Enhanced Purification of Similarly Charged Proteins with Self-Generated On-Column Resin pH Gradients

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Protein Purification

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Abstract

The presence of carboxyl groups in cation exchange (CEX) resins can often lead to complex pH shifts during protein purification processes. UNOsphere™ S, a strong methacrylate-based CEX resin, has a significant number of matrix-associated carboxyl groups. These groups respond to buffer changes by inducing an on-column pH gradient. This arms UNOsphere S with a resolving power unattainable by other S-type resins. Such a pH gradient can be exploited to separate protein charge variants and proteins with a pI difference of as little as 0.1 units. This article explains the phenomenon of on-column pH gradient formation with UNOsphere S and outlines some examples of protein purification utilizing this gradient.

Introduction

Cation exchange (CEX) chromatography is one of the most commonly used techniques to purify proteins. Binding to CEX resins depends on the isoelectric point (pI) of the protein, which is the pH at which it carries no charge. In most cases, proteins bind to a CEX resin at a pH below their pI, where they are positively charged. Elution can occur when the buffer pH is increased, causing the overall protein charge to decrease towards neutral and eventually become negative. Fine separations can be achieved by elution with a pH gradient rather than a step change in pH. In the past, this has been achieved by either applying a complex buffer mixture to the support or generating a pH gradient in front of the column via buffer mixing.

However, these approaches are limited by the availability of a suitable buffer (Tsonev et al. 2008) or by difficulties in validating a precolumn mixing system to reproducibly generate a pH gradient. Recently, significant pH transitions have been reported in CEX resins containing weak acid groups following both step and gradient changes in buffer salt concentration or pH (Ghose et al. 2002, Pabst and Carta 2007). In such resins, the functional ligand is a strong acid group such as sulfonic acid. However, a significant number of carboxylic acid groups are also present, typically associated with the matrix backbone. The buffering capacity of these weak acid groups induces pH transitions through changes in salt concentration or buffer pH.

UNOsphere™ S Resin is one such strong CEX support exhibiting a self-generated on-column pH gradient phenomenon. The resin is based on an acrylamido backbone, but it has a significant number of carboxyl (COO^-) groups similar to other methacrylate-based resins. These COO^- groups are solvent-accessible and therefore interact with ionic species present in the mobile phase. A reproducible pH gradient can result from the following: 1) step changes in buffer pH 2) step changes in salt concentrations, or 3) application of a salt gradient, leading to novel separations. Changes in some cases can generate pH steps, pH gradients, or mixed pH waves, depending on the conditions and buffer properties. These properties allow UNOsphere S to resolve proteins with a pI difference of as little as 0.1 units.

UNOsphere S is made from 80 µm acrylamide/vinylic copolymers, and is widely used in commercial manufacturing. In addition, the resin has a large pore size and exhibits high binding capacity and mass transfer, excellent base stability, and low backpressure under a high flow rate. This article provides the underlying mechanism as well as detailed information on the superiority of UNOsphere S over other CEX resins in resolving closely related proteins at the manufacturing scale.

BIO-RAD

Materials and Methods

Chromatography Resins

- UNOsphere S (Bio-Rad Laboratories): Contains sulfonic acid groups along with a significant number of carboxylic acid groups resulting from the manufacturing process
- Nuvia™ HR-S Prototype (Bio-Rad Laboratories): Based on a similar matrix but contains substantially fewer carboxylic acid groups compared to UNOsphere S
- SP-Sepharose FF (GE Healthcare): An agarose-based strong cation exchanger with minimal weak acid group content

The relevant physical properties of the resins are summarized in Table 1.

Table 1. Summary of resin properties.

Property	UNOsphere S	Nuvia HR-S prototype	SP-Sepharose FF
Particle size*, µm	80	70	90
Weak acid group content**	170	25	5
Strong acid group content**	140	145	195
Total acid group content**	310	170	200

* Values obtained from the manufacturer's literature.
** Acid group contents are given in µmol/ml packed column.
Table adapted from Pabst and Carta 2007, Table 2.

UNOsphere S Self-Generated pH Gradient Formation

The mechanism of UNOsphere S pH gradient formation has been described by Pabst and Carta (2007). A local equilibrium model was developed to predict the magnitude and duration of pH transients. It is based on the characteristics method and numerical simulations using an equilibrium-dispersive model.

Buffer Salt Concentration Analysis

The impact of buffer salt concentration on the quality of the UNOsphere S self-generated pH gradient was evaluated using lysozyme elution. The protein was applied to a column with UNOsphere S in buffer containing 20 mM NaCl at pH 5.5 with a salt gradient of 0–500 mM NaCl in 20 column volumes (CVs).

Buffer Analysis

The effect of buffer type on pH gradient formation was assessed as previously described (Pabst et al. 2008). Briefly, different ratios of acetate/phosphate buffers (2:1, 10:1, and 20:1) were prepared using buffer solutions with a fixed sodium (Na^+) concentration (0.04 M) and a step change of pH 4.5–7.

Column pH Transitions

A comparison of the on-column pH gradient formation for the three resins evaluated in this article (Table 1) was performed by assessing pH transitions during salt steps as previously described (Pabst and Carta 2007). Briefly, pH transitions were measured in response to a 0–5 M NaCl step gradient in NaOAc buffer (0.02 N) with a subsequent decrease to 0 M NaCl in the same buffer.

Resolution of Protein Mixtures

A mixture of α-chymotrypsinogen (pI = 9.1) and lysozyme (pI = 11) in 20 mM Na acetate buffer was separated on the three previously mentioned resins, using a salt gradient of 0–750 mM NaCl at pH 5.5. In another experiment, a mixture of proteins with very similar pIs (ovalbumin: pI = 4.7, bovine serum albumin: pI = 4.9, human serum transferrin: pI = 5.2) were separated on UNOsphere S as described in Pabst et al. (2008). Buffers containing a 10:1 ratio of acetate and phosphate at a total sodium concentration of 40 mM were used with a pH 4.5–7 step transition and varying mobile phase rates.

Separation of Modified Proteins

Native and PEGylated superoxide dismutase (SOD), as well as native and carbamylated carbonic anhydrase II (CA), were separated via pH gradient CEX chromatography as previously described (Ng et al. 2009). Isoelectric focusing (IEF) was carried out as previously described, and was performed under 8,000 V for 35,000 Vh.

Batch Testing for pH Lot-to-Lot Variability of UNOsphere S

The pH profiles of different batches of UNOsphere S were tested as described in Ng et al. (2009). Fractions were collected over the course of experiments and the pH of each fraction was assessed offline.

Results and Discussion

Mechanism of Self-Generated pH Gradient Formation

UNOsphere S is unique in the high level of COO^- groups within the resin. At a given pH, these COO^- groups are in equilibrium with Na^+ and protons (H^+) from the mobile phase (Figure 1A, step 1). Following a step change in Na^+ concentration or buffer pH (Figure 1A, step 2), the equilibrium shifts and the COO^- groups reversibly gain or lose protons, resulting in a local pH change (Figure 1A, step 3). This initiates the start of a pH gradient, which continues to develop until both ionic and/or pH equilibria are re-established. Due to the presence of a large number of COO^- groups in UNOsphere S, this gradient lasts for a relatively large number of CVs. Similar effects are seen when the buffer change comes from application of a gradient rather than a step. This provides a significant window for protein desorption, thereby enhancing the resolution.
A local equilibrium model was developed that accurately predicts these changes (Figure 1B) (Pabst and Carta 2007, Pabst et al. 2008).

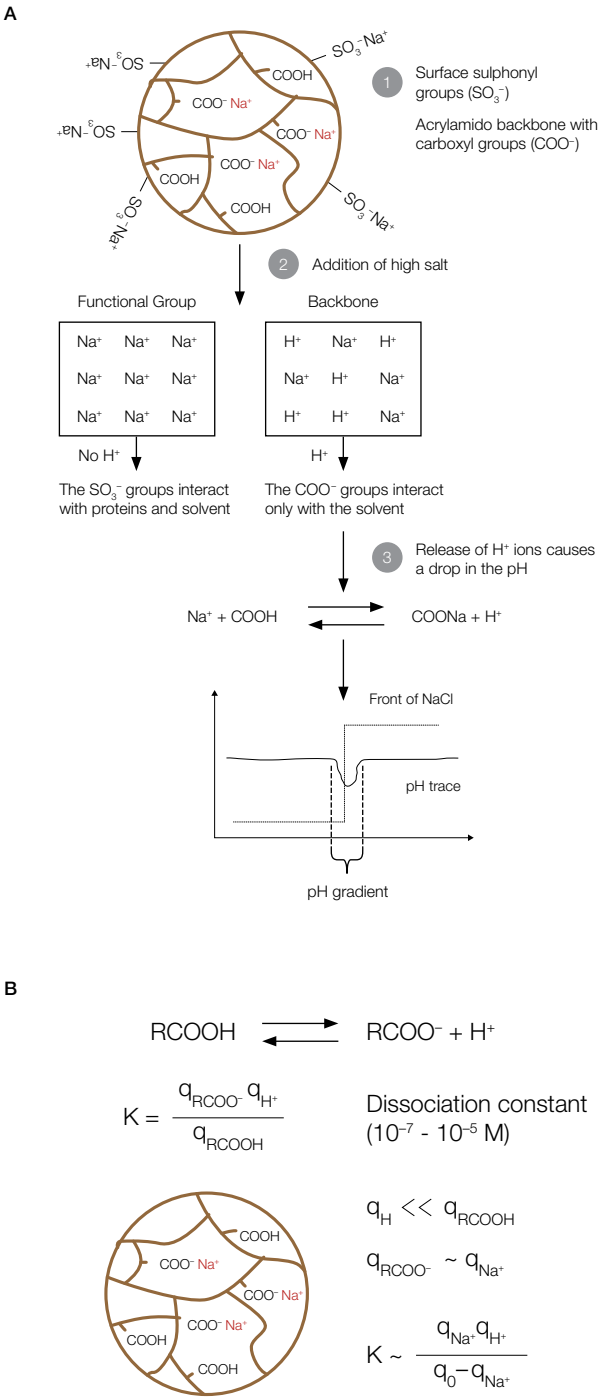


Fig. 1. Self-generated gradient formation in UNOsphere S. A, effect of NaCl increase on local equilibrium in UNOsphere S. **B**, ion exchange equilibrium model.

Factors Affecting the Self-Generated pH Gradient of UNOsphere S

The COO^- within UNOsphere S are solvent/buffer accessible and therefore interact with ionic species in solution to create the pH gradient. The buffer salt concentration and pH, as well as the type of buffer used, are the most important factors affecting the quality of the pH gradient generated and consequently, the resolution of proteins separated. The following examples demonstrate these effects.

Buffer Salt Concentration

Lysozyme was applied to a column packed with UNOsphere S in a buffer containing 20 mM sodium phosphate at pH 5.5 (Figure 2). A buffer salt gradient of 0–500 mM NaCl (red) in 20 CVs was applied to elute the lysozyme. As shown in Figure 2, the lysozyme (brown) does not start eluting until approximately 20 CVs after the completion of the salt gradient. The self-generated pH gradient (blue) is also shown. The pH decreases immediately after the start of the salt gradient due to the release of protons from the COO^- groups in response to the Na^+ concentration. As the number of protons released into the buffer increases, the pH continues to decline. Once the release of protons starts to decrease, the pH returns to 5.5. The lysozyme is eluted in the process of the pH gradient generation. The pH gradient generated here allows for the separation of impurities from the target protein. The theoretical values generated by the model in Figure 1B correlate with the actual experimental results.

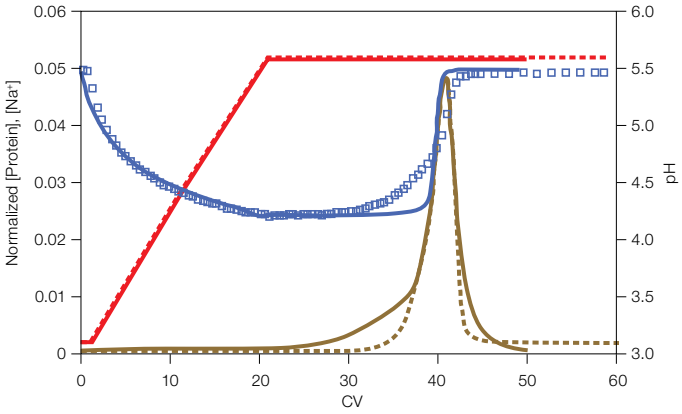


Fig. 2. Elution of lysozyme in a pH step generated by a salt gradient. Lysozyme (- -); lysozyme- model (—); Na^+ (- -); Na^+- model (—); pH (□); pH- model (—).

Buffer pH

A mixture of ovalbumin (pI ~5) and bovine serum albumin (pI ~5.5) in an acetate/phosphate buffer with a total Na^+ concentration of 40 mM at pH 4 was applied to a UNOsphere S column. A pH step change was then applied to the column at the same Na^+ concentration by using a buffer with the same constituents adjusted to pH 7 instead of 4. After application of the higher pH buffer, prolonged proton release prevented the pH from rising immediately to 7. Instead, the pH of the effluent gradually increased over approximately 18 CVs to pH 7 (Figure 3). This pH gradient results in baseline separation of the two proteins with a pI difference of only 0.5 units. Additionally, the conductivity of the buffer drops immediately after the introduction of the pH step, due to differences in H^+ and Na^+ mobility, and then increases gradually until it stabilizes at its initial level. Similar to the findings in Figure 2, the experimental and predicted profiles (Figure 1B) generally align with each other in this case.

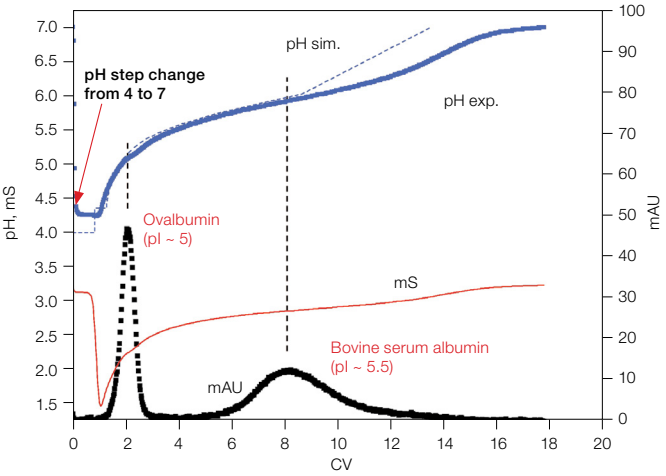


Fig. 3. Separation in a pH gradient generated by a buffer step at constant Na^+ (pH 4–7). Model pH (- -); experimental pH (—); protein elution pH (- -); Na^+ (—).

Type of Buffer

The degree of the self-generated on-column pH gradient formed depends on the buffers used. To illustrate this, a step change of pH 4.5–7 with 0.04 M Na^+ was performed with different acetate and phosphate buffer combinations. As shown in Figure 4, the various combinations produced different lengths of pH gradients in the UNOsphere S column. A longer pH gradient serves better in separating closely charged species.

Formation of the Self-Generated pH Gradient Is More Effective in UNOsphere S than in Other CEX Resins

In order to determine the extent to which other CEX resins show similar on-column pH gradient formation, pH transitions during salt steps were compared for UNOsphere S, a 70 µm prototype of Nuvia HR-S, and SP-Sepharose FF. The resin characteristics are shown in Table 1. The pH transitions were

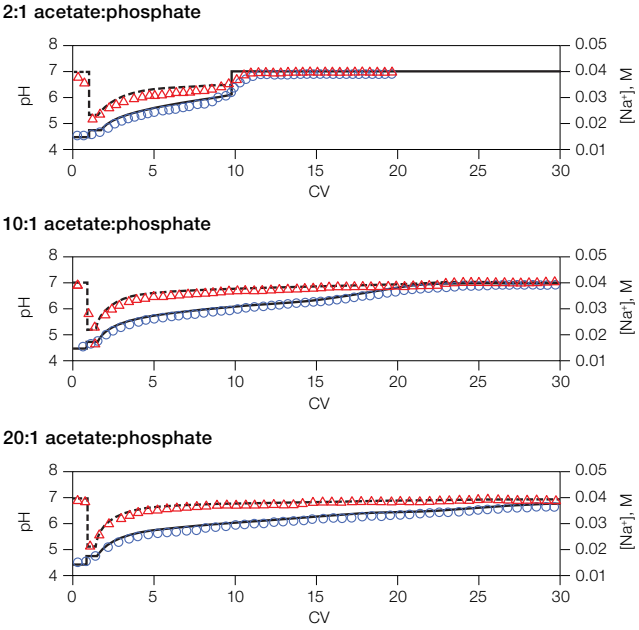
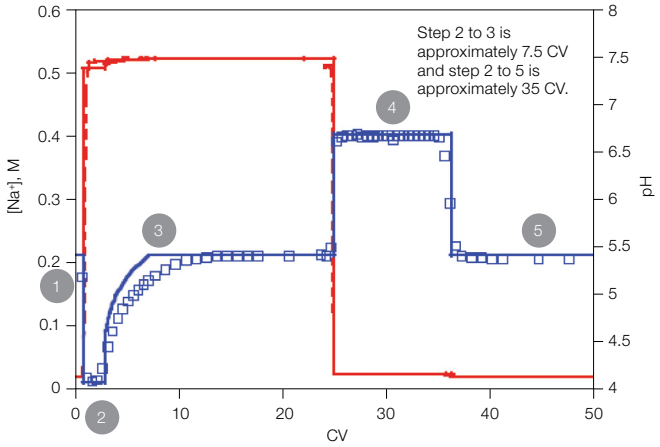


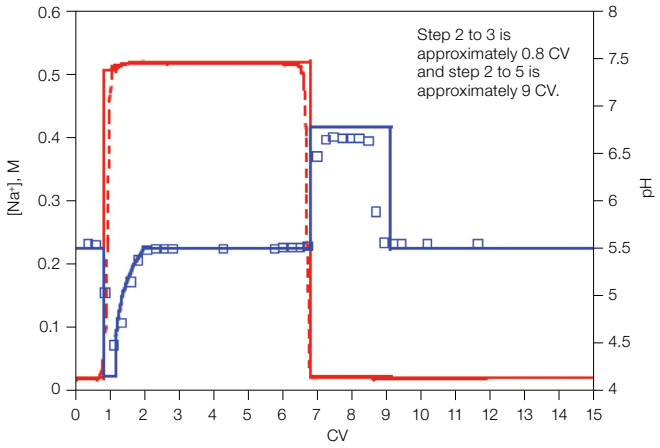
Fig. 4. Experimental and predicted Na^+ concentration profiles (Δ) and pH transitions (○) for pH 4.5–7 steps with buffers containing different acetate:phosphate mixtures and 0.04 M Na^+. Lines are based on the local equilibrium model. Experimental pH (○); model pH (—); experimental Na^+ (△); model Na^+ (- -).

studied in response to a 0 to 0.5 M NaCl step gradient in 0.02 N NaOAc buffer, followed by a decrease to 0 M NaCl in the same buffer. With all three resins, the pH decreased with increased salt concentration. The opposite effect on the pH was observed with a decrease in salt concentration. All resins showed a pH drop of 1.4 units from ~5.5 to ~4.1 immediately after the introduction of salt (Figures 5A, 5B, and 5C, step 1). However, the duration within which the pH returned to the operating pH of ~5.5 varied significantly. The duration of the pH drop for UNOsphere S was ~7.5 CVs (Figure 5A, steps 2 and 3), in contrast to 0.8 CVs for the Nuvia HR-S prototype and only 0.2 CVs for SP-Sepharose FF (Figures 5B and 5C). This difference between the duration of the pH gradients depends, at least in part, on the weak acid group content (the COO^- group) of the three resins. As shown in Table 1, UNOsphere S has the most COO^- groups in its backbone. These COO^- groups can contribute a high number of protons into the solution over a longer period of time, thereby increasing the duration of the pH gradient. In contrast, both the Nuvia HR-S prototype and SP-Sepharose FF have lower weak acid group content. UNOsphere S took ~10 CVs to return to the operating pH of ~5.5 after the decrease in salt concentration, which was considerably longer than the ~2 CVs for the Nuvia HR-S prototype and ~0.5 CVs for SP-Sepharose FF. These results indicate that although most S-type resins have residual COO^- groups and produce a pH gradient, to a certain extent, only UNOsphere S has sufficient COO^- groups to allow the formation of gradients with usable durations.

A. UNOsphere S



B. Nuvia HR-S prototype



C. SP-Sepharose FF

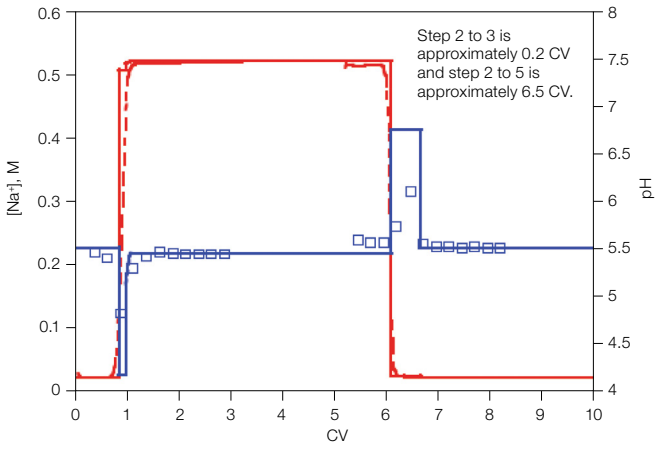
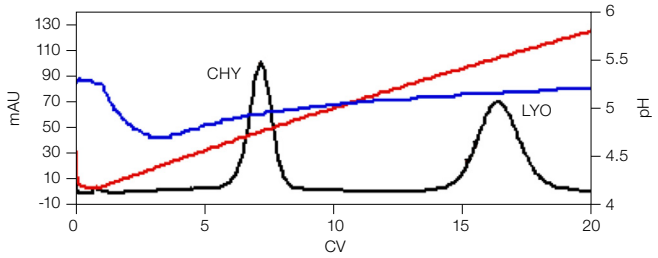


Fig. 5. Experimental and predicted pH and NaCl profiles for positive and negative 0–0.5 M NaCl steps in 0.02 N NaOAc, pH 5.5. The steps are: 1. pH 5.5 equilibration, 2. 0–0.5 M NaCl, 3. re-equilibration, 4. 0.5–0 M NaCl and 5. re-equilibration. Model Na^+ (—); experimental Na^+ (- -); model pH (—); experimental pH (□).

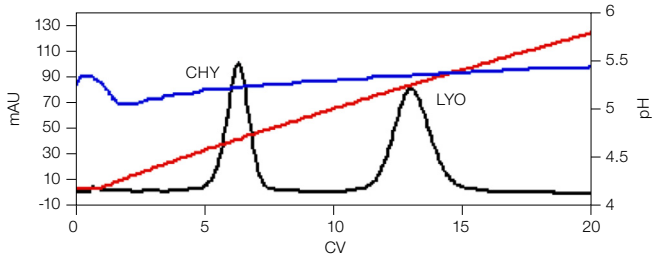
Enhanced Resolution of Proteins with the UNOsphere S Self-Induced On-Column pH Gradient Relative to Other Resins

A mixture of α-chymotrypsinogen (pI, 9.1) and lysozyme (pI, 11) in 20 mM Na^+ acetate buffer was applied to the above-mentioned resin columns (Table 1). A salt gradient of 0–750 mM NaCl at pH 5.5 was introduced for protein separation. Although the two proteins can be resolved on all three resins, UNOsphere S, due to its efficient pH gradient induction (dip in the blue pH line), provides the best resolution between the purified protein peaks (Figure 6). The peaks were ~10 CVs apart in the case of UNOsphere S, ~7 CVs for the Nuvia HR-S prototype, and 4 CVs for SP-Sepharose FF.

A. UNOsphere S



B. Nuvia HR-S prototype



C. SP-Sepharose FF

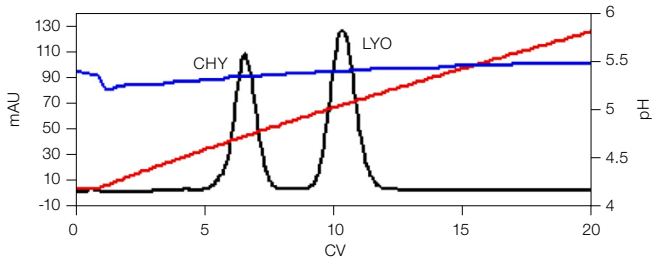


Fig. 6. Resolution of a mixture of α-chymotrypsinogen and lysozyme. pH (—); salt gradient (—); protein (—); CHY, α-chymotrypsinogen; LYO, lysozyme.

Examples of Protein Separation on the Self-Generated pH Gradient of UNOsphere S

Proteins with Very Close Isoelectric Points Can Be Separated in a Nearly Salt-Free System

The ability of UNOsphere S to resolve proteins with small differences in pI was evaluated through elution of ovalbumin (OVA, pH 4.8), bovine serum albumin (BSA, pH 4.9), and human serum transferrin (HST, pH 5.2). This was performed with pH 4.5–7 steps using buffers containing a 10:1 ratio of acetate and phosphate mixtures with a total sodium

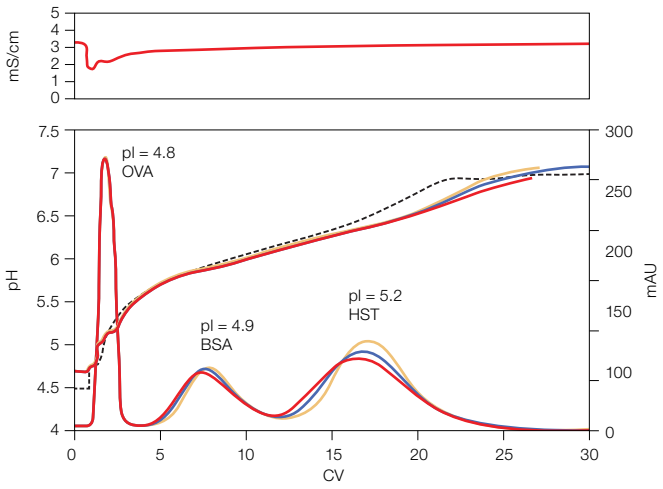


Fig. 7. Separation of the ovalbumin/albumin/transferrin mixture on UNOsphere S with pH 4.5–7 steps using a 10:1 ratio of acetate and phosphate buffers containing 0.04 M Na^+ at different mobile phase flow rates. Total protein load was 6 mg/ml of packed column at pH 4.5. BSA, bovine serum albumin; HST, human serum transferrin; OVA, ovalbumin. pH model (- -); 2 ml/min (—); 4 ml/min (—); 6 ml/min (—).

concentration of 40 mM at different mobile phase flow rates. The conductivity variation remained very low throughout the separation, between 2 and 3 mS/cm (Figure 7, top panel). Both conductivity and pH traces demonstrated little sensitivity to the flow rate. The three acidic proteins were well separated despite their close pIs, and eluted in order of increasing pI (Figure 7). OVA and BSA, which differ by a pI of 0.1 units, were separated by about 5 CVs.

Separation of the Native Superoxide Dismutase (SOD) Protein from Its PEGylated Form

Covalent binding of PEG to proteins (PEGylation) typically neutralizes one charge on the protein (for example, lysine) and alters its size to a certain extent. Separation of the conjugated protein from the unmodified native protein requires a resin with high resolution capability. Size exclusion chromatography (SEC) can separate the protein species by size. However, it is limited by small load volume and a long processing time, which makes this technique unsuitable for process purification. Therefore, the feasibility of applying pH gradient CEX chromatography to the purification of PEGylated proteins was tested and is described in detail in Ng et al. (2009). In that study, native SOD and PEG-SOD were run on a UNOsphere S self-generated gradient to assess whether they could be resolved to pure fractions.

PAGE analysis showed that native SOD appears as a single prominent band, whereas PEG-SOD is more heterogeneous, with a smear of higher molecular weight species (Figure 8A). Chromatography of SOD on UNOsphere S resulted in two major peaks (Figure 8B, peaks 1 and 2), which presumably correspond to two charge variants of the native protein, since only one band was visible by PAGE analysis (Figure 8C, lanes 1 and 2). PEG-SOD eluted as one major peak (Figure 8B, peak 3), which was confirmed to be that of PEG-SOD (Figure 8C, lane 3). To demonstrate the effectiveness of the on-column pH induced method, a 1:1 mixture of native SOD and PEG-SOD was applied to the column.

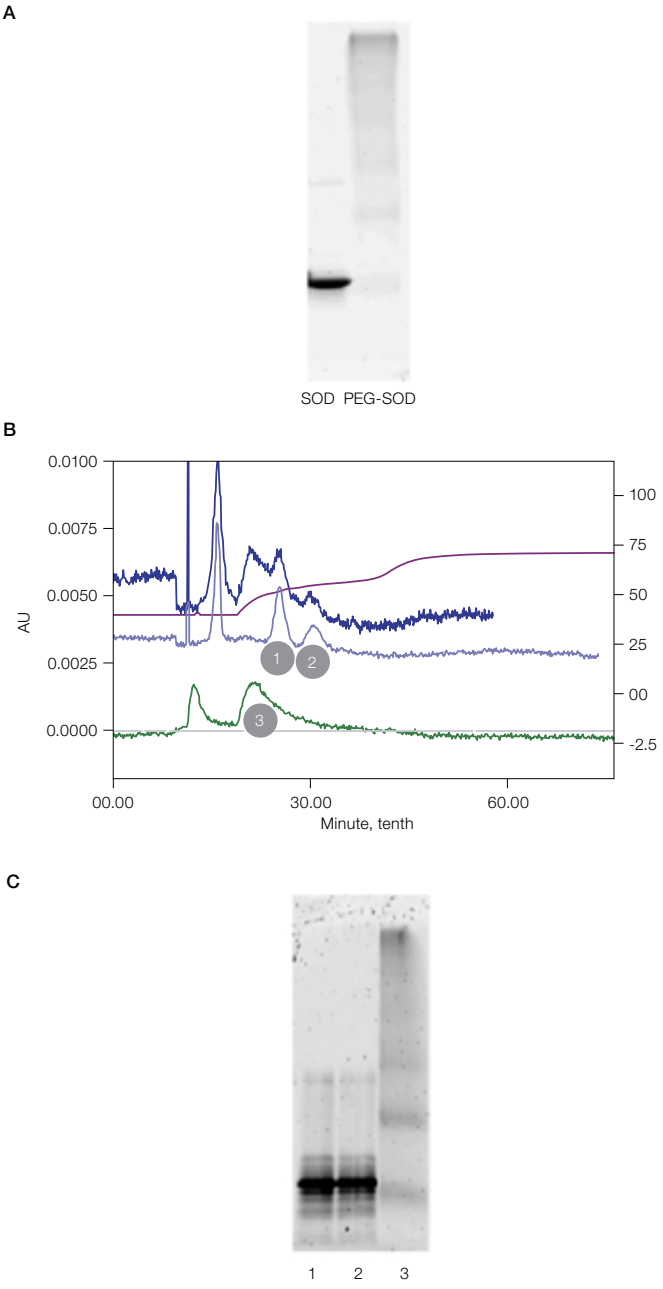


Fig. 8. Separation of native and PEGylated superoxide dismutase (SOD). **A**, SDS-PAGE analysis of SOD and PEG-SOD prior to chromatography. **B**, chromatographic profiles of a mixture of SOD and PEG-SOD, SOD only, and PEG-SOD only. **C**, SDS-PAGE analysis of pools from the fractions obtained by induced pH chromatography. Numbers in the SOD profile and the PEG-SOD profile (in **B**) correspond to the lane numbers listed in the gel scan. SOD and PEG-SOD mixture (—); SOD (—); pH gradient (—); PEG-SOD (—).

As shown in Figure 8B (dark blue trace), a pH-induced gradient resolved the mixture into three distinct peaks, each corresponding to its respective position as identified by chromatography of SOD and PEG-SOD separately. These results demonstrate partial resolution of the native and PEGylated protein using self-generated pH gradient chromatography with UNOsphere S. Further resolution may be possible with an even shallower pH gradient. Alternatively, additional purification of the low salt eluate can be accomplished with an orthogonal chromatography step.

Separation of Native and Carbamylated Carbonic Anhydrase II (CA)

A similar experiment to that described in the previous section was performed with native and carbamylated forms of carbonic anhydrase II (CA). A UNOsphere S column was equilibrated with a 10:1 ratio of 40 mM Na^+ acetate and 40 mM $NaPO_4$ (pH 5). The proteins were eluted with a 40 CV gradient to a 4:1 ratio of 40 mM Na^+ acetate and 40 mM $NaPO_4$ (pH 7.5). The chromatogram in Figure 9 shows two separate peaks at fractions 18 and 28 (Figure 9A) corresponding to the two forms of CA, which differ by a pI of 0.5. This was also demonstrated by IEF (Figure 9B).

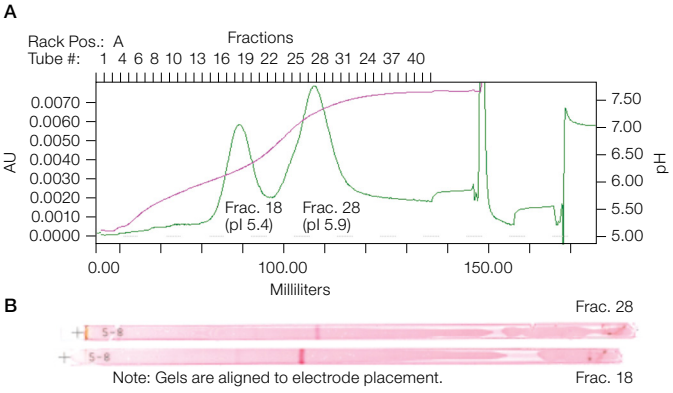


Fig. 9. Separation of native and carbamylated carbonic anhydrase II. **A**, chromatogram **B**, IEF. AU (—); pH (—).

Reproducible pH Gradient Generation with UNOsphere S

Protein separation can be vulnerable to nonreproducible pH changes. It is therefore crucial that the pH gradient induced by the CEX resin is the same each time a new manufacturing lot of resin is used. This becomes mandatory for process-scale purification of therapeutic proteins and antibodies. Previous results (Ng et al. 2009) demonstrate that UNOsphere S exhibits consistent pH transitions across three different batches of the resin tested (Figure 10). Minor differences seen in the elution profiles were within the experimental margin of error of ±5% of pH measurements. These results indicate that protein separation behavior on UNOsphere S is predictable and this resin can therefore be used for process-scale purification.

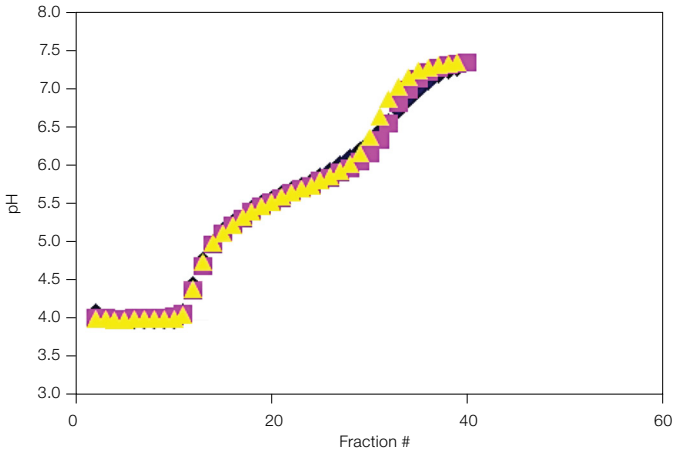


Fig. 10. Overlay of pH profiles from induced pH gradients in three batches of UNOsphere S. 2448 (◆); 2431 (■); 79734B (▲).

Conclusions

In summary, UNOsphere S resin shows superior self-generated pH gradient formation compared to other CEX resins. This capability provides a better resolving power over traditional pH gradient techniques and can be exploited to separate proteins with very close pIs. In addition, the large size of UNOsphere S and the reproducibility of the self-generated on-column pH gradient formation across multiple batches make this resin ideal for use in manufacture-scale protein purification.

Acknowledgements

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