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High-performance chromatofocusing using linear and concave pH gradients formed with simple buffer mixtures II. Separation of proteins☆

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Abstract

The separation of proteins using high-performance chromatofocusing with linear or concave pH gradients formed using simple mixtures of buffering species in the elution buffer is investigated experimentally. The separation achieved is comparable to that using polyampholyte elution buffers with these types of systems. More specifically, protein band widths at one half of the band height in the range between 0.1 and 0.025 pH units were observed, and good resolution was achieved of protein variants differing by a single amino acid residue in separation times of 30 min or less. An especially useful elution buffer is investigated that contains only four buffering species and that produces a linear pH gradient in the range between pH 9.5 and 6.0 when used together with a particular high-performance column packing made specifically for chromatofocusing. This elution buffer and column packing combination is evaluated by using it for the chromatofocusing of equine myoglobin and human hemoglobin variants. Additional applications are described in which a polyethyleneimine derivatized silica column packing and a pH gradient that is concave in shape are used for the separation of proteins in an *E. coli* cell lysate.

Keywords: Chromatofocusing; pH gradients; Buffer composition; Gradient elution; Proteins; Hemoglobins; Myoglobin

1. Introduction

In Part I it was demonstrated that retained, internally generated pH gradients that are linear or concave in shape, and potentially useful for the chromatofocusing of proteins, can be produced using high-performance column packings and simple mixtures of buffering species in the elution buffer provided that the buffering species employed do not adsorb onto the column packing, i.e., amine buffering species are employed that form only neutral or positive ions when using an anion-exchange column packing, and provided that the identities and concentrations of these buffering species are properly chosen. In this part, the application of high-performance chromatofocusing using these types of gradients will be evaluated with regard to the resolution and speed achieved when separating protein mixtures.

2. Experimental

The basic experimental methods and chromato-

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graphic equipment used are described in Part I. Human hemoglobin variants and equine myoglobin were obtained from Sigma (St. Louis, MO, USA). Chromatography was conducted using a 5×0.5 cm I.D. Mono P column (Amersham Pharmacia Biotech, Piscataway, NJ, USA) or a 25×0.46 cm I.D. Baker-bond WP-PEI column (J.T. Baker, Phillipsburg, NJ, USA). Feed samples were introduced into the chromatography columns using a Model 7010 multiport injection valve (Rheodyne, Rohnert Park, CA, USA) and, unless otherwise specified, a 20-μl sample loop was employed. An additional Model 7010 Rheodyne multiport injection valve was positioned such that it could be used to direct flow to the column or to isolate the column from the flow stream.

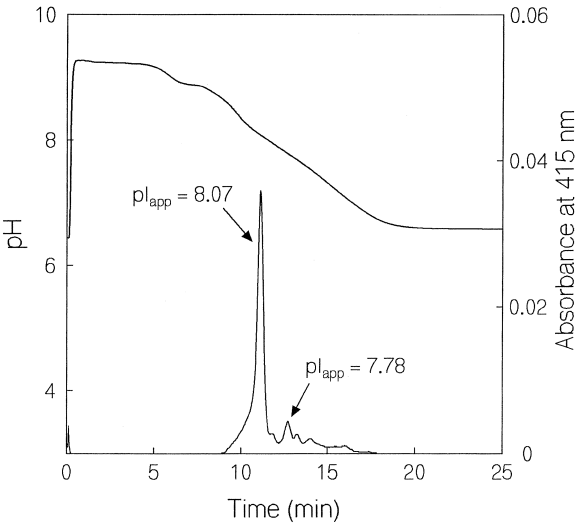

Fig. 1. Chromatofocusing of 0.005 mg of a preparation of equine myoglobin on a 5×0.5 cm I.D. Mono P column. The column was initially presaturated with a mixture of diethanolamine (DEA), Tris, imidazole and piperazine each at 2 m*M* at pH 9.4 and was eluted with the same mixture of species at pH 6.5 and at a flow-rate of 1.0 ml/min.

3. Use of a polyether column packing derivatized with amine functional groups

3.1. Characteristics of protein bands

Fig. 1 shows the chromatofocusing of a commercial preparation of equine myoglobin obtained from Sigma (Product No. M 1882) using the same weak-base, polyether column packing and simple buffer system as was used to produce gradient D in Fig. 7 of Part I. As shown in the figure, one main band is observed in the chromatogram having an apparent isoelectric point (i.e., the pH at which the band exits the column; pI_{app}) of 8.07 while a minor band having an apparent isoelectric point of 7.78 is also evident. As also shown, the band width at one half the band height for myoglobin is approximately 0.07 pH units, which implies that complete resolution of proteins having band broadening properties similar to myoglobin and differing by 0.14 pH units in apparent isoelectric point should be achieved within the separation time shown of 15 min. This is consistent with the observation that myoglobin is completely resolved from the minor impurity present, which differs in apparent isoelectric point from myoglobin by 0.29 pH units. Similar band widths for myoglobin have been observed for chromatofocusing using polyampholyte elution buffers and either low-pressure [1–3] or high-performance chromatography [4,5]. This indicates that when the buffering species are chosen as described in Part I, the use of simple mixtures of buffering species yields resolutions of proteins comparable to, if not greater than, those achieved when polyampholyte buffers are employed.

3.2. Resolution of proteins

Human hemoglobin A_0 exists as a tetramer of two α and two β globin chains, each containing one heme group with one iron ion. Hemoglobin S_1 is identical to the A_0 form except that in the S_1 form valine is substituted for glutamic acid at residue 6 in the β globin chains. Figs. 2 and 3 illustrate the separation of a mixture of human hemoglobins S_1 and A_0 obtained by mixing together preparations of the individual hemoglobin variants (product Nos. H 0267 and H 0392 from Sigma). More specifically, the figures show the effects of the liquid phase flow-rate and the concentrations of the buffering species on the separation obtained. In addition, Tables 1 and 2 illustrate average values of para-

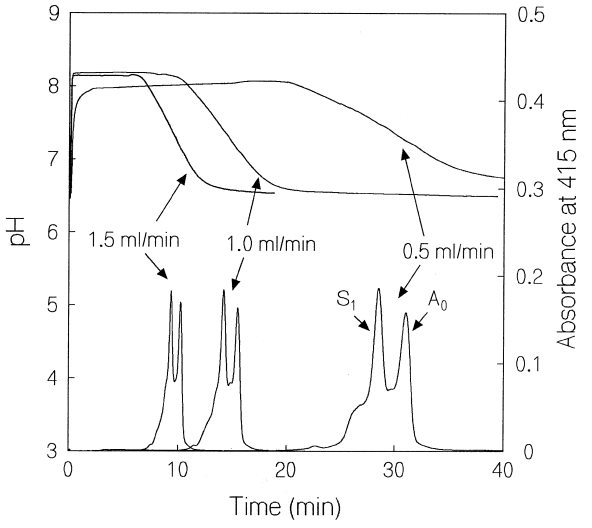

Fig. 2. Effect of flow-rate on the chromatofocusing of 0.1 mg of a mixture of preparations of the normal (A_0) and sickle cell (S_1) variants of human hemoglobin on a 5×0.5 cm I.D. Mono P column. The column was initially presaturated with a mixture of DEA, Tris, imidazole and piperazine each at 2 m*M* at pH 8.2 and was eluted with the same mixture of species at pH 6.5 and at the flow-rates indicated.

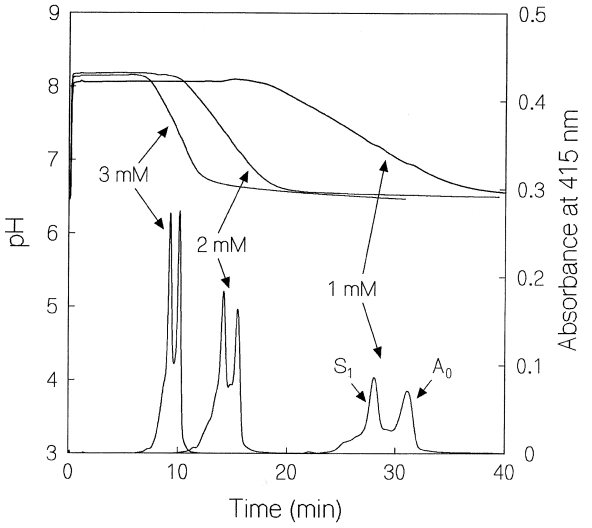

Fig. 3. Effect of buffering species concentration on the chromatofocusing of 0.1 mg of a mixture of preparations of the normal (A_0) and sickle cell (S_1) variants of human hemoglobin on a 5×0.5 cm I.D. Mono P column. The column was initially presaturated with a mixture of DEA, Tris, imidazole and piperazine each at the concentrations indicated at pH 8.2 and was eluted with the same mixture of species at pH 6.5 at a flow-rate of 1 ml/min.

meters related to the gradient and the separation for sets of duplicate experiments. In general, the apparent isoelectric points determined for identical experiments of the type shown in Figs. 2 and 3 were repeatable to within 0.02 pH units, which gives an indication of the overall reproducibility of the experiments performed. The separation of the A_0 and S_1 variants shown in the figures appears to be comparable to the separations obtained with low pressure chromatofocusing using polyampholyte buffers [6] and with low-pressure or high-performance ion-exchange chromatography using an externally generated pH gradient [7–9], which are the other major chromatographic methods that have been applied to these variants.

One characteristic evident from Tables 1 and 2 is that the effect of varying the flow-rate and the buffering species concentrations on the gradient shape is consistent with Eqs. (1) and (2) of Part I and the numerical calculations shown in Fig. 2 of Part I, i.e., the slope of the gradient at its midpoint is approximately proportional to both these parameters. Figs. 2 and 3 and Tables 1 and 2 also indicate that the apparent isoelectric points of human hemoglobins S_1 and A_0 do not vary with the liquid-phase flow-rate, but do vary when the concentrations of the buffering species are changed, although the difference in apparent isoelectric points between the two variants is only very weakly dependent on the buffering species concentrations. In particular, it can be seen that the apparent isoelectric points of hemoglobins S_1 and A_0 vary from 7.20 and 6.95, respectively, when the buffering species concentrations are 1 m*M*, to 7.57 and 7.30, respectively, when the buffering species concentrations are 3 m*M*. This can be compared to the actual isoelectric points as determined by isoelectric focusing of 7.25 and 7.10 for the S_1 and A_0 forms, respectively.

As shown in Figs. 2 and 3, the hemoglobin variants are reasonably well resolved from each other in the column effluent, although baseline resolution is not achieved in these cases. Additional chromatofocusing experiments using the hemoglobin variants individually indicate that the band widths at one half the band height for the A_0 and S_1 variants are 0.13 and 0.10 pH units, respectively (data not shown). However, the band shapes determined in these experiments tended not to be purely Gaussian in

Table 1
Effect of mobile phase flow-rate on resolution between hemoglobins A_0 and S_1[a]

Flow-rate (ml/min)	Slope of pH gradient at midpoint (pH units/min)	Distance between peak maxima (min)	Distance between peak maxima (pH units)	Resolution
0.5	0.10	2.52	0.25	1.20
1.0	0.20	1.28	0.26	1.23
1.5	0.29	0.87	0.26	1.16

[a] The column was initially presaturated with a mixture of DEA, Tris, imidazole and piperazine each at 2 m*M* and at pH 8.2 and was eluted with the same mixture of species each at 2 m*M* and at pH 6.5 and at the flow-rate shown.

shape but instead had a broadened baseline width likely due to the presence of a number of minor impurity bands. This accounts for the fact that complete resolution is not achieved in Figs. 2 and 3 despite the fact that the sum of the band widths at one half the band height for the two variants is slightly less than their difference in apparent isoelectric points of approximately 0.26 pH units.

Tables 1 and 2 also indicate the quantitative resolution achieved as the flow-rate and buffering species concentrations vary. The resolution shown in the tables is defined as:

$$R = \frac{\Delta t}{2\sigma_{A_0} + 2\sigma_{S_1}} \quad (1)$$

where Δt denotes the difference in retention times for the two variants measured at the peak maximum and σ_i denotes one half the band width measured at 60% of the band height for the two variants, i.e., σ_i is the standard deviation for a Gaussian band shape. Tables 1 and 2 in particular indicate that the resolutions obtained are in the range from 1.2 to 1.4, which is again consistent with the fact that the measured sum of the band widths at one half the band height is slightly less than the observed difference in retention times. However, as just mentioned, baseline resolution is not achieved despite the resolutions being greater than unity likely because of the presence of minor impurities which broaden the baseline bandwidth as compared to a purely Gaussian band shape.

Although there appears to be some scatter in the data shown in Tables 1 and 2, these data nevertheless appear to indicate a small but discernable increase in resolution as the flow-rate or buffering species concentration decrease. As shown in Table 1, the increase in resolution as the flow-rate decreases is smaller than might be expected on the basis that the mobile phase flow-rate varies by a factor 3 in the table. This appears to be due to the fact that the Van Deemter plot measured for myoglobin using the Mono P column (i.e., the plot of the reduced plate height as a function flow-rate for isocratic conditions as shown in Fig. 4) exhibits a *y*-intercept of 10. Although this *y*-intercept is typical of the upper limit observed for a well designed high-performance liquid chromatography system when relatively short

Table 2
Effect of buffering species concentration on resolution between hemoglobins A_0 and S_1[a]

Concentration of buffering Species (m*M*)	Slope of pH gradient at midpoint (pH units/min)	Distance between peak maxima (min)	Distance between peak maxima (pH units)	Resolution
1.0	0.09	2.79	0.25	1.43
2.0	0.20	1.28	0.26	1.23
3.0	0.30	0.90	0.27	1.36

[a] The column was initially presaturated with a mixture of DEA, Tris, imidazole and piperazine each at the concentration shown at pH 8.2 and was eluted with the same mixture of species at pH 6.5 and at a flow-rate of 1 ml/min.

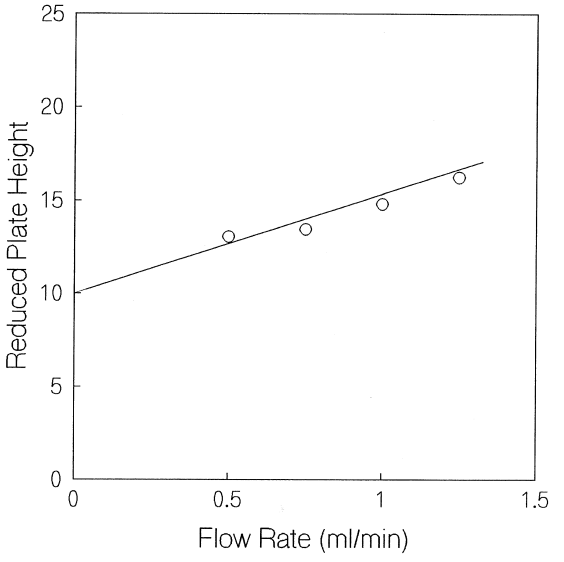

Fig. 4. Reduced plate height [i.e., $L\sigma^2/(t^2 d_p)$] as a function of the mobile phase flow-rate for the isocratic elution of myoglobin under unretained conditions. To perform the experiment, the column was presaturated and eluted with a 100 m*M* imidazole buffer at pH 6.5.

columns are employed [10], its relatively high value causes the increase in plate height with flow-rate to have only a small effect on the overall column efficiency for the range of flow-rates attainable for this column packing.

The results in Table 1 can be compared to those of Sluyterman and Wijdenes [1] and Wagner and Regnier [5] who found essentially no influence, and a large influence, respectively, of flow-rate on resolution for chromatofocusing performed with polyampholyte buffers, which seems to indicate that this effect depends strongly on the specific conditions used. In addition, the observed increase in resolution shown in Table 2 as the concentration of the buffering species decreases is in agreement with a similar observation by Sluyterman and Wijdenes [1], although a definitive comparison is again obscured by the scatter in the resolutions shown in Table 2, and by the fact that Sluyterman and Wijdenes made only qualitative observations of the resolution achieved in their system.

The results described in this section and in Part I indicate that nearly linear pH gradients in the range between pH 9.5 and 6.0 can be produced using a Mono P high-performance column and amine buffering species. However, as also described in Part I, amine buffering species with pK_a values below 5 tend to be unsuitable for use in chromatofocusing since they are generally large organic molecules with physical properties that make it difficult to achieve a pH gradient of a desired shape. For this reason, the alternative strategy of having a reduced liquid phase buffering capacity in the low pH range so that a concave pH gradient is formed is generally preferred under these conditions. In fact, this was the procedure followed by Bates and Frey [11] to separate the A and B variants of β-lactoglobulin using low-pressure chromatofocusing with a concave pH gradient that spanned the pH range between 5.0 and 3.9. An additional example of the use of concave pH gradients in the low pH range is given in the following section where a high-performance silica column packing is employed.

4. Application of a polyethyleneimine derivatized silica column packing

To further demonstrate the use of the chromatofocusing method discussed here, experiments were conducted using a Bakerbond WP-PEI high-performance column containing 5-μm silica particles with 300 Å pores and functionalized with polyethyleneimine. A clarified cell lysate from an *E. coli* fermentation where the cells were genetically altered to produce glucose–galactose binding protein (GGBP) was obtained from colleagues at the University of Maryland who are investigating this protein [12] and used as a feed sample. This feed material was selected so that a large number of different proteins would elute in a relatively small pH range. To produce the feed material, cells from an *E. coli* fermentation run were collected, lysed by sonication, and clarified by centrifugation. Due to the small concentrations of proteins in the feed material in the range of apparent isoelectric points investigated, a relatively large feed sample containing 5 ml of the clarified liquid was injected into the column, which was initially presaturated and then eluted using buffers as described in the figure caption.

Fig. 5 illustrates the results of the chromatofocus-

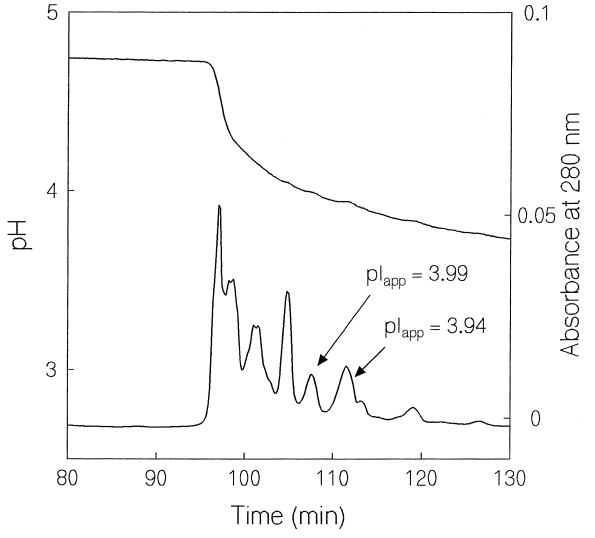

Fig. 5. Chromatofocusing of proteins from a clarified *E. coli* cell lysate on a 25×0.46 cm I.D. WP-PEI column. The column was initially presaturated with 5 m*M* piperazine at pH 5 and was eluted with a mixture of 5 m*M* piperazine and 3.5 m*M* *N*-methylpiperazine at pH 3.8 and a flow-rate of 1.0 ml/min. The volume of feed sample injected was 5 ml.

ing experiment just described. Note that an initial pH decline in the form of a weakly retained pH front from the presaturation pH of 5.2 to an intermediate plateau at pH 4.7 occurred during the early portion of the experiment but is not illustrated in the figure since the time scale was selected to emphasize the eluate behavior on the retained pH profile. Initial pH transitions of this type result from having different amine buffering species in the presaturation and elution buffers. These transitions are considered in detail by Bates and Frey [11] and occur as either unretained or weakly retained fronts depending on whether the amine buffering species used are completely unadsorbed or are weakly adsorbed, respectively. Note also that initial pH transitions of this type are also observed when polyampholyte buffers are employed [13].

Fig. 5 indicates that high-resolution separations can be achieved even when the pH profile employed is concave in shape instead of linear provided that the profile has an appropriate slope in the region where the proteins of interest elute from the column. In particular, eight distinct protein bands are evident in the figure, with components differing by as little as 0.05 pH units in apparent isoelectric point being completely resolved from each other. As also shown in the figure, the buffering species chosen for use resulted in a concave pH gradient, with the highest resolution occurring on the flattest portion of the pH profile. Note finally that high resolutions were achieved in spite of the fact that a large feed sample of 5 ml was used in the experiment shown.

The resolution shown in Fig. 5 where eluites differing by 0.05 pH units in apparent isoelectric point are well separated is comparable to the maximum resolution of 0.045 pH units reported by Wagner and Regnier [5] for a similar type of column packing used for chromatofocusing with a polyampholyte buffer. However, the resolution reported by Wagner and Regnier was based on a calculation that used the measured width of one protein band, as opposed to Fig. 5 where this resolution was actually achieved. Furthermore, although the resolution shown in Fig. 5 would initially appear to be less than the maximum resolution observed for capillary isoelectric focusing where separations of components differing in actual isoelectric point of 0.02 pH units have been reported [14], it is often the case that the difference in apparent isoelectric points observed during chromatofocusing is larger than the corresponding difference in actual isoelectric points by roughly a factor of two. This is illustrated by the results shown in Figs. 2 and 3 for the hemoglobin variants where the apparent isoelectric points differ by 0.26 pH units whereas the actual isoelectric points differ by 0.15 pH units, and by the results reported by Bates and Frey [11] for the A and B variants of β-lactoglobulin where the apparent isoelectric points differ by 0.21 pH units whereas the actual isoelectric points differ by 0.13 pH units. This suggests that the resolution shown in Fig. 5 may in fact nearly rival the highest resolutions reported for capillary isoelectric focusing.

5. Conclusions

This study demonstrates that simple mixtures of buffering species can be substituted for the polyampholyte elution buffer normally used in chromatofocusing in order to produce a high-performance chromatography method which employs retained,

gradual pH gradients for separating proteins. Furthermore, if the identities and concentrations of the buffering species are properly chosen as described in Part I, resolutions comparable to, if not greater than, those obtained using polyampholyte buffers can be obtained. In particular, in past work by other workers involving high-performance column packings and polyampholyte buffers, the band width at one half the band height for proteins are in the range between 0.025 and 0.1 pH units, and separation times of 2 h or less are typically observed. Similar band widths and separation times were observed in this study using simple mixtures of buffering species in the elution buffer instead of polyampholyte buffers. It was also observed that high resolution separations could be achieved even when the pH profile was concave instead of linear in shape.

6. Nomenclature

d_p	Particle diameter, cm
L	Column length, cm
R	Resolution
Δt	Difference in time between two eluting bands, s
σ	Standard deviation of a Gaussian band, s

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