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High-performance cation-exchange chromatofocusing of proteins

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Abstract

Chromatofocusing using high-performance cation-exchange column packings, as opposed to the more commonly used anion-exchange column packings, is investigated with regard to the performance achieved and the range of applications possible. Linear or convex gradients in the range from pH 2.6 to 9 were formed using a variety of commercially available column packings that provide a buffering capacity in different pH ranges, and either polyampholytes or simple mixtures having a small number (three or fewer) of buffering species as the elution buffer. The resolutions achieved using cation-exchange or anion-exchange chromatofocusing were in general comparable, although for certain pairs of proteins better resolution could be achieved using one type of packing as compared to the other, evidently due to the way electrostatic charges are distributed on the protein surface. Several chromatofocusing methods were investigated that take advantage of the acid–base properties of commercially available cation-exchange column packings. These include the use of gradients with a composite shape, the use of very low pH ranges, and the use of elution buffers containing a single buffering species. The advantages of chromatofocusing over ion-exchange chromatography using a salt gradient at constant pH were illustrated by employing the former method and a cation-exchange column packing to separate β-lactoglobulins A and B, which is a separation reported to be impossible using the latter method and a cation-exchange column packing. Trends in the apparent isoelectric points determined using cation- and anion-exchange chromatofocusing were interpreted using applicable theories. Results of this study indicate that cation-exchange chromatofocusing is a useful technique which is complementary to anion-exchange chromatofocusing and isoelectric focusing for separating proteins at both the analytical and preparative scales.

Keywords: Chromatofocusing; pH gradients; Cation-exchange chromatofocusing; Proteins

1. Introduction

Ion-exchange chromatography (IEC) is a versatile method for separating proteins based on exploiting differences in electrostatic charge. In the standard technique for carrying out IEC, proteins are eluted using a salt gradient with the liquid-phase pH being nearly constant.

Although IEC performed this way is widely used, it nevertheless exhibits several potentially limiting characteristics. First, according to the most commonly used isotherms for ion-exchange equilibrium, such

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as the steric mass-action isotherm [1], the relative adsorption affinity of most pairs of proteins is highest at low salt concentrations, while in IEC using a salt gradient proteins generally elute at high salt concentrations. Second, it has been reported that the resolution of proteins in IEC is highest when the pH of the liquid phase is near the isoelectric point (p*I*) of proteins being separated since this condition again enhances the relative adsorption affinity [2]. However, IEC performed with a salt gradient generally employs a fixed liquid-phase pH, so that it is not possible to operate the process near the p*I* of each protein if several proteins are being separated. Finally, IEC employing a salt gradient can be difficult to optimize since the resolution is determined not only by the characteristics of the salt gradient but also by the liquid-phase pH.

Chromatofocusing mitigates many of the shortcomings of IEC and combines elements of both IEC and isoelectric focusing (IEF). In particular, chromatofocusing employs a retained, internally produced pH gradient formed at low ionic strength to elute proteins at a pH, termed the ''apparent'' isoelectric point (pI_{app}), which is generally near the true p*I* of a protein. Since it was first developed by Sluyterman and co-workers [3–6], chromatofocusing has been demonstrated to be useful for separating proteins due to its high resolving power and its ability to retain the protein native state. Chromatofocusing is particularly useful for separating isoforms of proteins and proteins with minor differences in p*I*, making this technique an effective alternative to IEF for these cases, especially for preparative-scale applications.

To perform chromatofocusing, an ion-exchange column is first equilibrated with a starting buffer at a pH chosen so that all the components to be separated in the feed sample are adsorbed onto the column packing. The feed sample is then introduced, and the column is subsequently eluted with a stepwise change to an elution buffer, whose composition and pH are chosen so that none of the components of interest in the feed sample are adsorbed. For the case where a weak-base, anion-exchange column packing is used, the pH of the presaturation buffer is higher than the elution buffer. As a result of the buffering capacity of the column packing and the adsorption characteristics of the buffering species, a retained pH gradient (i.e., a gradient which travels down the column more slowly than an unadsorbed eluite) is formed inside the column. Proteins in the feed sample then elute from the column at characteristic positions on the gradient.

Although Sluyterman and Wijdenes [4] pointed out in their earliest work on chromatofocusing that both anion- and cation-exchange column packings can be used, the packings most commonly employed nowadays are weak-base anion exchangers, such as the commercial high-performance column (Mono P HR 5/5) and low-pressure stationary phases (PBE 118 and PBE 94) specifically designed for chromatofocusing by Amersham Biosciences [7]. Cation-exchange chromatography employing an unretained pH gradient formed by external mixing is a relatively common technique and has been used for hemoglobin variant analysis [8] and, more recently, for purifying antibody fragments [9]. However, in these methods the pH gradient is generally produced by mixing two buffers of different ionic strength so that a combined pH and ionic strength gradient is employed. Only a few published studies, all of which have employed low-pressure column packings, have been performed which use cation exchangers for true chromatofocusing in which a pH gradient is formed dynamically inside the column at low ionic strength. One of these studies was that of Francina et al. [10], who employed a carboxymethyl derivatized cellulose packing (CM-52 cellulose) and a gradient between pH 6.8 and 8.5 to also separate human hemoglobin variants. However, the pH gradient employed by these workers was irregular in shape and was obtained by sequentially introducing three polyampholyte eluents at different pH values into the column. Another study was performed by Hearn and Lyttle [11], who attempted to replicate the performance of a polyampholyte buffer by employing a carboxymethyl-derivatized cross-linked dextran packing (CM-Sephadex) and a large number of adsorbed and unadsorbed buffering species. However, these workers did not separate proteins using this pH gradient so that its effectiveness for this application is unknown.

In summary, the use of high-performance cation-exchange chromatofocusing is under-exploited, probably because of the uncertainties associated with optimizing the performance of commercially avail-

able packings suitable for this purpose. The goal of this study is to describe several high-performance chromatofocusing systems which employ cation-exchange column packings, and to characterize their performance in terms of the resolution and separation speed achieved and the range of applications possible.

2. Experimental

The buffering species used in this work are diethanolamine (DEA), imidazole, Trizma base [Tris, tris(hydroxymethyl)aminomethane], piperazine, citric acid, itaconic acid, sodium phosphate, formic acid, 3-(*N*-morpholino)propanesulfonic acid (MOPS), and *N*-[tris(hydroxymethyl)methyl]glycine (tricine), all obtained from Sigma (St. Louis, MO, USA). To produce the elution and presaturation buffers, mixtures of these species were titrated with HCl or NaOH to the desired pH, and the mixture then vacuum filtered through a 0.2 μm membrane filter. Polybuffer 74 and Polybuffer 96 were purchased from Amersham Biosciences (Piscataway, NJ, USA). After the polybuffer was diluted to the desired concentration, it was vacuum filtered as described above. The columns used were a 25×0.4 cm I.D. ProPac WCX-10 analytical column which was a gift from Dionex (Sunnyvale, CA, USA), and a 10×0.46 cm I.D. PolyCAT A column obtained from PolyLC (Columbia, MD, USA). The Dionex column contained 10-μm pellicular particles composed of ethylvinylbenzene crosslinked with 55% divinylbenzene and derivatized with poly(acrylic acid). The nonporous nature of this column packing makes it possible to separate proteins at a higher resolution and speed in comparison to the use of porous particles. In addition, the packing particles were tentacle-type, which is reported to have favorable properties such as enhancing adsorption equilibrium and minimizing nonspecific hydrophobic interactions [12,13]. The PolyCAT A column consisted of 5-μm porous silica particles derivatized with poly(aspartic acid). The proteins used (see Table 1) were ovalbumin, bovine serum albumin (BSA), β-lactoglobulin A (Lac A), β-lactoglobulin B (Lac B), recombinant sperm whale myoglobin, and β-glucosidase, all obtained from Sigma, and human hemoglobin variants A, C, S, and F obtained from Perkin-Elmer Life Sciences (Boston, MA, USA).

Table 1
Physical properties of proteins used in this study

Protein	Source	M_r	p*I*
Serum albumin	Bovine	66 000	4.9
Ovalbumin	Chicken	44 000	4.7
Lactoglobulin (Lac)	Bovine		
Lac A		36 000	5.1
Lac B		36 000	5.2
Hemoglobin (Hb)	Human		
Hb A		65 000	7.1
Hb F		65 000	7.15
Hb S		65 000	7.25
Hb C		65 000	7.5
Myoglobin	Sperm whale	18 000	8.3
β-Glucosidase	Almonds	135 000	7.3

The chromatography equipment used was a Model P4000 SpectraSystem pump and a Model UV2000 Spectrasystem UV–Vis absorbance detector (Thermo Separation Products, San Jose, CA, USA). The chromatography system was equipped with a low-dead-volume, in-line pH sensing cell (Sensorex, Stanton, CA, USA) and a Model 701A Ionanalyzer (Orion, Beverly, MA, USA) to record the pH of the column effluent. Sample introduction and the formation of a stepwise change from the presaturation buffer to the elution buffer at the column inlet was accomplished using two Model 9010 valves (Rheodyne, Rohnert Park, CA, USA).

3. Elution with polyampholytes

3.1. Use of Polybuffer 74 to produce a pH gradient in the acidic range

Fig. 1 shows the chromatofocusing of a mixture of ovalbumin, Lac A and BSA using the WCX-10 cation-exchange column. To perform the experiment, the column was first presaturated with 25 m*M* formic acid titrated with NaOH to pH 4 and, after introducing the feed sample, the column was then eluted with 10% (w/v) Polybuffer 74 titrated with NaOH to pH 6.2. As shown, a nearly linear pH gradient was produced. The figure also indicates that ovalbumin (p*I*=4.7) is completely separated from Lac A (p*I*=5.1), but that baseline separation of Lac A and BSA

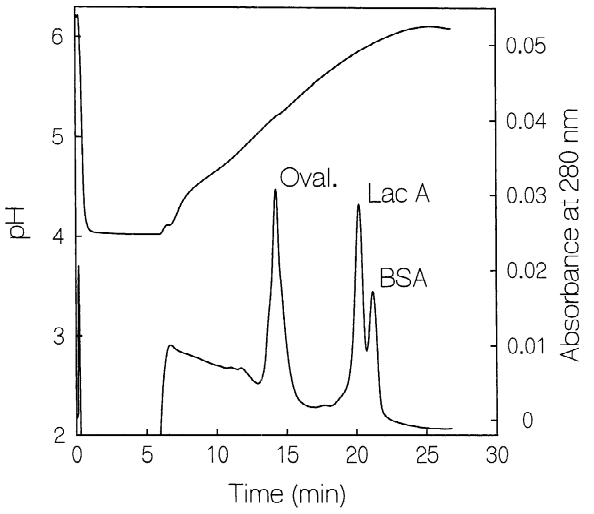

Fig. 1. Separation of a sample containing 0.01 mg each of ovalbumin, β-lactoglobulin A, and BSA using the WCX-10 column. The column was presaturated with 25 m*M* formic acid at pH 4.0 and eluted with 10% Polybuffer 74 titrated to pH 6.2 with NaOH. The flow-rate used was 0.2 ml/min.

(pI=4.9) is not achieved. It can also be seen that Lac A elutes at a lower pH than BSA although the isoelectric point for Lac A is higher than that for BSA.

The results in Fig. 1 indicate that the apparent isoelectric point of a protein is not determined solely by the true isoelectric point, but is instead influenced by other factors. A simplified relation between pI and pI_{app} has been developed by Sluyterman and Elgersma [3] and can be written as:

$$pI_{app} - pI = -\frac{\phi}{4.6} + \frac{\ln(K_d)}{\phi\,|dz/dpH|} \quad (1)$$

In Eq. (1), K_d is the distribution coefficient for the protein, defined as the mass of protein per unit volume in the adsorbed phase divided by the same quantity in the liquid phase, dz/dpH is the rate of change of the protein charge with pH, and ϕ is the logarithm of the ratio of hydrogen ion concentration in the fluid and adsorbed phases, which is equivalent to the dimensionless Donnan potential described by Sluyterman and Elgersma. Eq. (1) can be used qualitatively to interpret trends in measured values for pI_{app} by noting that ϕ is negative for the case of a cation exchanger, and nearly constant for a given gradient since the concentrations of charged groups in the liquid and adsorbed phases tend to vary in the same direction with pH. Furthermore, for a cation exchanger, the first term on the right side of Eq. (1) is positive and contributes to making $pI_{app} > pI$, while, if $K_d > 1$, the second term is negative and contributes to making $pI_{app} < pI$. Eq. (1) therefore suggests that the value of dz/dpH largely determines the sign of $pI_{app} - pI$, especially for proteins with similar retention times and hence similar values for K_d. The results in Fig. 1 agree with this simplified analysis since the available data on the effect of pH on both the characteristic binding charge for ion-exchange equilibrium and the total protein charge [2,14–16] indicate that dz/dpH tends to be large for BSA and Lac A and more moderate in value for ovalbumin, while the values of $pI_{app} - pI$ are 1.03, 0.76, and 0.5 pH units, respectively, for these three proteins.

Fig. 2A illustrates the chromatofocusing of a mixture of Lac A and B with all of the conditions used the same as in Fig. 1. The resolution (defined as the difference in retention times for the peak centers divided by the sum of twice the standard deviations

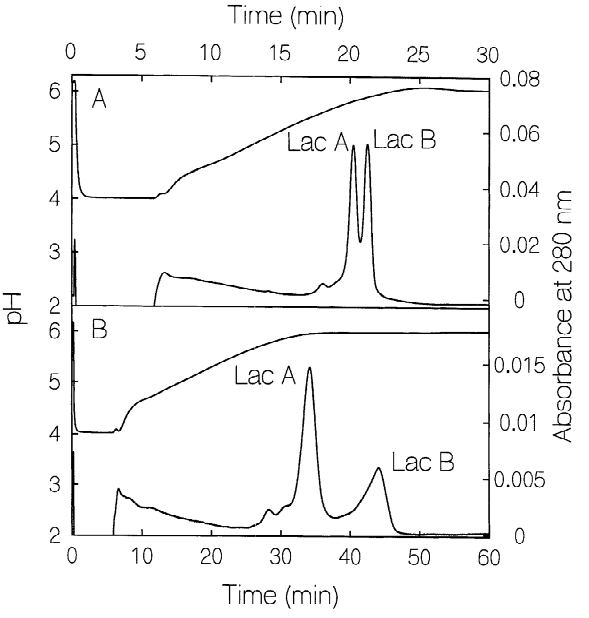

Fig. 2. Separation of 0.02 mg of a sample of β-lactoglobulins A and B. Elution buffer: (A) 10% Polybuffer 74 and (B) 5% Polybuffer 74. Other conditions as in Fig. 1.

of the two peaks) achieved in the figure is approximately 0.95, which is significantly less than that achieved using high-performance anion-exchange chromatofocusing [17]. This difference in resolution can be explained by the charge distribution on the surface of β-lactoglobulin. In particular, the 64th and 118th amino acid residues are Asp and Val in the A form, but are Gly and Ala in the B form, respectively, so that there is a charge difference at the 64th residue when comparing the two forms [18]. Furthermore, the 64th amino acid residue resides on a loop between two neighboring strands of β sheets near several other such loops, so that this residue is located on the protein surface in a region having a net negative charge, as illustrated in Fig. 3. Consequently, the region containing the 64th amino acid residue on the A and B forms of β-lactoglobulin interacts strongly with the surface of an anion-exchange column packing through attractive electrostatic forces, but only weakly with the surface of a cation-exchange column packing and principally through repulsive electrostatic forces. Similar arguments based on the distribution of charged residues on the surface of a protein have been used to rationalize trends in ion-exchange adsorption equilibrium by a number of previous investigators [19,20] which suggests the explanation just given is reasonable.

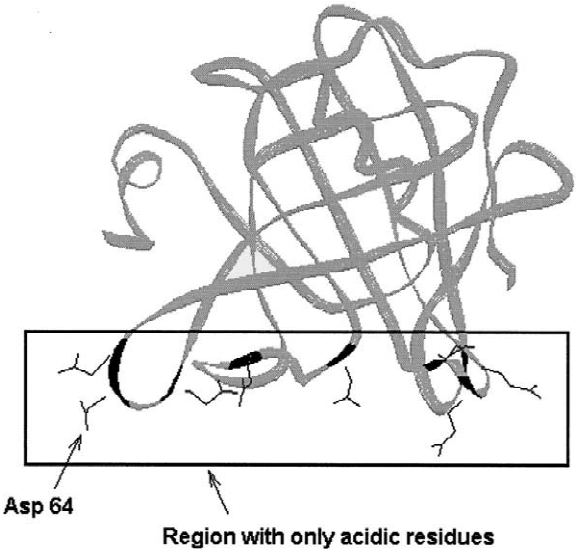

Fig. 3. Structure of β-lactoglobulin A indicating the location of residue 64. Also shown in black are the positions of acidic residues and their side chains in a region containing eight acidic and no basic residues.

Although Lac A and B can be separated by both cation- and anion-exchange chromatofocusing, it has been reported by at least two investigators that these two variants can be separated by anion-exchange chromatography, but not by cation-exchange chromatography, when elution is performed using a salt gradient at a fixed liquid-phase pH [2,9]. This can be explained by extending arguments proposed by Yamamoto and Ishihara [2] which state that although the total number of charges involved in the interaction between a protein and the column packing decreases when the pH of the liquid phase approaches the protein pI, the relative difference between the number of charges for two proteins with similar pI values, and therefore the relative adsorption affinity for these two proteins, increases. The observation that Lac A and B can be separated by both cation- and anion-exchange chromatofocusing therefore appears to result from the fact that during chromatofocusing proteins are generally nearer their isoelectric point than can be accomplished in IEC using a salt gradient. Consequently, not only is the relative adsorption affinity enhanced in chromatofocusing by the small charge on the protein, but for the case of Lac A and B it is apparently enhanced beyond that observed in IEC using a salt gradient because the small charge permits a larger fraction of the protein surface to interact with the column packing, particularly for the tentacle-type packing used.

In order to further increase the resolution between Lac A and B, a more dilute elution buffer was employed so that the corresponding increase in apparent isoelectric points would cause the two proteins to elute on a shallower portion of the gradient. Fig. 2B illustrates results obtained with all the conditions the same as in Fig. 2A, except that a 5% (w/v) solution of Polybuffer 74 was used. As shown, the observed apparent isoelectric points of Lac A and B increase to 5.98 and 6.00, respectively, as compared to the values of 5.85 and 5.93 in Fig. 2A. The increase in pI_{app} is consistent with the lower ionic strength used, which increases the absolute value of ϕ in Eq. (1) so that the first term on the right side of the equation, which is presumed to

dominate the second term in this case, gets larger. The resolution achieved in Fig. 2B is 2.0, which is much larger than the resolution achieved by Mhatre et al. [9] who used an externally produced, combined pH and ionic strength gradient and a strong-acid cation exchanger to separate the same two proteins.

3.2. Use of Polybuffer 96 to produce a pH gradient in the basic range

In Fig. 4, Polybuffer 96 was used to produce a pH gradient in the basic range on the WCX-10 column and to separate human hemoglobin variants A, F, S, and C. In the experiment shown, the column was first presaturated with a buffer composed of 2 m*M* each of DEA, Tris, imidazol and piperazine titrated with HCl to pH 6, and then eluted with 4% (w/v) Polybuffer 96. The presaturation conditions were chosen as suggested by the studies of Frey [21] which indicate that a stable, monotonically changing pH gradient can be produced on a column with minimal buffering capacity, which is the case for the WCX-10 column under the basic conditions employed, if the column is presaturated with buffering species of the proper charge type to adsorb onto the packing. As shown in the figure, a smooth pH gradient was produced with an initial steep pH rise resulting from the replacement of the chloride ion in the presaturation buffer by negative ions from the Polybuffer, as confirmed by the fact that this front is located where an unadsorbed tracer elutes (data not shown). The figure also shows that the A, S, and C variants of hemoglobin are well separated, although baseline separation of A and F forms is not achieved.

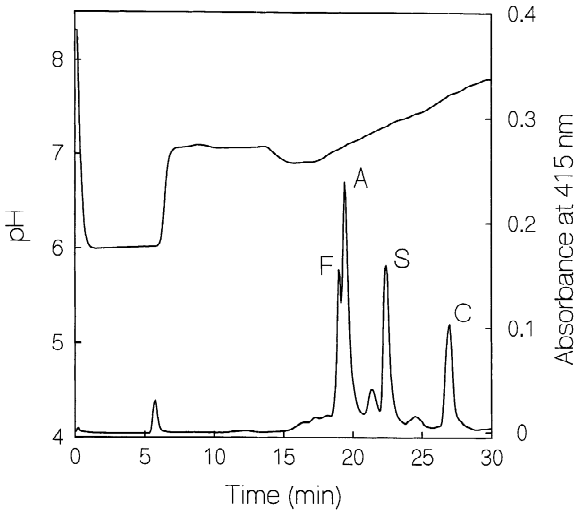

Fig. 4. Separation of 0.04 mg of a sample of human hemoglobins A, F, S, and C. The column was presaturated with a pH 6 buffer composed of DEA, Tris, imidazole and piperazine, each at 5 m*M*. The elution buffer was 4% Polybuffer 96. Other conditions as in Fig. 1.

The apparent isoelectric points of the A, F, S, and C forms of hemoglobin in Fig. 4 are 7.09, 7.06, 7.3 and 7.64, respectively, which are very near their true isoelectric points of 7.1, 7.15, 7.25, and 7.5. This differs from observations of Francina et al. [10] who also used cation-exchange chromatofocusing and observed that the apparent isoelectric points of these hemoglobin variants were much higher than the corresponding p*I* values, although these workers evidently had difficulty in producing a monotonically changing gradient. This difference in the observed values of pI_{app} is likely due to the corresponding difference in the Donnan potential since Francina et al. employed a very dilute solution containing 1% (w/v) polyampholyte. A dilute elution buffer corresponds to a large value for the Donnan potential so that the first term on the right side of Eq. (1) dominates, while for the results shown in Fig. 4 the two terms are evidently of comparable value so that p*I* and pI_{app} are nearly equal.

The resolution achieved between the A and S forms in Fig. 4 is 3.6, which is the same resolution reported by Kang and Frey [22] for these two variants using high-performance anion-exchange chromatofocusing. This similarity can be explained by the fact that the charged amino acid residue that differs between the A and S forms of hemoglobin is the sixth residue on the β chain, which is located on an α helix at the protein surface, while the second, seventh and eighth residues on this chain are histidine, glutamic acid, and lysine, respectively. If the N-terminus is also included, then the first eight residues on the β chain of hemoglobin A include two acidic and three basic groups, with two of the basic groups having pK_a values near 7. This region can therefore have either a positive or negative net charge depending on the pH so that it can interact with either a cation or anion exchanger with equal facility. Fig. 4 also indicates that the S and C forms

of hemoglobin are better separated with a resolution of 4.9, and the A and F forms are more poorly separated with a resolution less than unity, as compared to the previous study by Kang and Frey which employed an anion-exchange column packing. This indicates that for some hemoglobin variants anion-exchange chromatofocusing leads to better resolution, while the opposite holds true for other pairs of hemoglobin variants, again likely due to the way electrostatic charges are distributed on the surfaces of these proteins.

Fig. 5 illustrates the use of conditions similar to those in Fig. 4, except that the elution buffer was titrated to pH 9 and the feed sample was a commercial preparation of recombinant sperm whale myoglobin. As shown, a minor component of the sample was not retained by the column packing, while the major component eluted at pH 7.6, which is lower than the protein isoelectric point of 8.3. These results are therefore similar to those reported by Sluyterman and Wijdenes [4], who observed that sperm whale myoglobin eluted early in relation to its pI when using an anion-exchange column packing so that $pI_{app} > pI$. This trend is consistent with the low value of dz/dpH that applies to myoglobin [23], which in turn implies that the second term on the right side of Eq. (1) dominates the first term, so that myoglobin tends to elute ahead of its isoelectric point pH in both anion- and cation-exchange chromatofocusing.

4. Elution with simple mixtures of buffering species

Kang and Frey [22] have recently reported methods for producing nonself-sharpening, retained pH fronts suitable for chromatofocusing using a small number of buffering species, as well as computer-aided design methods for selecting the identities and concentrations of the buffering species used. In the present case, to produce a pH gradient in the acidic range with simple buffer mixtures, the WCX-10 column was first presaturated with a buffer composed of 10 mM each of citric acid, itaconic acid, and NaH_2PO_4, titrated with NaOH to pH 4. The column was subsequently eluted with the same buffer titrated with NaOH to pH 6.2. Fig. 6 illustrates the separation of a mixture of ovalbumin, Lac A and BSA using this procedure. As shown, the three proteins are well separated, with a resolution between ovalbumin and Lac A of 3.6 and a res-

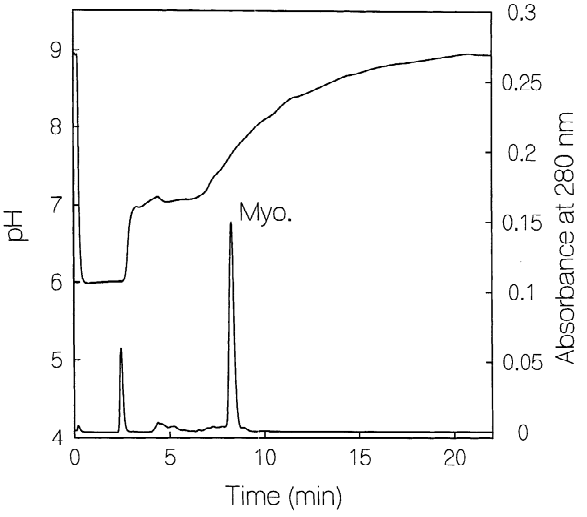

Fig. 5. Chromatofocusing of 0.005 mg of a commercial preparation of sperm whale myoglobin. The elution buffer was titrated with NaOH to pH 9 and the flow-rate was 0.5 ml/min. Other conditions as in Fig. 4.

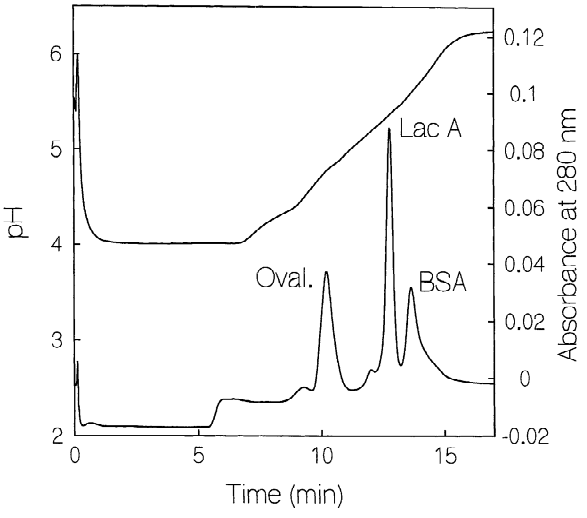

Fig. 6. Separation of a sample containing 0.01 mg each of ovalbumin, β-lactoglobulin A, and BSA. The presaturation buffer was a mixture of 10 mM each of citric acid, itaconic acid, and NaH_2PO_4, titrated to pH 4 with NaOH. This buffer titrated to pH 6.2 was used to elute the sample from the column. Other conditions as in Fig. 1.

olution between Lac A and BSA of 1.3. A comparison of Fig. 6 with Fig. 1 where a polyampholyte buffer was used indicates that the resolution obtained between ovalbumin and Lac A is greater in Fig. 1, while the resolution obtained between Lac A and BSA is greater in Fig. 6. These differences in resolution may result from the tendency of polyampholyte buffers to form weak association complexes with proteins, and thereby to change the apparent isoelectric points as well as the separation achieved in the two figures.

In order to enhance the usability of chromatofocusing, it is desirable to simplify the liquid-phase composition as much as possible. One example of this is provided by the work of Logan et al. [24], who produced a gradient in the range from pH 5 to 9.5 on a weak-base anion exchanger using two buffering species. For the case where a gradient is desired in the range from pH 4 to 6, the use of citric acid as the only buffering species in the liquid phase yields a similar opportunity to simplify the buffer system. In particular, citric acid has three pK_a values that are evenly spaced apart (i.e., 3.1, 4.8, and 6.4) so that a uniform liquid-phase buffering capacity is produced in the range from pH 3.1 to 6.4. Furthermore, the particles in the WCX-10 column incorporate tentacles composed of poly(acrylic acid) [25] in which case the carboxylic acid groups are somewhat less acidic than those in a typical carboxymethyl-based cation exchanger [26]. This higher pK_a value for the carboxylic acid group, especially when combined with the Donnan equilibrium effect which produces a lower equilibrium pH in the column packing as compared to the liquid phase, suggests that the maximum buffering capacity for the WCX-10 column likely occurs when the liquid phase is slightly acidic, e.g., near pH 6. The result is that the ratio of the liquid- and adsorbed-phase buffering capacities for this system should tend to vary linearly with pH in the acidic pH range so that, as described by Kang and Frey [22], a correspondingly linear pH gradient should be formed. Fig. 7 shows experimental results which demonstrate this behavior for the WCX-10 column. As illustrated, a linear pH gradient from pH 4 to 6.2 is produced and used to completely resolve Lac A and BSA in less than 4 min. This resolution is achieved despite the broadened appearance of the BSA band as compared to Figs. 1 and 6, which evidently results from a flattening of the pH gradient at high pH levels in Fig. 7.

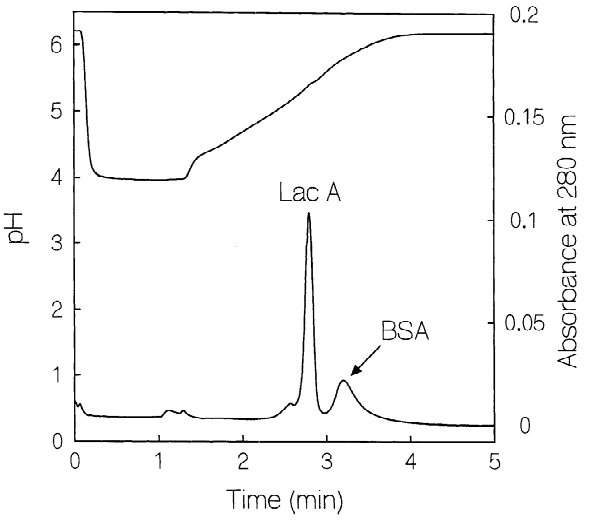

Fig. 7. Separation of a sample containing 0.025 mg each of β-lactoglobulin A and BSA. The presaturation buffer was composed of 10 m*M* citric acid, titrated to pH 4.0 with NaOH. This buffer titrated to pH 6.2 was used to elute the sample from the column. The flow-rate used was 1.25 ml/min. Other conditions as in Fig. 6.

5. Chromatofocusing using a two-section pH gradient produced with a single elution buffer

In traditional IEC it is common practice to employ a gradual salt gradient in order to elute the protein of interest, and to then follow this gradual gradient with a stepwise gradient to a high salt concentration to rapidly co-elute strongly bound contaminants in a single band. Similar types of two-section gradients for chromatofocusing can be produced by taking advantage of the relation between the velocity of a pH level and the buffering capacities of the liquid and adsorbed phases, as described by Kang and Frey [22] and Bates et al. [27]. According to these workers, an increase in the liquid-phase buffering capacity results in an increase in the velocity of a pH level in the column. If this increase occurs at the end of an otherwise gradual pH gradient, i.e., a nonself-sharpening pH front, then a composite gradient is formed in which a self-sharpening, stepwise section follows a gradual section. Under these conditions,

the stepwise section will be located approximately at the pH corresponding to the pK_a of the buffering species used to increase the liquid-phase buffering capacity over that required for an entirely gradual gradient.

Fig. 8 shows the separation of Lac A and B employing the strategy just described in which a mixture of citric acid, MOPS, and tricine was titrated to pH 4.1 or 8.5 and used as the presaturation or elution buffer, respectively. As illustrated, a linear, ascending section of the resulting pH gradient was produced and used to elute Lac A and B with the resolution achieved being comparable to that achieved in Fig. 2A. This gradual section was then followed by a stepwise section where various impurities in the feed sample co-elute in a highly focused band.

Fig. 9 shows that a pH gradient extending to as low as pH 2.6 can be produced using the cation-exchange column packing PolyCAT A, which consists of aminopropyl-silica derivatized with poly-(aspartic acid) [28]. Since poly(aspartic acid) bound to silica in this manner contains roughly equal amounts of side chain carboxylic acid groups and α-carboxylic acid groups, and since the acidity of the latter group is relatively high due to its proximity to an amide linkage, the PolyCAT A column provides a buffering capacity at pH values lower than can be provided with the poly(acrylic acid)-based WCX-10 column. As shown in the figure, a linear pH gradient between pH 2.6 and 5 was produced using citric acid as only buffering species present. As in Fig. 8, a stepwise change in pH follows the linear part of the gradient, although in this case the decrease in adsorbed-phase buffering capacity near pH 5, rather than an increase in liquid-phase buffering capacity at this pH, is responsible for the formation of the stepwise section. Fig. 9 also illustrates the chromatofocusing of a feed sample consisting of a commercial preparation of β-glucosidase. As shown, a number of peaks corresponding to sample impurities are resolved, several of which elute at very low pH values.

The results in Fig. 9 indicate that the use of a cation exchanger is a convenient method to accomplish the chromatofocusing of proteins with very low isoelectric points, although the propensity of the Si–O–C moiety in the PolyCAT A column packing to undergo hydrolysis at low pH prohibits elution at less than pH 2.5. However, it is likely that related types of polymer-based column packings that do not

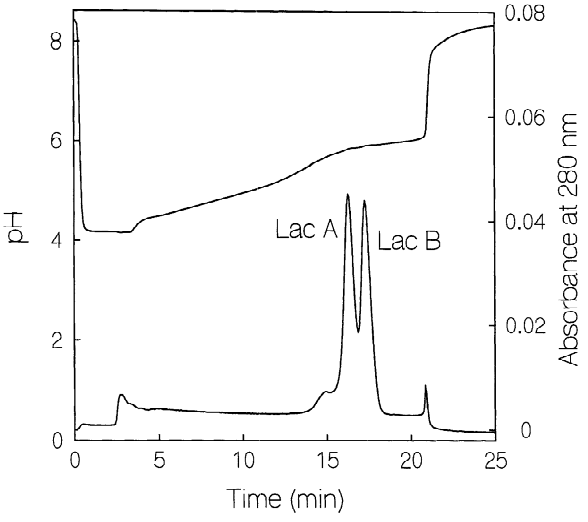

Fig. 8. Separation of 0.05 mg of a sample of β-lactoglobulins A and B. The presaturation buffer was a mixture of 2 m*M* each of citric acid, MOPS, and tricine, titrated to pH 4.1 with NaOH. This buffer titrated to pH 8.5 was used to elute the sample from the column. The flow-rate used was 0.5 ml/min. Other conditions as in Fig. 6.

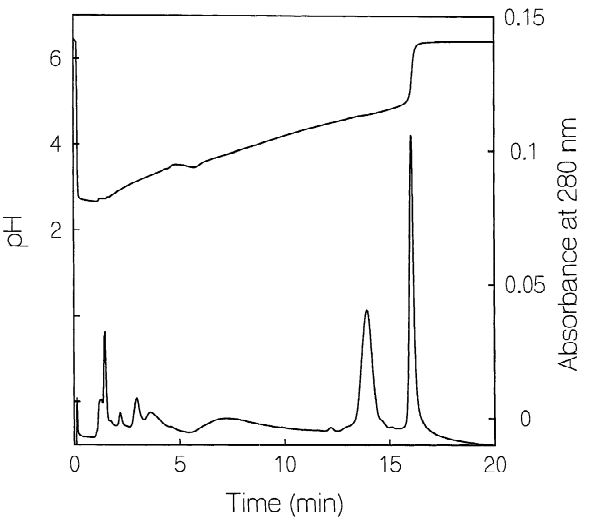

Fig. 9. Chromatofocusing of 0.5 mg of a commercial preparation of β-glucosidase using the PolyCAT A column. The presaturation buffer was composed of 10 m*M* citric acid, titrated to pH 2.6 with NaOH. This buffer titrated to pH 6.5 was used to elute the sample from the column. The flow-rate used was 1.5 ml/min.

contain siliceous bonds will permit the use of even more acidic elution buffers. Previous work by other investigators on anion-exchange chromatofocusing in an extremely low pH range has been conducted using a polyethyleneimine-agarose column packing [29]. However, this previous work employed multiple stepwise changes in pH at the column inlet to form the pH gradient, evidently due to the lack of a buffering capacity for the column packing used. The method illustrated in Fig. 9 may therefore be a useful alternative for accomplishing chromatofocusing in the very low pH range.

6. Preparative applications

All of the experiments described in this study were performed using analytical-scale columns and with proteins at low concentration. However, it is useful to consider briefly aspects of larger-scale preparative operation, possibly performed using low-pressure packings, where recovery of native protein is an objective. Although certain of the gradients used in this study extend to low pH levels where acid-induced denaturation might be expected to occur, in general proteins are stable conformationally near their p*I* regardless of its value [30]. Since in this study proteins typically eluted near their p*I* values, the common aphorism [3] that chromatofocusing tends to preserve a protein's native structure likely applies. In addition, although protein solubility is typically lowest near the p*I*, there are several additives such as glycerol or taurine that have been advocated to enhance the solubility during chromatofocusing under mass-overloaded conditions [31]. Consequently, although protein recoveries were not determined in this study, they would likely be comparable to those observed when a similar pH range is employed in preparative anion-exchange chromatofocusing. Examples of relevant studies in this regard are those of Ofman and Wanders [32] and Jacob et al. [33], the latter of whom investigated the purification of yeast hexokinase PI (p$I = 5.0$) and observed an activity recovery of 80% with chromatofocusing, as compared to 65% with standard ion-exchange chromatography using an externally produced pH gradient. Another consideration is that gradual gradients can be produced in chromatofocusing without the need for blending buffers externally to column, which should facilitate large-scale operation. Finally, when it is desired to use buffers with low toxicity, such as might be the case for the large-scale manufacturing of therapeutic protein drugs, cation-exchange chromatofocusing using simple buffers has advantages over anion-exchange chromatofocusing because in the former case all of the buffering species employed can be natural metabolic products, such as citric acid.

7. Conclusions

This study demonstrates that commercially available, high-performance cation-exchange column packings can be used for the chromatofocusing of proteins. Linear pH gradients in the range from pH 2.6 to 5 were produced using a simple buffer mixture as the eluent and a poly(aspartic acid) derivatized column packing. Similar pH gradients in the somewhat higher range of pH 4 to 6.2 were produced also using a simple buffer mixture as the eluent and a tentacle-type column packing based on poly(acrylic acid). Nearly linear pH gradients extending to as high as pH 9 were produced by presaturating the latter column with a buffering species that adsorbed onto the packing, and then eluting with a polyampholyte buffer. Using these methods, pH gradients in a variety of useful shapes were produced in the range from pH 2.6 to 9.

The resolution achieved using high-performance cation-exchange chromatofocusing was evaluated for separations involving β-lactoglobulin variants, human hemoglobin variants, sperm whale myoglobin, ovalbumin, β-glucosidase, and bovine serum albumin. The results indicate that for some separations the resolution achieved using polyampholytes is better than that achieved using simple buffer mixtures, while the reverse holds true for other separations, possibly due to differences in how the buffering species associate with proteins in the two cases. Furthermore, for some separations anion-exchange chromatofocusing yielded better resolution than cation-exchange chromatofocusing, while again the reverse holds true for other separations, probably because of how charged amino acid residues are distributed on the protein surface. In general, how-

ever, it was observed that a given pair of proteins can be separated using both anion- and cation-exchange chromatofocusing to an extent greater than is possible for anion- and cation-exchange chromatography using salt gradients at a fixed pH, as illustrated by the separation of β-lactoglobulins A and B. This suggests that a relatively large portion of the entire protein surface participates in binding with the column packing during chromatofocusing, likely due to the fact that in chromatofocusing proteins are only weakly adsorbed during their entire transit time through the column. In contrast, for ion-exchange chromatography with a salt gradient, proteins tend to be more highly charged so that the binding site is more narrowly localized on the protein surface. It was also observed that for both anion- and cation-exchange chromatofocusing, the relation between the apparent and true isoelectric points for a protein can be interpreted qualitatively using the theoretical relation developed by Sluyterman and co-workers with the dominate factor being the derivative of the characteristic binding charge with respect to pH.

Three chromatofocusing methods were investigated which take advantage of the acid–base properties of commercially available, cation-exchange column packings. In one method, several types of carboxylic-acid derivatized column packings were used to produce a composite pH gradient in the acidic range consisting of a initial gradual section which accomplished the high-resolution separation of a target protein, followed by a stepwise section which quickly eluted strongly bound impurities in a highly focused single band. In a second method, a column packing based on poly(aspartic acid) was used to form a pH gradient that spans an extremely low pH range and to separate highly acidic proteins. In a third method, an elution buffer containing only citric acid as the buffering species was used to produce a linear pH gradient in the range from pH 4 to 6.

Results obtained in this study indicate that cation-exchange chromatofocusing is a useful method which is complementary to anion-exchange chromatofocusing and isoelectric focusing for separating proteins. Furthermore, under certain circumstances, cation-exchange chromatofocusing may have intrinsic advantages over these latter two methods. For example, it is generally more convenient to perform cation-exchange rather than anion-exchange chromatofocusing in the acidic range using simple buffer mixtures since unadsorbed buffering species must be used in this case, and there is a greater selection of acidic buffering species with low pK_a values as compared to basic buffering species.

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