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Application of Well-Controlled pH Gradients at Variable Isocratic Salt Concentrations to IEX Chromatography

The ion exchange technique described in this article enables the formation of controlled, externally generated pH gradients over the pH range 2–12 on either anionic or cationic stationary phases.[1] The pISep kit (**CryoBioPhysica**, Rockville, MD) consists of a software package and two buffers, acidic and basic, composed of small zwitterions with overlapping pKas. The pISep software computes column volume and time-based protocols for the development of multishape, multistep, and multislope pH gradients on ion exchange (IEX) chromatography columns. An extension of the pISep technology allows the formation of fully controlled, externally generated pH gradients in the presence of arbitrary levels of NaCl ranging from 0 to 1.0 M. The ability to add salt while retaining control over the formation of pH gradients provides much improved flexibility for the separation of proteins sensitive to extremes of pH. It also provides fast, efficient scouting of pH gradients at varying isocratic salt concentrations in order to determine the optimal binding and separation conditions for a particular set of proteins.

In IEX with salt, the accepted method of scouting for a set of optimal separation conditions (pH and salt) for a particular set of proteins on a specific stationary phase is to systematically vary the isocratic pH of a salt gradient with a defined slope until satisfactory separation resolution is achieved. Following this, the gradient slope can be gradually decreased in an attempt to gain a modest additional improvement in resolution. In theory, an alternative strategy would be to use a fixed slope pH gradient at varying isocratic levels of salt. The final optimizing steps would then involve systematic flattening of the chosen pH gradient. This cumbersome procedure requires changing the buffer chemistry every 1.5–2.0 pH units in order to scout a different segment of the pH range.[2,3] Since the pISep buffer composition possesses strong, relatively uniform buffering capacity throughout the pH range 2–12, the scouting process can be greatly simplified. Achieving computer control over the formation of pH gradients in the presence of NaCl requires the creation of a two-dimensional manifold equation, which will determine the proportion of acidic and basic pISep buffers required to generate a specific pH in the presence of a specific salt concentration. This article describes such a manifold and demonstrates its practical value for the optimization of protein separations.

Results and discussion

Control of pH gradients in the presence of salt

The titration curve %A, acidic + %B(1–A%), basic buffer vs pH is fitted to Eq. (1):

$$A\% = C_n\,pH^n + C_{n-1}\,pH^{n-1} + \ldots\ldots\ldots + C_2\,pH^2 + C_1\,pH + C_0;\ C_0 \text{ to } C_n \text{ fitting constants} \qquad (1)$$

In practice, when n ≥ 7 the calculated A% for a specific pH is accurate to at least 0.1% over the fitted pH range and precise to better than 0.1%. As detailed in Eq. (1), HPLC/FPLC (fast-performance liquid chromatography) protocols for computer-controlled formation of pH gradients can be routinely calculated by the pISep software. Often, optimized pISep pH gradients separate mixtures of proteins with much higher resolution than even the most optimized salt gradients since the retention factor, *k*, is much more sensitive to changes in pH than to changes in ionic strength. On the other hand, there are frequent occasions when the presence of salt during a pH gradient fractionation is essential. These include 1) low solubility of a target protein in a particular pH range, 2) denaturation or loss of activity of a protein at acidic or alkaline pH where adding salt can shift the effective pI of the protein into a pH range where the protein remains functional, and 3) increasing the chromatographic resolution of unresolved isoforms by the addition of salt engendered by unequal shifts of the pH (apparent pI) at which each isoform elutes as a function of salt concentration.

The data presented here will demonstrate cases 2 and 3. To achieve accurate estimates of pH in the presence of salt and to extend the strategy of Eq. (1), we assume that, over the full range 0–1 M salt concentration, the A% value can be expressed as:

$$A\% = A_m\,(pH)*[NaCl]^m + A_{m-1}\,(pH)*[NaCl]^{m-1} + \ldots\ldots\ldots + A_2\,(pH)*[NaCl]^2 + A_1\,(pH)*[NaCl] + A_0\,(pH) \qquad (2)$$

Where each of the $A_j\,(pH)$ $0 \le j \le m$ are of the form of Eq. (1), pH range is 2.4–10.8, and salt range is 0–1 M.

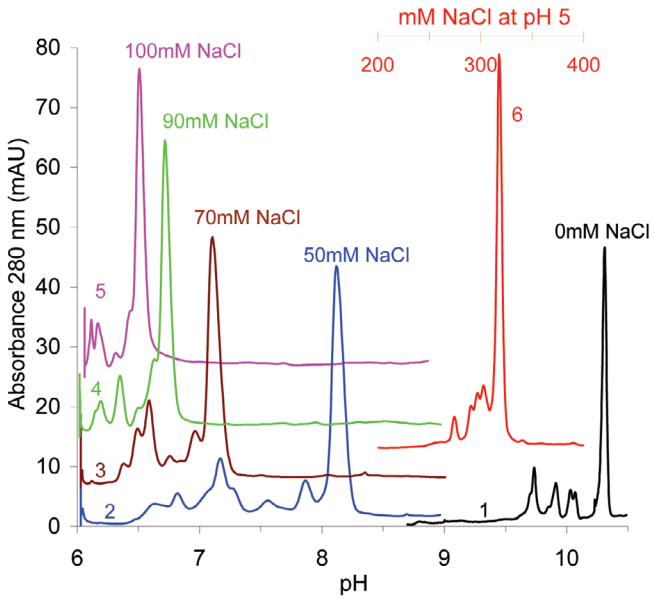

Figure 1 *Comparative CEX purifications of RNase A by pISep pH gradients at varying isocratic concentrations of salt (curves 1–5) and by a salt gradient at isocratic pH (curve 6). Slope of the pH gradients: 0.075 pH units per column; isocratic concentrations of salt: 0, 50, 70, 90, and 100 mM. Buffer A: pISep pH 2.4, buffer B: pISep pH 10.8. Salt gradient: 15.4 mM NaCl per column volume from 0 to 500 mM NaCl. Buffer A: 20 mM acetic acid/NaOH pH 5, buffer B: 20 mM acetic acid/NaOH, 500 mM NaCl, pH 5. Column: Mono S HR 5/5 (**GE Healthcare**, Piscataway, NJ) with column volume 0.98 mL; flow rate: 1 mL/min; injected RNase A: 390 µg, detection: 280 nm.*

In practice, Eq. (2) is calculated as follows: A series of titration curves, each obtained at a different [*NaCl*], are fit to Eq. (1). Using these solutions to Eq. (1), sets of A% values, each set at a unique pH value, are calculated over the range of [*NaCl*] values. Each of these isocratic pH A% value sets is further fit to a particular function of [*NaCl*]. Empirically, this works very well using a polynomial fit of the form of Eq. (2) with $m = 5$. In the final step, each of the $A_j\,(pH)$ is plotted against the pH and fit to:

$$A_j\,(pH) = C_{k,j}pH^n + C_{k-1,\,j}pH^{n-1} + \ldots\ldots\ldots + C_{2,j}pH^2 + C_{1,j}pH + C_{0,j};\ C_{0,j}\ \text{to}\ C_{k,j}\ \text{fitting constants} \quad (3)$$

This provides us with the manifold A% = A%(*pH*, [*NaCl*]). The pISep software uses these equations to calculate the value of A% at any pH within the pH range 2.4–10.8, while the two pISep buffers contain an arbitrary concentration of salt in the range 0–1 M. The estimated pH and NaCl values over the fitted pH and salt ranges are accurate to at least 0.1 pH units and 5 mM NaCl.

Apparent pI shift as a function of salt concentration

Figure 1 illustrates several of the important advantages of forming pH gradients in the presence of salt (curves 1–5) as compared to a salt gradient at isocratic pH (curve 6). Upon storage, RNase A forms acidic, deaminated isoforms that can be separated from the very basic, covalently intact protein with an isoelectric focusing (IEF) pI of 9.45. When purified by a pISep pH gradient from pH 8.7 to 10.5 (curve 1), the intact RNase A exhibits an apparent pI of 10.31, 0.86 pH units above its electrophoretic pI. A detailed explanation for this frequently observed discrepancy between IEF pI and pISep pH gradient IEX pI is provided by the augmented electrostatic interaction theory.[1] It is sufficient to say here that due to their natural structural organization, protein molecules almost always orient only a subset of their charges to interact with the IEX stationary phase, causing the observed shift of the IEF pI. In chromatogram 1, at least eight acidic isoforms are identifiable and totally resolved from the intact RNase A and relatively well separated with respect to each other. Although these results demonstrate that pISep works well near its alkaline limit of buffering capacity, RNase A as well as many other proteins are often configurationally and covalently compromised by such an extreme pH. An effective way to deal with this is to shift the elution pH range toward a more neutral pH by the addition of salt. The pISep purification of RNase A in the presence of 50 mM NaCl from pH 6 to 9 shows a well-resolved, intact RNase with an apparent pI of 8.1 (curve 2). Three of the nine acidic isoforms identifiable in chromatogram 1 are still well resolved. In the pH gradient elution of RNase A in the presence of 100 mM NaCl, three of the four isoforms are well separated from the intact form, which is probably convoluted with one of the deamidated isoforms and elutes at an apparent pI of 6.5 (curve 5). The addition of 100 mM salt lowers the apparent pI of the intact RNase by four pH units, yet causes only a minor loss of purity. It should also be noted that in chromatograms 1–5, the shifts of the apparent pI of the RNase A isoforms as a function of salt concentration differ from one isoform to another. Because the apparent pI of each form shifts along its own unique curve, this scouting procedure can determine the optimal conditions for the separation of each form. Chromatogram 6, a salt gradient elution of RNase A at pH 5, shows that RNase A is eluted between 250 and 315 mM salt. In the salt gradient, two of the five identifiable acidic isoforms are resolved, whereas the other three still compromise the purity of the dominant form.

Effects of the binding pH and the pH gradient slope

An obvious goal of chromatography is to separate a complex mixture of isoforms with the highest resolution possible. One of the more interesting general aspects affecting resolution in conventional salt gradient IEX is that the movement of a protein band through a column does not depend solely on the gradient slope and the final eluent concentration, but also on the concentration of binding and the starting concentration of the gradient. This is because the time to elution is an integral of the inverse of $1 + k$ where k, the retention factor, is a com-

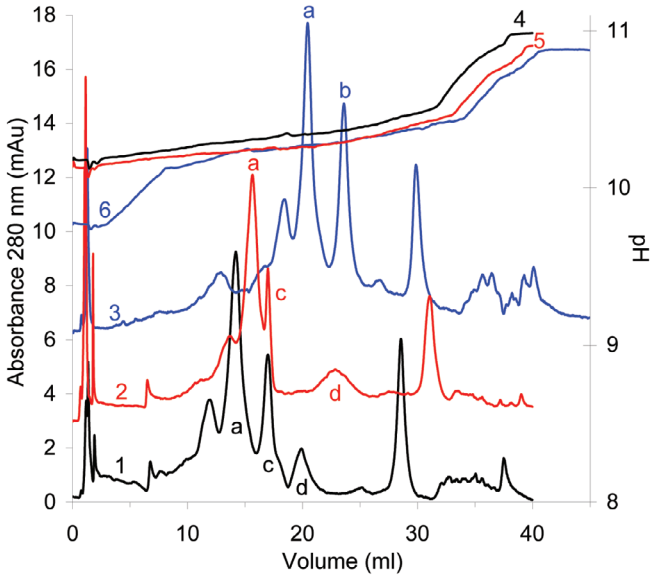

*Figure 2 Effect of the initial pH and slope of the pH gradient on the selectivity and resolution of CEX separations of a monoclonal antibody with pISep pH gradients. MAb separations starting at different pH: 9.7, 10.15, and 10.2; curves 1, 2, and 3. Multistep, multislope pH gradient profiles—pH units per column volume and pH range—curve 4: 0.015 pHU/CV pH 10.15–10.25, 0.006 pHU/CV pH 10.25–10.35, 0.27 pHU/CV pH 10.35–10.9; curve 5: 0.015 pHU/CV pH 10.1–10.25, 0.006 pHU/CV pH 10.25–10.35, 0.27 pHU/CV pH 10.35–10.9; curve 6: 0.25 pHU/CV pH 97–10.1, 0.025 pHU/CV pH 10.1–10.2, 0.035 pHU/CV pH 10.2–10.35, and 0.27 pHU/CV pH 10.35–10.9. Column: ProPac® WCX (**Dionex Corp.,** Sunnyvale, CA), 250 × 4 mm, flow rate: 1 mL/min, 50 µg of MAb injected, detection: 280 nm.*

plex function of both pH and ionic strength. *Figure 2* illustrates the effect of both the initial binding pH and the slope of the pH gradient on the separation of MAb isoforms with relatively high alkaline pIs in the pH range 9–10.4. Chromatograms 1, 2, and 3 begin at pH 9.7, 10.15, and 10.2, but their respective pH gradients maintain nearly identical slopes thereafter.

The hyperexponential dependence of *k* on pH significantly increases the mobility of all MAb isoforms as the initial pH is raised. Separation 1 starts at the highest binding pH and, as expected, most MAb isoforms display increased mobility compared to their mobility in elutions 2 and 3. In contrast to their varying mobility, the resolution of most MAb isoforms remains virtually unaltered. Exceptions are the two isoforms labeled *c* and *d* in chromatogram 1 eluted together in chromatogram 3 as an apparently single isoform labeled *b*. Due to the increased mobility at the highest binding pH, isoforms *c* and *d* are well separated from isoform *a* in separation 1. In contrast, because of the decrease in mobility at a lower binding pH in chromatogram 2, isoform *c* is almost convoluted with isoform *a*. Nevertheless, here, isoform *d* is baseline resolved as a consequence of the flatter pH gradient in the pH range 10.25–10.35 (curve 5). Since changing the loading pH is easy and unlikely to seriously affect productivity, this approach is extremely useful as a way to adjust the selectivity and the resolution of the IEX protein separations using controllable pH gradients.

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