separation cell would cause dispersion in the middle of the channel, thus decreasing resolution. Separation occurred in the presence of possible heating as its effect was relatively low considering the small cell width and low current. As will be shown later, the heat conduction in the cell is mainly through conduction, not convection, so the differential equation governing the temperature rise due to Joulean heating is derived from Fourier's law. Neglecting the end effects at the electrode reservoirs, this equation may be put in the form of Poisson's equation:

$$k \cdot \frac{\partial^2 T}{\partial x^2} + k \cdot \frac{\partial^2 T}{\partial y^2} = -EI \tag{1}$$

where T = temperature (°C), k = thermal conductivity of buffer ($5.6 \cdot 10^{-3}$ W cm^{-1} °C^{-1}), x,y = distance coordinates in cell normal to electric field (cm), E = electric field (V/cm) and I = current (A). Eqn. 1 describes the local temperature rise above the constant temperature at the walls. This partial differential equation was solved with IMSL simulation software employing a finiteelement algorithm on a PC XT[18]. This mode indicates that the temperature rise at the center of the channel is 0.012°C operating at 0.2 mA, the conditions shown in Fig. 3.

The isoelectric point at the center of the channel shifts towards the cathode with the decrease in pH encountered at the center of the channel. A temperature rise at the center of the channel decreases the pH from that at the wall by $-3.4 \cdot 10^{-4}$ units (0.012°C · 0.028 unit °C^{-1} = $-3.4 \cdot 10^{-4}$ units). The pH gradient used in these experiments was 0.14 units cm^{-1}, so the position of the isoelectric point shifted towards the cathode by 24 μm ($3.4 \cdot 10^{-4}$ units/0.14 units cm^{-1} = 24 μm). This effect increased with increase in current as heat generation is proportional to EI. Operating at 25 V cm^{-1} (the results of which are shown in Fig. 2) raised the temperature at the center 0.30°C above that at the wall. The resolution decrease owing to the shift towards the cathode, yet two very sharp peaks were observed, indicating that this effect is not significant. By measuring the bandwidth in Fig. 2, peaks A and S were concentrated roughly by a factor of 20; in Fig. 3, peak A was concentrated by a factor of 5.

Another possible manner in which Joulean heating may decrease resolution is through natural convection. The cell shown in Fig. 1 is laid horizontally so that a density gradient, parallel to the gravitational vector, does not develop between the cold and hot ends, limiting gross natural convection and mixing; however, as a result of the temperature difference between the cell center and the wall (derived from eqn. 1), a density gradient forms parallel to the gravitational vector which is perpendicular to the voltage gradient. This density gradient could cause mixing if buoyant forces override viscous forces. A measure of this ratio is the Grashof number, N_{Gr}:

$$N_{Gr} = \frac{g\ \beta\ (\Delta T)\ d^3}{\nu^2} = 1 \tag{2}$$

where g = gravitational constant (9.8 m s^{-2}), β = volume coefficient of expansion ($3.0 \cdot 10^{-4}$ K^{-1}), ΔT = temperature difference between the cell center and the wall (0.012°C at 5 V cm^{-1}), d = distance between the cell center and the wall (2.5 mm) and v = kinematic viscosity ($8.63 \cdot 10^{-7}$ m^2 s^{-1}). For $N_{Gr} < 2000$, heat transfer is mainly through conduction and convective forces are absent. At higher temperatures, convection begins to form a pattern of hexagonal cells called Benard cells, in which the fluid circulates from the lower hot region to the cool upper plate[19]. Benard cells will not develop until $N_{Gr} > 1700$, which from eqn. 2 would require temperature difference several orders of magnitude greater than operation at 5 V cm^{-1} (see eqn. 1).

Eqn. 2 predicts that the cell cannot be held vertically, with the hot end above the cool end or *vice versa*. In this case, d in eqn. 2 would be 28 mm, other values remaining unchanged. N_{Gr} would then be $(28/2.5)^3 \cdot 1 = 1405$, much greater than the value for a horizontal cell. Stability is decreased when using a vertically held cell even if the hot end is held above the cooler end. Because of the small diameter of the cell, natural convection due to Joulean heating is limited, but a temperature rise at the center of the channel will tend to shift the center of the peak towards the cathode. A limitation to free-solution IEF results from this shift due to Joulean heating. Capillaries are especially efficacious in free-solution IEF because the heat generated may be removed more effectively with smaller diameter cells and natural convection decreases with d^3 (see eqn. 2).

These experiments indicate that IEF in thermally engendered pH gradients may be accomplished in a capillary without density-stabilized pH gradients. Increasing the diameter of the capillary to raise the capacity will result in decreased resolution because the rise in the center temperature is proportional to R^2, causing the peak to be shifted towards the cathode. Even with a decreased resolution in wider cells open-channel IEF may still have promise on a preparative scale.

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