Nylon-6 Capillary-Channeled Polymer Fibers as a Stationary Phase for the Mixed-Mode Ion Exchange/Reversed-Phase Chromatography Separation of Proteins

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Abstract

Capillary-channeled polymer (C-CP) fibers extruded from nylon-6 are used as the stationary phase for the ion-exchange/reversed-phase mixed-mode chromatographic separation of a three protein mixture. The nylon-6 C-CP fibers are packed collinearly in a 250 × 1.5-mm i.d. column with an interstitial fraction of ~0.6. The effects of four displacing salts at three different pHs are studied with regards to protein retention time, peak width, selectivity, and resolution for a synthetic mixture consisting of myoglobin, ribonuclease A, and lysozyme to determine the optimum mobile phase conditions. The net charge model is found to be inadequate in fully explaining the retention behavior, as the proteins are retained by anion and cation-exchange interactions, as well as hydrophobic interactions with the stationary phase. It is found that pH and displacing salt strength had a significant influence on the retention properties and resolution of the proteins.

Introduction

Ion-exchange chromatography (IEC) is one of the primary methods for the separation of proteins due to its ability to resolve and purify the proteins while maintaining their biological activity during the separation process (1–4). In IEC, binding is a result of the interaction of a charged solute to a stationary phase of opposite charge (4,5). Elution is achieved with the employment of a gradient from low to high salt concentrations, thus increasing the concentration of competing ions in the mobile phase and attenuating the electrostatic interaction between the charged solute and the oppositely charged stationary phase (1,2,5,6). Retention and selectivity in IEC protein separations are governed by pH, ionic strength, addition of organic solvents, and stationary phase chemistry (7). The protein is eluted when the mobile phase composition reaches the appropriate ionic strength and/or pH (8). Typical IEC supports are derived from silica, polystyrene, and polyacrylate beads with pore sizes ranging from 30 to 100 nm, with the smaller pore sizes having a higher ion exchange capacity, though at the expense of increased backpressure and hindered mass transfer (6). Popular bonded phases of IEC supports consist of sulfonic acid and carboxylic acid groups for cation exchange and quarternary and polyalkylamine groups for anion exchange (8).

While in the case of inorganic ions, electrostatic interactions are solely responsible for retention, organic ions can be retained by electrostatic and hydrophobic interactions (6). There are well known situations where mixed-mode IEC/reversed-phase separations can be achieved (9–11). Solutes that have a charge opposite that of the stationary phase are predominately retained by the ion exchange process, whereas neutral solutes are retained primarily by hydrophobic processes (7). Of course polyfunctional organic molecules can be retained by a combination of interactions. In the case of biomacromolecules, properties of proteins such as isoelectric point (pI), molecular weight, and hydrophobicity must be considered when choosing the appropriate ion-exchange column (8).

The elution characteristics and retention properties of proteins in the mixed-mode IEC/RP mode are affected by the eluting salt type and organic composition. The effect of displacement salt strength for the separation of proteins has been studied extensively (3,12–17). Typically, the strength of displacing salts in anion exchange chromatography follows the trend of $CH_3COO^- < Cl^- < HPO_4^{-2} < SO_4^{-2}$ and for cation exchange $K^+ < Na^+ < NH_4^+ < Ca^{+2} < Mg^{+2}$ where the more highly charged displacers have stronger elution strengths. The elution strength of competing ions with the same ionic charge is dependent on the polarizability and size of the ion, as well as their respective affinities for the stationary phase (6). The elution properties of proteins cannot always be predicted based on the strength of displacing salts alone, especially when hydrophobic interactions are involved. Moreover, the use of different displacing salts or the use of different types of exchange resins can alter protein retention

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or selectivity (or both) (1,17). It is also possible for the salt to complex with the protein or alter the tertiary structure of the protein causing changes in the selectivity (2).

The pH of the mobile phase can have a significant influence on the retention and selectivity of proteins in IEC. According to the "net-charge" model, at the pI the net charge on a protein is zero and, in principle, will not be retained on ion-exchange columns (17). Below the pI, the protein is predominately in its cationic form and, therefore, will be retained by cation exchange ligands, while above the pI the protein is in its anionic form and will be retained on an anion-exchange phase (17). However, the pI of a protein alone cannot be used to determine the extent of bonding to the ion exchanger. The three-dimensional nature of a protein dictates that only groups of a certain charge will be exposed and thus able to interact with the ion exchange phase. As such, the interaction of the protein with the ion exchange phase is dependent on the surface charge of the exposed amino acids (and its density) rather than the net charge of the protein (1,6). These factors make it possible for many proteins to have retention on either anion or cation exchange columns (1). Staby et. al. (18,19) have studied the pH dependence on the retention factor of five proteins by comparing their responses over a pH range of 6 to 9 on strong anion-exchange resins. They found that the retention factor of the proteins followed the general trend of increased retention as pH increased, but the response was not proportionate and varied according to the identities of the IEC resin and protein in question. More importantly, they found that proteins could be retained on the anion-exchange resins at pH values below that of the protein's pI. This was attributed to localized charge on the exterior of the protein. This conclusion was supported by previous studies of Kopaciewicz et al. (17) who found retention of proteins up to one pH unit below the pI on strong anionic-exchange mono-Q columns.

Due to the instability of silica under alkaline pH conditions ($\geq$ 8), synthetic polymer supports have become increasingly popular for IEC separations. Two commonly used polymer bead supports are the hydrophobic poly(styrene-divinylbenzene) and the hydrophilic polymethylacrylate and polyvinylalcohol beads (6). While the pH stability of these phases is quite high, exposure to organic solvent can cause the beads to swell, further reducing the specific permeability of particle-packed columns (6). In addition, the microporosity inherent to most of these polymer phases restricts mass transfer in the case of macromolecules such as proteins. An alternative to microparticulate supports is the use of natural and synthetic fiber stationary phases that have the characteristic of high specific permeability as well as being available from a wide range of base materials (i.e., different chemical selectivities).

Ladisch overcame the pressure limitations imposed by microparticulate columns by employing rolled stationary phases (RSP) made from woven textile fabrics consisting of a 60:40 cotton–polyester blend (20,21). The cotton portion of the RSP was derivatized with (diethylamino)ethyl to give the fabric anion-exchange capabilities. The authors were able to successfully separate cytochrome C and β-lactoglobulin A in less than 10 min, demonstrating the viability of polymer fiber stationary phases for the ion-exchange separation of proteins.

Chen and co-workers used poly(vinyl alcohol) fibers deriva-

tized with quarternary ammonium groups to successfully separate a mixture of inorganic (F^-, NO_2^{2-}, NO_3^{3-}, PO_4^{3-}, and SO_4^{2-}) anions (22). Different from the case of organic ions, inorganic ions are separated solely by the ion-exchange (i.e., electrostatic) processes. The fibers had a diameter of 36 μm and were packed into stainless steel columns (250 × 1.8-mm i.d.). The authors demonstrated the ability to fully resolve the anion mixture employing relatively high linear velocities ranging from ~6 to 20 mm/s. The separation efficiency decreased with increasing linear velocity as might be expected, but the fiber packed columns generated a pressure drop of only about a tenth of that common to columns packed with 15-μm diameter particles when operated at the same flow rate. These characteristics suggest the further use of fiber phases in preparative-scale and high speed separations.

This laboratory has introduced the use of capillary-channeled polymer (C-CP) fibers as combined stationary/support phases for liquid chromatography separations. The general advantages of using C-CP fiber stationary phases include low capital cost, ease of column preparation, high specific permeability, and the ability to choose from different base polymers to affect different separation characteristics (23–28). Fortuitously, the base polymer materials used to date (polypropylene, polyester, and nylon) exhibit insignificant swelling upon exposure to buffers and organic solvents. While studies of column hydrodynamics have been undertaken with regards to small molecule separations (24,25), the practical application of the C-CP fiber columns is best realized in the separation of proteins and other macromolecules (26–28).

A comparison of a number of chromatographic figures between polypropylene (PP) and polyester (PET) C-CP fibers and a commercial C4-derivatized silica column has recently been published (29). Five proteins [ribonuclease A (RNase A), cytochrome c, lysozyme, myoglobin, and bovine serum albumin (BSA)] were used to investigate the separation characteristics under typical RP gradient conditions. Column performance was compared under standard (identical) and optimized RP chromatographic conditions. The packed-bed column was operated as prescribed by the column manufacturer. The gradient compositions utilized with the C-CP fiber columns are similar to those used with conventional columns with gradient rates of ~1%/min, but employing flow rates in the 1 to 6 mL/min range. One particularly unique feature observed there is the lessening of the percentage of organic modifier necessary to elute the proteins from the fiber phases with increased linear velocity. The potential contribution of the different stationary phases to protein denaturation was evaluated through a spectrophotometric enzymatic activity assay, with the PET fiber column showing superior activity retention. The repeatability of retention times under both sets of conditions for six consecutive injections of lysozyme on each C-CP fiber column is ≤ 1.5% RSD. The column-to-column reproducibility of retention times for three columns of each fiber type is also ≤ 1.5% RSD. The overall performance of the C-CP fiber columns was comparable to the conventional column used in these studies. Basic characteristics demonstrated suggested further developments in the areas of ultra-fast protein separations and preparative-scale protein chromatography.

The use of nylon-6 C-CP fibers is demonstrated for the ion-

exchange/reversed-phase mixed-mode separation of a synthetic three-protein mixture consisting of myoglobin, ribonuclease A, and lysozyme. The nylon-6 monomer (Figure 1A) consists of amide groups, which provide different chemical functionality than found in a polyolefin, for example. Of course, within the extruded fiber system there is discontinuity of polymer chains that exposes amine and carboxylate functional end groups as shown in Figure 1B. The identity of the functional groups are a function of the pH of the solvent environment, fortuitously resulting in a zwitterionic surface in the pH range of 4 to 8 (30,31), providing both anion and cation-exchange capabilities. At the same time, the alkyl backbone provides the fiber with slightly hydrophobic characteristics. The results of preliminary studies in the use of the nylon-6 C-CP fiber phases for mixed-mode protein separations are presented here. The effects of displacing salt type and pH have been studied to determine their influence on retention time, peak width, selectivity, and resolution. It is believed that the trends observed here, when coupled with results of other studies in protein separations (29), suggest a great deal of potential with regards to high-speed protein separations across a range of separation modes.

Experimental

Column construction

The nylon-6 C-CP fibers were obtained from Fiber Innovation Technology (FIT) (Johnson City, TN). The individual fibers are extruded with eight capillary channels running parallel with the fiber axis, having asp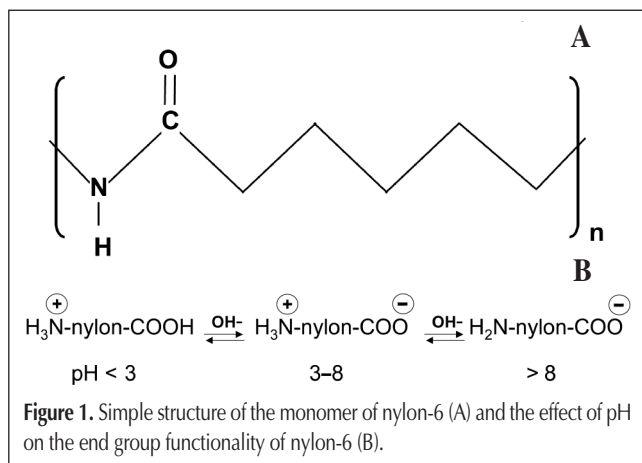ect ratios that permit spontaneous fluid movement (32,33). In column format, the channel walls of adjacent fibers interdigitate to form a monolithic structure. These fibers were produced with a maximum cross sectional diameter of 40 μm as determined by scanning electron microscopy (SEM). Ion exchange capacity measurements were performed on the nylon-6 C-CP fiber in a manner similar to that of Staby et. al (19). Approximately 0.75 grams of the nylon-6 C-CP fiber were washed with ~ 30 mL of 1M NaCl (pH 2). The fibers were then transferred to a beaker containing 30 mL of 1M NaCl (pH 2) and titrated with 0.1M NaOH to pH 13. Titration of the nylon-6 C-CP fibers showed the exchange capacity of the nylon-6 fiber to be ~8 μmol/g.

The nylon-6 C-CP fiber columns were packed by winding the fibers onto a spool and then doubling the fiber over a monofilament and pulling the fiber bundle through 250 × 1.5-mm i.d., 316 stainless steel tubing. The ends of the fibers were then cut flush with the end of the tubing and frits and end-fittings were installed. The packing process is described in greater detail in previous publications (23,24,26,27). The nylon-6 C-CP fiber packed column was produced with an interstitial fraction of ~ 0.6, as previous studies have shown this to be the optimum value with regards to hydrodynamics and separation quality (24). The stainless steel tubing (10 μm porosity frits) and column end-fittings were purchased from Valco Instruments (Houston, TX).

Apparatus

The chromatographic system consisted of a Waters (Milford, MA) Model 1525 high-performance liquid chromatography pump with a Rheodyne (Rohnert Park, CA) six port injector valve fitted with a 1 μL injection loop and a Waters 996 photodiode array detector. The chromatograms were recorded using Waters Millennium 4.0 data acquisition system and processed using Microsoft Excel (Seattle, WA) and Sigmaplot 8.0 (Point Richmond, CA). The data presented is the mean of triplicate injections, with the error bars in each plot representing a variance of one standard deviation. It should be noted that, throughout the course of these experiments, as the roles of pH and eluting salt type were evaluated, the variation in retention times under a single set of conditions were less than 2% RSD.

Methods

Buffer A for all experiments was 10mM piperazine-1,4-bis (2-ethanesulfonic acid) in 18.2MΩ/cm water with the pH adjusted to the appropriate value with 1M NaOH. Buffer B consisted of buffer A and 1M of the monovalent eluting salts (sodium acetate and sodium chloride) or 0.5M of the divalent ions (sodium phosphate and sodium sulfate) to keep the ionic strength constant. Stock solutions of reagent-grade myoglobin, ribonuclease A, and lysozyme (Aldrich, Milwaukee, WI) were prepared in buffer A at a concentration of 100 μg/mL each. The molecular weight and pI of the proteins are listed in Table I. The salt gradient was from 0 to 100% in 15 min at a flow rate of 1.3 mL/min, equating to a linear velocity of ~21 mm/s, which is ~2× higher than the common linear velocities employed with microparticulate supports, but far lower than what has been found to be optimal for RP separations on the C-CP fiber columns (4,34–36).

Figure 1. Simple structure of the monomer of nylon-6 (A) and the effect of pH on the end group functionality of nylon-6 (B).

Table I. Molecular Weight and Isoelectric Points (pI) of the Three Proteins Separated on Nylon-6 C-CP Fiber Columns

Protein	Molecular Weight	pI
Myoglobin (equine)	17,800	7.0
Ribonuclease A (bovine)	13,700	8.7
Lysozyme (hen egg-white)	14,300	11.0

Results and Discussion

Effect of salt type and pH on retention time and peak width

The type and strength of displacing salts used in IEC can have a profound effect on the selectivity, resolution, and speed of protein separation (3,8,14,17). Four salts at three pH values were compared to determine their effect on the chromatographic properties for the separation of myoglobin, ribonuclease A, and lysozyme. The chromatograms in Figure 2 illustrate the effects of displacing salt strength (pH 8) on the three protein mixture. At this pH, the nylon surface should be zwitterionic, though approaching overall anionic character. As can be seen, the type of displacing salt has an effect on the retention time (t_r) of each protein and, thus, the overall selectivity of the separation. The elution of the three proteins was in order of increasing isoelectric points, suggestive of a cation-exchange separation.

Figures 3A–3C show the effects of salt type and pH on the retention time of myoglobin, ribonuclease A, and lysozyme, respectively. Myoglobin (pI ~7) is only slightly retained, though clearly differentiated from the solvent (unretained) peak, which elutes at t_0 ~0.2 min. Figure 3A depicts the retention time of myoglobin being only slightly affected by changes in salt type and pH. At these retention times (0.39–0.46 min), it is probable that the effect of salt type will be minimal (due to the dwell volume of the LC system) with the pH being a major contributing factor to the retention properties. The largest difference in retention time (~10%) is at pH 6, where myoglobin is more cationic in overall charge. Thus, this decrease in retention is most likely due to the greater affinity with the solution phase (1). The longest retention time of myoglobin is found at pH 7. With the pI of myoglobin being ~7, the net charge of the protein should be zero. However, proteins at their isoelectric point still have charged groups on their periphery, allowing for electrostatic interactions. At a pH value of 8, myoglobin is predominately in its anionic form and appears to be only slightly retained with a minimal effect of displacing salt. This would mostly be due to the change in the nylon fiber surface charge from zwitterionic to anionic (30). The retention at this pH would suggest only minimal extents of hydrophobic interaction.
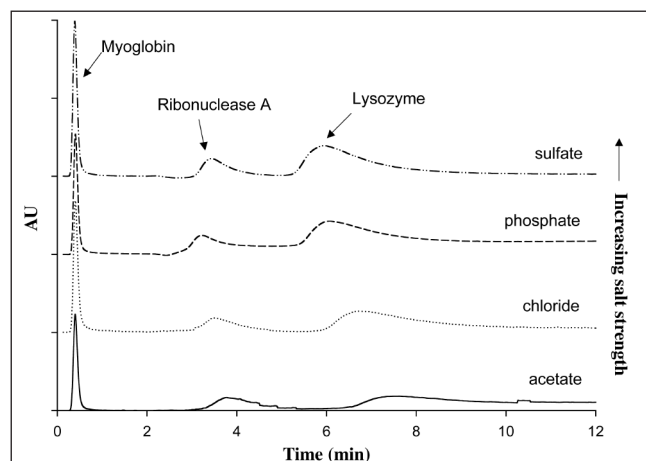
Clearly, retention properties of proteins can only be estimated by their pI as the binding strength is governed by surface charge, charge density, and stationary phase hydrophobicity rather than solely the net charge of the protein (1,5,17).

Figures 3B and 3C depict proteins with higher pI's, ribonuclease A (pI ~ 8.7) and lysozyme (pI~11.0), which are more strongly retained. Both graphs follow the trend of longest retention time at pH 7, with the pH 6 having the shortest retention time. This decrease in retention is most likely due to the change
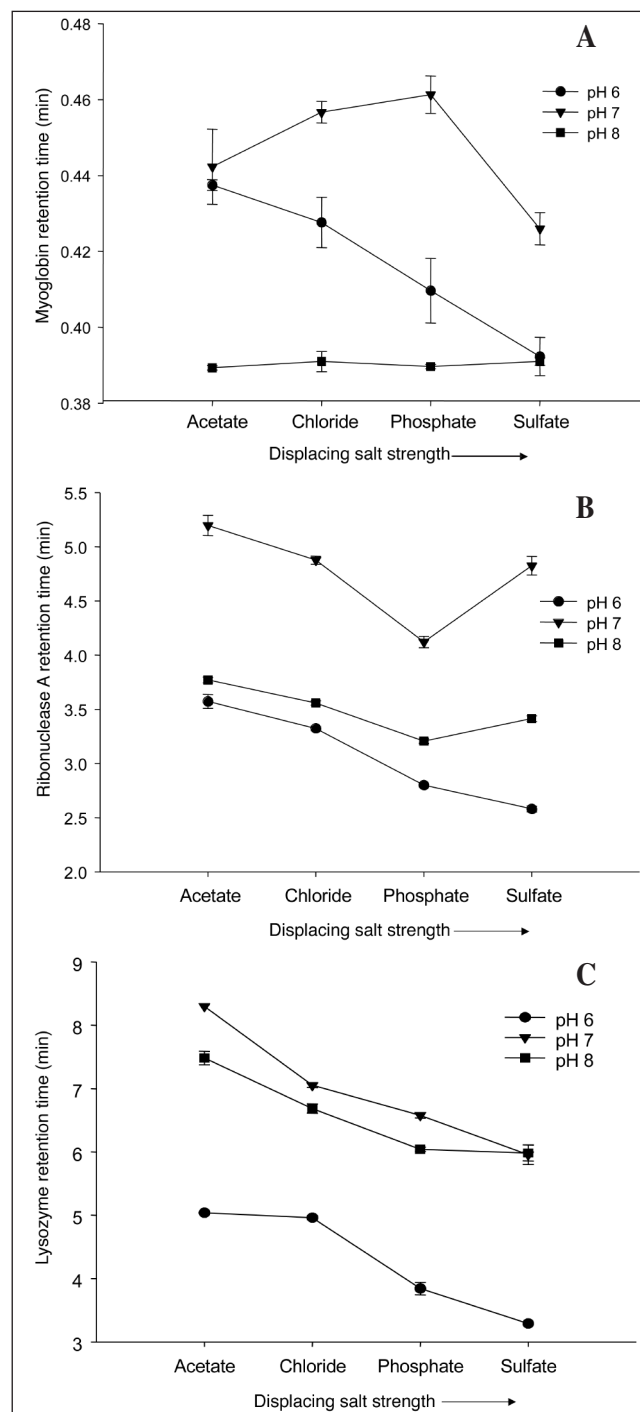

Figure 2. Effect of eluting salt type on the IEC/reversed-phased mixed-mode separation of myoglobin, ribonuclease A, and lysozyme on a nylon-6 C-CP packed column (250 × 1.5-mm i.d.). Gradient elution 0–100% B over 15 min, pH 8, flow rate 1.3 mL/min.

Figure 3. Effects of salt type and pH on the retention time of myoglobin (A), ribonuclease A (B), and lysozyme (C) on a nylon-6 C-CP packed column. Gradient elution 0–100% B over 15 min, flow rate 1.3 mL/min.

in the nylon surface charge, which is more cationic at a pH of 7 (30). The retention times also decrease as the displacing salt strength increases, with the exception of sodium sulfate. This general trend emphasizes the fact that it is not only the interaction of the protein with the surface but also the interaction of the protein with the bulk solution that are of importance (1). Different from the other two proteins, a discontinuity exists in the retention time of myoglobin at pH 7 (Figure 3A) with the generally strong phosphate mobile phase showing greater retention. This suggests some form of solution-phase chemistry that decreases the affinity of the protein for the aqueous-phosphate environment. Similar to myoglobin, ribonuclease A was also retained at a net charge close to zero (pH 8), which is presumably due to the localized anionic regions of the protein structure and hydrophobic interactions between the proteins and the nylon-6 C-CP fiber stationary phase.

The addition of organic solvents, ranging from 1% to 10%, can be used to minimize the extent of hydrophobic interactions of the proteins with stationary phases (1,3,17,37). With the addition of 5% isopropanol to the mobile phase, only lysozyme displayed a reduction in retention time. This would suggest that electrostatics are the primary mode of interaction for all of the proteins, and that lysozyme would appear to have the largest extent of hydrophobic interactions (though still minor). The greatest

effect of isopropanol occurred with the use of the chloride salt with the retention time reduced by ~ 50 s and the least effect when the sulfate salt was used, with the retention time decreasing by ~ 21 s.
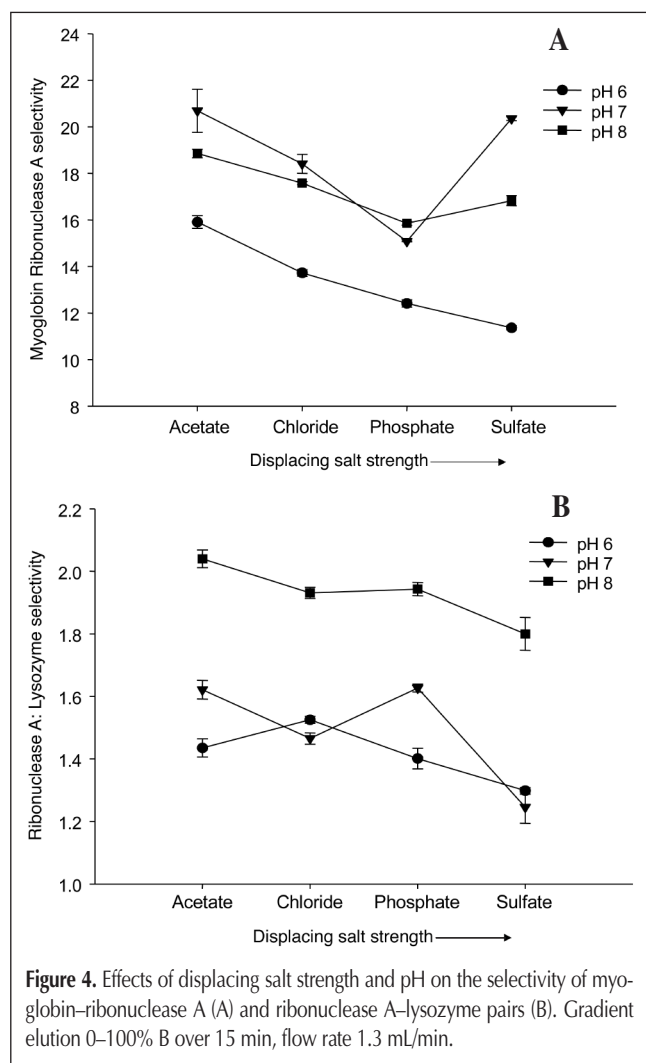
The strength of displacing salt can also affect the peak width of the eluting proteins as the desorption kinetics are the limiting aspect in the relatively high velocity C-CP fiber separations. The effects of pH and displacing salt strength on the peak width of all proteins are similar to the effects on their retention time (Figure 3A–3C). More specifically, the elution conditions that result in increased retention also display the largest peak widths, while the stronger elution conditions result in faster elution times and smaller peak widths. As the residence time of a protein on a hydrophobic surface increases, so too does the potential extent of denaturation (37). This in turn increases the onset and breadth (i.e., peak width) of ionic strength values necessary to affect elution. It is difficult to compare these results to the effects of pH and displacing salt on other IEC/RP mixed-mode stationary phases due to the fact that each protein behaves differently in a given mobile phase environment and with different column chemistries.

Effect of salt type and pH on the selectivity

Changes in pH can have a profound effect on the selectivity of an ion-exchange separation (6). Figure 4 illustrates the effects of salt type and pH on the selectivity for the protein pairs in the mixture. Seen in Figure 4A, pH values of 7 and 8 result in higher selectivity factors ($a = k_2/k_1$, where $k = t_R - t_0/t_0$) of myoglobin and ribonuclease A than at pH 6. An increase in displacing salt strength decreases these values. The exception is an increase in selectivity using sodium sulfate at pH values of 7 and 8. This is due to sodium sulfate causing an increase in retention time of ribonuclease A while the retention time of myoglobin decreases. At pH 6, the selectivity values are lowest and consistent with the trend of displacing salt strength, which is expected because the proteins are in their least anionic form. Figure 4B shows the selectivity values for the ribonuclease A–lysozyme pair. The highest selectivity values are found at pH 8 and decrease ~12% across the suite of eluting salt strengths. The pH of the mobile phase has a more profound effect on the selectivity of the proteins than the type of displaying salt employed. This is the result of the change in the net charge of ribonuclease A, and the fact that the nylon-6 surface is mostly anionic. In this situation, the attractive forces would be lessened, and so small differences in environment might have greater effects.

Effect of salt type and pH on resolution

The resolution [$R_s = 2(t_2 - t_1)/w_1 + w_2$] obtained for a pair of solutes is a reflection of the combined effects of selectively and peak width. As such, the pH and strength of displacing salt can have a profound effect on the resolution of protein mixtures. Figures 5A and 5B depict how resolution varies as a function of pH and salt type on the nylon-6 C-CP fiber packed column. As seen in Figure 5A, the resolution of the myoglobin–ribonuclease A pair at pH 8 is relatively insensitive to salt displacement strength due to minimal changes in the retention time of myoglobin. When the pH is reduced to 7, an increase in salt strength is accompanied by an increase in resolution. At this pH, the acetate salt gives the lowest resolution (~4), while the sulfate
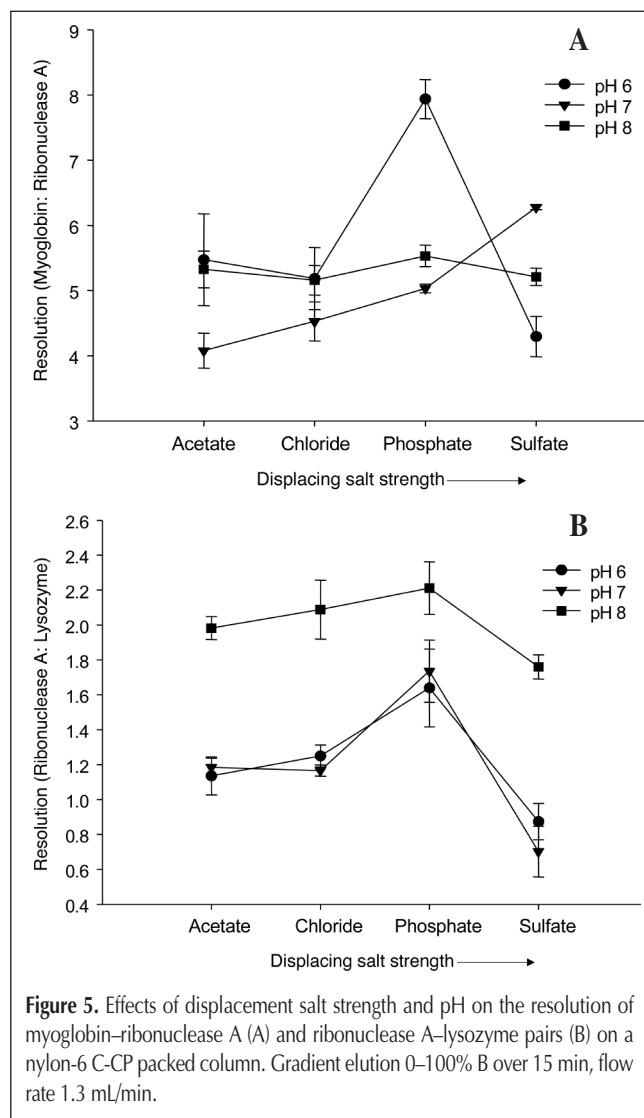

Figure 4. Effects of displacing salt strength and pH on the selectivity of myoglobin–ribonuclease A (A) and ribonuclease A–lysozyme pairs (B). Gradient elution 0–100% B over 15 min, flow rate 1.3 mL/min.

Figure 5. Effects of displacement salt strength and pH on the resolution of myoglobin–ribonuclease A (A) and ribonuclease A–lysozyme pairs (B) on a nylon-6 C-CP packed column. Gradient elution 0–100% B over 15 min, flow rate 1.3 mL/min.

Conclusion

The use of nylon-6 C-CP fibers as the stationary phase for the IEC/RP mixed-mode chromatographic separation of proteins has been demonstrated. The nylon-6 C-CP fiber-packed columns enabled the separation of a three-protein mixture at much higher linear velocities (~21 mm/s) compared to microparticulate packed columns. In addition, the nylon-6 C-CP fibers do not require derivatization, thus minimizing the time-consuming steps of stationary phase preparation. The effects of four displacing salts at three different pHs were studied with regards to protein retention time, peak width, selectivity, and resolution using a mixture consisting of myoglobin, ribonuclease A, and lysozyme. It was generally found that an increase in pH resulted in increased resolution, similar to protein IEC studies by other research groups (3,5). The results reported here generally follow the elution order of displacing salt strengths (1). The net charge model was found to be inadequate for the ion-exchange separation of proteins on nylon-6 C-CP fiber-packed columns due to some level of hydrophobicity and the specificity of local charges of protein structures. It is difficult to compare the results of nylon-6 C-CP fiber columns used in this study to commercial columns due to the differing behavior of each protein in a given mobile phase environment and with the different column chemistries.

Given the complexities associated with mixed-mode separations, clearly much work remains toward assessing the practicalities of these separations on C-CP fiber columns. Due to the retention of the proteins being predominately electrostatic interactions, the ability to retain and resolve proteins similar in nature (e.g., molecular weght, hydrophobicity) is possible and has already been achieved in this laboratory. Future studies will focus on a wider range of pH and displacing salts and the determination of the effects of changes in the protein tertiary structure on the retention properties. In addition, the use of nylon-6 C-CP fiber packed columns will be evaluated for the separation of acidic proteins under cation-exchange conditions. The study of higher flow rates (linear velocities) and shorter gradient times will allow the optimization of elution conditions toward rapid protein separations. Studies are also currently underway using nylon-6 C-CP stationary phases for the hydrophobic interaction separation of proteins.

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gives the highest resolution (~5.5) due to the influence of the ribonuclease A peak width. When the pH is further lowered to 6, the resolution remains relatively constant for the acetate and chloride salts. With the phosphate salt, there is a significant increase in resolution (~8) attributed to the narrower peak widths of both myoglobin and ribonuclease A. The resolution decreases ~50% to ~4.2 when sulfate salt is substituted due to the sulfate salt increasing the peak widths. The use of phosphate salt, at pH 7, results in the best resolution for myoglobin and ribonuclease A, under these chromatographic conditions.

The resolution of the ribonuclease A–lysozyme pair (Figure 5B) is more strongly affected by changes in pH. The resolution of the solutes at pH 6 and 7 are similar with increasing salt strength, with the highest resolution obtained using sodium phosphate and the lowest resolution with sodium sulfate. The resolution is significantly improved at pH 8. The increase in resolution is primarily due to a reduction in peak width while changes in selectivity play only a minor role. The general trend observed was resolution increased with increasing displacing salt strength, with the exception of sulfate, which was significantly lower than the other salts used.

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