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Displacement chromatography of proteins using a self-sharpening pH front formed by adsorbed buffering species as the displacer[1]

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Abstract

The preparative-scale separation of two proteins into adjoined, pure bands was accomplished using a novel, hybrid chromatography method which employs chromatofocusing using a self-sharpening pH front and displacement development. The method eliminates the use of a traditional displacer for accomplishing displacement chromatography, and was used to separate the A and B forms of β-lactoglobulin using a strong-base anion-exchange column packing and a buffer system composed of acetic acid and either 3-(*N*-morpholino)propane-sulfonic acid (MOPS) or 2-(*N*-morpholino)ethanesulfonic acid (MES). Sample loads up to 150 mg of protein were applied to a 75×7.5 mm column to produce a displacement train composed of highly pure protein bands with greater than 90% recovery of protein. A discussion is given of the chromatographic behavior of proteins under concentration overloaded conditions for the case where a self-sharpening pH front formed using adsorbed buffering species is used to desorb proteins from an anion-exchange column packing.

Keywords: Displacement chromatography; Preparative chromatography; Chromatofocusing; Mobile phase composition; Proteins; Lactoglobulins

1. Introduction

1.1. General considerations

Tiselius [1] and Claesson [2] were among the first to investigate displacement chromatography. To perform the technique, a column containing an adsorbent packing is first equilibrated with a mobile phase, and then a feed slug containing the components to be separated is introduced. Next, a component, usually termed the displacer, is dissolved into the mobile phase, and the resulting solution is pumped through the column. Provided that the displacer has an affinity for the column packing higher than any of the feed components, a displacement train of adjoining rectangular bands containing the separated feed components will be produced in the column effluent. Finally, the displacer is removed from the column using a regenerant solution, and the column is re-equilibrated with the mobile phase in preparation for the next cycle.

Displacement chromatography differs from the more common technique of gradient elution chromatography in several ways. Most importantly, in gradient elution chromatography the solutes being

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separated do not form adjacent rectangular bands, but instead form a sequence of individual bands, each having a different velocity, and consequently diverging from each other as they migrate through the column. In addition, in displacement chromatography the displacer remains behind the solute bands, whereas in gradient elution chromatography the component constituting the solvent gradient passes through these bands.

Liao et al. [3] were the first to perform displacement chromatography of proteins. Further work by Cramer and co-workers has emphasized protein displacement chromatography using ion-exchange column packings, primarily because of the importance of this class of separations in the biotechnology industry [4–6]. Of special interest to these latter workers is the development of novel displacers, including dendritic polymers, protected amino acids and related types of low-molecular-mass species, in order to address several of the key disadvantages of displacement chromatography; namely, the need for finding a displacer suitable for a given application, the fact that the displacer can potentially contaminate the feed components being purified, and the difficulties associated with removing the displacer from the column after the displacement step.

The novel displacers introduced by Cramer and co-workers show much promise in alleviating many of the disadvantages associated with displacement chromatography by expanding the range of compounds suitable for use as a displacer, and by expanding this range into the class of low-molecular-mass components which can be readily separated from proteins using size-exclusion methods. In order to expand the range of applications for displacement chromatography even further, this study presents an alternative technique in which a sequence of adjacent, rectangular protein bands is produced without the use of a traditional displacer. Instead, a pH change traveling through the column as a retained front, and formed using adsorbed buffering species, serves the purpose of a displacer by inducing the formation of a displacement train downstream from the front.

In addition to being a novel form of displacement chromatography, the technique described in this study can also be considered as a new variation of chromatofocusing. Chromatofocusing was originally developed by Sluyterman and co-workers [7,8] and involves separating proteins using a retained, gradual pH gradient formed inside a weak-base ion-exchange column using a polyampholyte elution buffer. Subsequent work by Hearn and Lyttle [9], Hutchens et al. [10], and Frey and co-workers [11–13] investigated the use of multicomponent mixtures of well-defined buffering species in place of the polyampholyte buffer originally used in the technique. Furthermore, Frey et al. [11] developed optimized buffer systems which eliminate the need for using a weak-base ion-exchange adsorbent with a buffering capacity in order to form a retained pH gradient. However, in contrast to the present study, none of these previous investigations have addressed the behavior of proteins when they are eluted by a single, retained stepwise pH front under concentration overloaded conditions where competitive adsorption effects must be accounted for.

Additional related previous work by other investigators includes studies of ‘‘heater-displacer’’ gas-phase chromatography, in which a traveling heater is used to perform displacement chromatography in the gas phase [14], and the study of Carta et al. [15] in which the separation of amino acids is accomplished using NaOH dissolved in water as a displacer. It should be noted that neither of these two techniques is suitable for protein purification since they both involve severe conditions (i.e., extremes in temperature or pH) which are incompatible with retaining native protein structures. Finally, the technique of ‘‘ampholyte displacement’’ chromatography has been employed to separate proteins by eluting a column presaturated at an initial condition with a high concentration of polyampholyte buffer with or without a change in pH [16]. However, in contrast to the present study, ampholyte displacement chromatography does not cause the formation of a displacement train of proteins and generally exhibits behavior characteristic of traditional chromatofocusing combined with elution chromatography using an ionic strength gradient.

1.2. Protein behavior in a retained pH gradient

Fig. 1 illustrates the effluent pH and buffering species concentration profiles for a typical chromatofocusing system discussed by Frey and co-workers

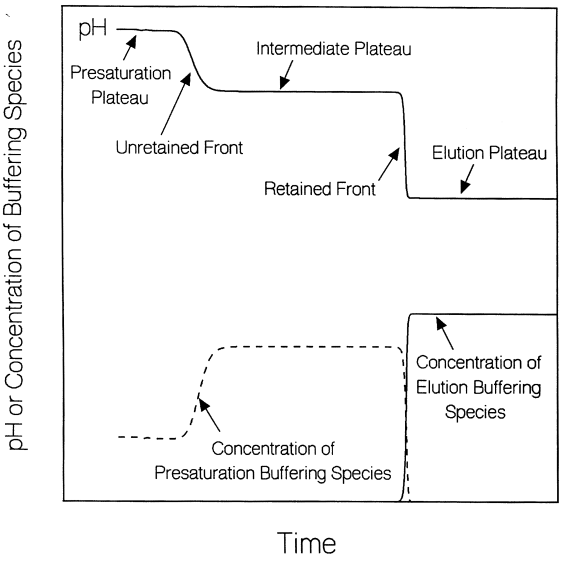

Fig. 1. Effluent concentration and pH profiles for a column exhibiting one retained and one unretained pH front.

[11–13]. For the case shown, the column was presaturated with a single buffering species having a charged form of the proper type to adsorb onto the ion-exchange column packing, e.g., anion-forming buffering species such as acetic or formic acids would typically be used with an anion-exchange column packing. The column was then eluted by a sudden change to an elution buffer at a lower pH which contains a different buffering species with a pK_a lower than that of the presaturation buffering species, but also with a charged form of the proper type to become adsorbed. As shown, this results in the formation of two pH fronts, the first of which is unretained by the column, i.e., it has the velocity of an unadsorbed tracer. The second pH front which forms inside the column is a retained, self-sharpening front (i.e., a front which assumes a ''constant pattern'' shape as it propagates through the column) associated with the replacement of one adsorbed buffering species with another. As also indicated in the figure, the concentration of the buffering species on the intermediate plateau and the concentration of the buffering species in the elution buffer are nearly the same and, as discussed by Frey [12], would be exactly so if stoichiometric ion-exchange is the only adsorption mechanism.

The top part of Fig. 2 illustrates qualitatively the

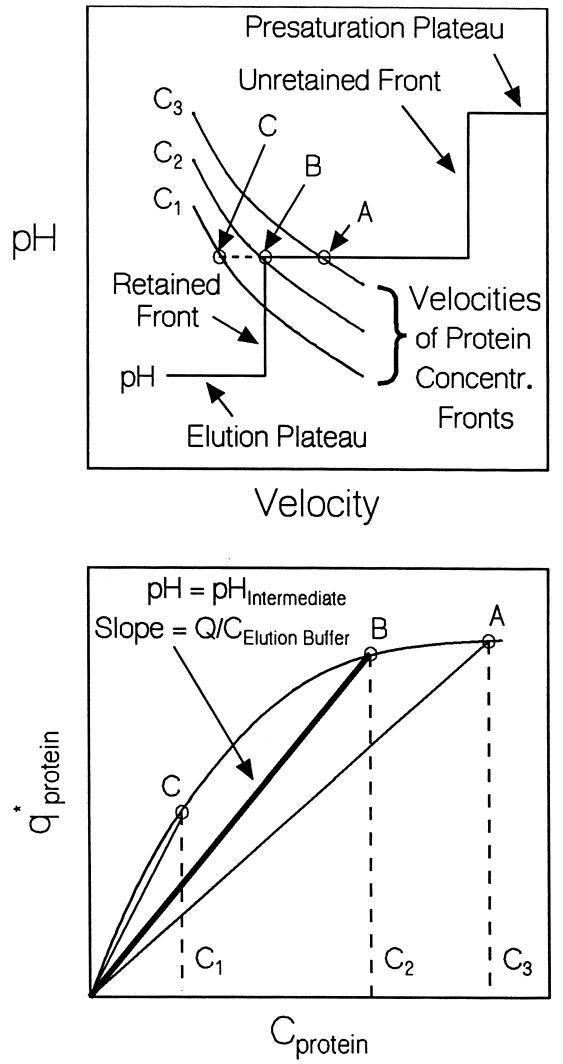

Fig. 2. Top: Superposition of the velocities of protein concentration changes and effluent pH profile, the latter presented in terms of velocities of features on the pH profile. Bottom: Corresponding protein adsorption isotherm.

behavior of a protein in the pH gradient shown in Fig. 1, with the gradient presented in terms of the velocity of pH values under local-equilibrium conditions, i.e., with mass-transfer resistances ignored so that self-sharpening pH fronts appear as vertical steps. Since the horizontal axis in the top part of Fig. 2 is the velocity of features on the pH profile, the figure in effect illustrates the axial pH profile in the column, in contrast to Fig. 1 which illustrates the pH

as a function of time at the column outlet. Also shown in the top part of Fig. 2 are curves denoting the velocities of stepwise, protein concentration fronts, as a function of pH, with the concentration on the front going from the value of zero to the final concentrations indicated. In general, the velocity of a stepwise change in adsorbate concentration, i.e., the velocity of a concentration front, is given by [17]:

$$\nu_{\Delta C} = \frac{\nu_{\text{fluid}}}{1 + \frac{1-\alpha}{\alpha}\epsilon + \frac{1-\alpha}{\alpha}(1-\epsilon)\left[\frac{\Delta q^*}{\Delta C}\right]} \qquad (1)$$

where the quantity in square brackets on the right side of Eq. (1) is the concentration change of the adsorbate across the front in the adsorbed phase divided by that in the fluid phase. Since equilibrium can be assumed to apply on either side of a concentration front, the concentration in the adsorbed phase is written as q^* to denote an equilibrium value associated with C.

Eq. (1) applies not only to a protein concentration front, but also to a buffering species concentration front provided that C and q are interpreted in this latter case as the sum of the concentrations of the individual charged forms of the buffering species in the fluid and adsorbed phases, respectively. Furthermore, although it can be expected that the adsorption selectivity of various buffering species will be influenced to some degree by interactions with adsorbed proteins, it is also generally true that the adsorption capacity of the column packing for the buffering species is largely unaffected by the simultaneous adsorption of proteins as a result of the steric considerations described below. Consequently, the effect of proteins on the pH profile, even at relatively high protein concentrations, is likely to be small, so that the pH profile can be approximated to a reasonable degree using relations developed by Frey [12] based on the application of Eq. (1) to the buffering species.

As shown in the bottom part of Fig. 2, for a given pH the concave shape of a typical protein adsorption isotherm yields a velocity for a concentration front that increases as the size of the concentration change on the front increases, i.e., $\Delta q^*/\Delta C$ decreases as C increases. Furthermore, consider the case where the steric effects described below apply so that, as mentioned above, the amount of buffering species adsorbed is only moderately affected by the simultaneous adsorption of protein. If the adsorption of the neutral forms of the buffering species are neglected under these conditions, then since Eq. (1) applies to the buffering species concentration front that coincides with the pH front, it follows that the velocity of the retained pH front in Fig. 1 is given approximately by the right side of Eq. (1) with the quantity in square brackets replaced by $Q/C_{\text{Elution buffer}}$, where Q is the ion-exchange capacity of the column packing and $C_{\text{Elution buffer}}$ is the concentration of the buffering species in the elution buffer.

Consider first the case of a dilute protein at the concentration C_1 in Fig. 2, in which case the curve labeled C_1 in the upper part of the figure is the velocity of a band containing a trace concentration of protein as a function of pH. If this curve intersects a vertical section of the pH profile, then the protein band velocity downstream from the pH front is less than the velocity of the front, while the opposite situation applies upstream from the pH front, so that the protein tends to become focused on the front. Consider next the case where the protein concentration in a rectangular band is high enough so that the velocities of the stepwise concentration changes associated with sides of the band are greater than the velocity of the pH front, as illustrated by the concentration C_3 in Fig. 2. Under these conditions, a rectangular protein band would tend to move ahead of the pH front into a region of constant pH where it would be subject to the influences of nonlinear adsorption dynamics [17]. In particular, although the downstream side of such a band would propagate unaltered as a self-sharpening front, the upstream side would exhibit so-called proportionate pattern behavior (i.e., the side would broaden in proportion to the length propagated), so that eventually both the average concentration and the average velocity of the band would decrease until the latter was less than the velocity of the retained pH front. This implies that bands containing protein concentrations greater than C_2 are unable to propagate intact on the intermediate pH plateau.

The discussion just given indicates that, for the case shown in Fig. 2, a protein will focus into a narrow band on the retained pH front as long as the amount of protein in the feed slug does not cause the band concentration to exceed C_2. If more than that

amount of protein is present in the feed slug, a rectangular band having the liquid-phase protein concentration C_2 will be formed which extends onto the intermediate plateau so that the upstream side of the band still coincides with the retained pH front, and the band still has the same velocity as the front. Furthermore, since the protein concentration C_2 in Fig. 2 is determined by the velocity of the pH front, and since as mentioned previously the velocity of the pH front is given by Eq. (1) written for the buffering species concentration change at that front (see Fig. 1), it follows that the concentration C_2 is determined by the concentration of the buffering species in the elution buffer, as illustrated graphically in the bottom part of Fig. 2. In addition, by analogy with traditional modes of displacement chromatography, it also follows that if the band velocity curves for two proteins under trace concentration conditions intersect a single, retained stepwise pH front, a sequence of rectangular bands each containing a single protein will result when the feed slug contains a large amount of protein.

2. Experimental

2.1. Equipment

Solvent delivery was performed by a Thermo Separations P4000 Gradient Mixing Pump whose wetted parts consisted of stainless steel which had been passivated to render it corrosion resistant. Passivation was performed by the pump manufacturer and consisted of pumping 20% (w/w) nitric acid in deionized water through the pump for 20 min at room temperature, followed by rinsing with deionized water, and then rinsing with 50% (v/v) methanol in deionized water. It was deemed unnecessary to repeat the passivation procedure during the course of the experiments performed here since no evidence of corrosion was ever observed on any of the wetted parts of the pumping system.

A Rheodyne Model 7010 injection valve with a 5-ml sample loop was used to introduce the sample onto the column, and a Rheodyne Model 9010 polyether ether ketone (PEEK) valve was employed as a column switching valve. The column effluent was monitored by a Thermo Separations UV2000 UV–Vis absorbance detector, and the pH of the effluent was measured by a Sensorex sealed combination pH electrode seated in an in-line flow cell having 50 μl of dead volume, and connected to an Orion Model 701A Ionalyzer pH meter. The analog outputs of the pH meter and absorbance detector were directed to a Strawberry Tree Datashuttle, which was connected to an 80286-based IBM PS/2 computer running the Strawberry Tree Workbench data acquisition software.

2.2. Materials

A TSK-Gel Q-5PW (75×7.5 mm) strong-base anion-exchange column was used for the displacement experiments. Fractions obtained from the column effluent were analyzed on a Pharmacia Mono-Q HR 5/5 strong-base anion-exchange column. The mobile phase buffering species were 3-(*N*-morpholino)propanesulfonic acid (MOPS), 2-(*N*-morpholino)ethanesulfonic acid (MES), sodium acetate, sodium hydroxide and sodium chloride, all obtained from Sigma. Bovine serum albumin (BSA) and β-lactoglobulins A and B (Lac-A and Lac-B) were also obtained from Sigma. All buffers were vacuum filtered through 0.2-μm nylon filter membranes.

2.3. pH displacement procedures

The TSK-Gel Q-5PW column was equilibrated with presaturant buffer before each run. Presaturation of the column was assumed to have been completed when the column effluent stabilized at the presaturant pH. Protein solutions were prepared in the presaturant buffer, and filtered through a Whatman 0.2-μm polysulfone filter upon introduction into the sample loop. The 5-ml sample loop was first purged with 15 ml of air, and then with 10 ml of sample, prior to the injection of the sample into the column. The injection of 10 and 15 ml of sample into the column was accomplished by filling the sample loop and injecting it two and three times in succession, respectively. During the injection procedure, presaturation buffer was pumped through the column. After injection of the sample, the column was washed with 15 ml of presaturant buffer and then eluted with the elution buffer. The UV absorbance of the column effluent was monitored at 305 nm during the elution

step, and effluent fractions were taken in 1-min intervals. Column regeneration was performed by washing the column with a pH 4.5 sodium acetate buffer containing 0.5 *M* NaCl, followed by the presaturation step. Information obtained from the column manufacturer [18] indicated that this regeneration procedure was compatible with the column and column packing used, as was confirmed by the fact that reproducible results were obtained in this study over an extended time period. The pumping system was flushed thoroughly with deionized water after each set of experiments. No corrosion was observed on any of the wetted parts of the system during the course of the experiments described here.

2.4. Fraction analysis

Effluent fractions were analyzed on the Pharmacia Mono-Q HR 5/5 strong-base anion-exchange column using gradient elution. A sodium acetate gradient from 75% buffer A and 25% buffer B to 25% buffer A and 75% buffer B in 30 min at 1.0 ml/min was used, where buffer A was a 25 m*M* sodium acetate solution adjusted to pH 6.0 with acetic acid, and buffer B was a 1.0 *M* sodium acetate solution also adjusted to pH 6.0 with acetic acid. Before analysis, collected fractions were diluted by a factor of 10 by adding Buffer A. A 20-μl aliquot from each diluted fraction was injected onto the column, and the column effluent absorbance was monitored at 278 nm. Peak areas were measured through numerical integration of the digitally recorded data, and converted to concentrations using a calibration curve that related area to mass in a linear manner.

3. Results and discussion

The following two sections describe experimental investigations of the chromatographic behavior of proteins under overloaded conditions when retained pH gradients are used. In particular, Section 3.1 investigates the behavior of a single protein under overloaded conditions when it is desorbed using the retained front of a pH gradient, i.e., the slower moving front illustrated in Fig. 1. The results obtained not only provide a basis for the subsequent section concerning the formation of a protein displacement train, but they also pertain to the case where it is desired to recover a single protein from a mixture of proteins under conditions where the band velocity curve for the protein to be purified intersects the retained pH front as in Fig. 2, while the corresponding curves for the impurities do not. In this case, the highly desirable situation occurs where the protein to be purified is contained in an isolated rectangular band of constant width located on the intermediate pH plateau, while the impurities elute from the column either on the presaturation or elution pH plateaus.

3.1. Band shape for a single protein

Fig. 3 illustrates the effluent pH and UV absorbance profiles that result when a TSK-Gel Q-5PW anion-exchange column is presaturated with a 0.05 *M* NaOH solution titrated with MOPS to pH 7.5, and then eluted at 1.0 ml/min with a 0.05 *M* NaOH solution titrated with acetic acid to pH 4.5. Various amounts of BSA as specified in the figure were injected into the column. Fig. 4 shows similar results for the case where the column was presaturated with

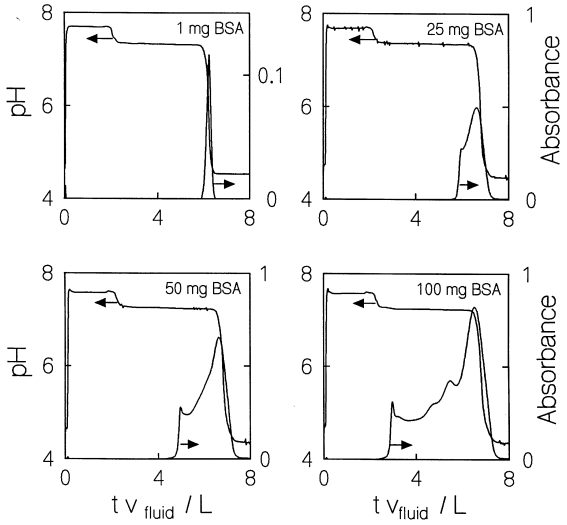

Fig. 3. UV absorbance and pH effluent profiles for the chromatography of various amounts of BSA in a 1- or 5-ml feed slug using a TSK-Gel Q-5PW column packing. The column was presaturated with 0.05 *M* NaOH titrated with MOPS to pH 7.5 and eluted with 0.05 *M* NaOH titrated to pH 4.5 with acetic acid. The flow-rate was 1.0 ml/min and the UV absorbance was measured at 305 nm.

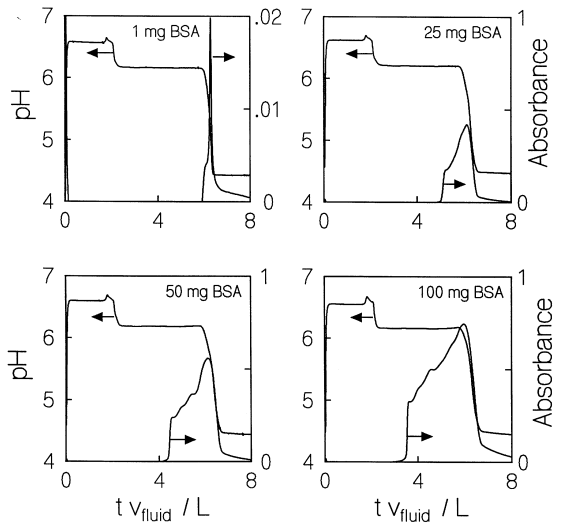

Fig. 4. UV absorbance and pH effluent profiles for the chromatography of various amounts of BSA in a 1- or 5-ml feed slug using a TSK-Gel Q-5PW column packing. The column was presaturated with 0.05 *M* NaOH titrated with MES to pH 6.5 and eluted with 0.05 *M* NaOH titrated to pH 4.5 with acetic acid. The flow-rate was 1.0 ml/min and the UV absorbance was measured at 305 nm.

a 0.05 *M* NaOH solution titrated with MES to pH 6.5, and then eluted under the same conditions as in Fig. 3.

As shown in Figs. 3 and 4, when 1 mg of BSA was injected into the column, the protein eluted as a narrow band localized on the retained (i.e., slower moving) pH front. This indicates that the band velocity curve for BSA under dilute conditions intersects the retained pH front when both the pH profile and the band velocity curve are plotted as in Fig. 2. Note that the horizontal axes in Figs. 3 and 4 correspond to a dimensionless time written as tv_{fluid}/L, where t is the time from the start of the elution step, L is the column length and v_{fluid} is the interstitial fluid velocity.

Figs. 3 and 4 indicate that when the amount of BSA in the feed slug is increased, eventually a protein band forms that is approximately rectangular in shape, in agreement with the discussion of Fig. 2. However, rather than being a truly rectangular band in this case, the figures indicate that quasi-rectangular bands form having a concentration maximum located on the retained pH front. This concentration maximum appears to result from the fact that BSA becomes progressively more weakly adsorbed as well as less concentrated as the pH decreases on the retained pH front. Depending on the properties of the protein, and if the retained pH front is broad enough, then on the retained front the protein will become subject to the same type of focusing effects that normally occur when chromatofocusing is carried out at low protein concentrations using a gradual pH gradient, thereby yielding the maximum in concentration shown.

The protein band shapes illustrated in Figs. 3 and 4 were very reproducible, with the band shapes themselves depending mainly on the shape of the pH profile and the amount of protein injected into the column, as shown in the figures. The band shapes were also relatively insensitive to the flow-rate used in the range between 0.2 and 1.0 ml/min when the UV absorbance was plotted as a function of the dimensionless elution volume, i.e., as a function of tv_{fluid}/L (data not shown). Since the band shapes illustrated in Figs. 3 and 4 were reproducible, the minor peaks shown are likely due to the presence of impurities in the BSA feed material, such as the dimer form of BSA. These minor peaks were not further investigated in this study.

The recovery of BSA for the experiment involving 100 mg of protein in Fig. 3 was determined by collecting the eluted protein in several fractions. These fractions were then diluted five-fold with the presaturation buffer, and the amount of BSA in each fraction was determined by measuring the UV absorbance at 280 and 305 nm. The relation between the concentration of BSA and the UV absorbance was determined by assuming the BSA feed material had no impurities present, and then dissolving known masses of this material in the presaturation buffer to make several mixtures having a known BSA concentration. The UV absorbance of these mixtures at the two wavelengths was measured by passing them through the UV absorbance detector until a steady reading was obtained. The recoveries of BSA determined in this way were 93% and 103% using the UV absorbances at 280 and 305 nm, respectively, which indicates that essentially complete recovery of BSA was achieved as far as could be determined within the experimental accuracy.

Although the results in Figs. 3 and 4 indicate that for certain conditions a protein band formed under

overloaded conditions will be quasi-rectangular in shape with a concentration maximum located on the pH front, it is likely that in many cases this will be acceptable if the ultimate goal of the process is to form a displacement train of proteins on the intermediate pH plateau. This is due to the fact that when conditions are not ideal for forming a truly rectangular band, the quasi-rectangular bands that tend to form generally have downstream sides sufficiently steep, and a plateau region adjoined to downstream side sufficiently wide, to induce the formation of a displacement train of more weakly adsorbed proteins located downstream.

Figs. 3 and 4 indicate that there is a slight broadening of the retained pH front as the amount of protein injected into the column increases. One likely reason for this broadening is that the protein concentration in the bands which contain 50 and 100 mg of BSA is relatively high, i.e., approximately 20 mg/ml, which causes a large difference in viscosity to exist between the fluid in the protein band and the fluid immediately upstream from the band. A viscosity difference of this size can lead to the phenomenon of viscous fingering, which would tend to broaden the upstream side of the protein band [19]. As an additional consideration, it follows from Eq. (1) together with the known liquid phase concentrations of the buffering species and protein and the velocities of the retained pH front and the protein band in Fig. 3, that there are approximately 150 molecules of the buffering species for every protein molecule in both the adsorbed and liquid phases for the conditions that apply to the figure. Since the net charge on an individual BSA molecule varies from approximately -12 at pH 7 to approximately $+7$ at pH 4.5 [20], it follows that although the majority of the buffering capacity is supplied by the buffering species, the protein present may nevertheless be participating in acid–base equilibrium to an extent sufficient to cause the minor effects on the retained pH front shown.

The relatively small effect of large amounts of protein on the width of the retained pH front in Fig. 3 can be further rationalized by considering the steric effects which occur when proteins adsorb onto an ion-exchange column packing [5]. These steric effects are largely due to the fact that the charged region on the protein surface responsible for adsorption interacts directly with a relatively small number of charged sites on the column packing. However, due to their large size, proteins generally extend over a much larger number of adsorption sites, most of which are therefore blocked from interacting with other proteins and thus occupied by small ions. Since adsorbed buffering species are necessarily employed in the method described here, these species tend to always be present in sufficient amounts to determine the pH profile even at high protein concentrations. This generally justifies the use of Fig. 2, with the pH profile determined in the absence of proteins, to predict behavior qualitatively when proteins are present. Furthermore, the fact that the elution times for the retained pH fronts in Figs. 3 and 4 are insensitive to the amount of protein loaded is an additional indication that the adsorption capacity of the column for the two buffering species is relatively unaffected by even large amounts of adsorbed proteins.

3.2. Formation of a displacement train of proteins

An approximately 50% mixture of the A and B forms of β-lactoglobulin was used to evaluate the ability of a self-sharpening pH front to separate two proteins by forming two adjacent rectangular bands under concentration overloaded conditions. β-Lactoglobulins A and B were chosen for study due to the fact that these proteins are readily available, and because their properties have been extensively studied. In particular, it is known that the A and B forms of β- lactoglobulin each consist of a dimer of two polypeptide chains, and differ by only two amino acid residues – aspartic acid and valine at residues 64 and 118 in A, and glycine and alanine at these residues in B. These two proteins also have nearly identical molecular masses, a net charge differing by at most one charge unit, and isoelectric points of 5.21 and 5.34 for the A and B forms of the protein [21,22]. Furthermore, the separation of β-lactoglobulins A and B has been used previously to demonstrate protein displacement chromatography [3,23], and is particularly relevant to the biotechnology industry where it is often the case that a desired protein product must be separated from structural variants, such as its deamidated or oxidized forms [24]. Finally, the concentration maximum observed

for BSA in Figs. 3 and 4 occurs to a much smaller degree when β-lactoglobulins A and B are employed, which facilitates the interpretation of the results obtained.

The top part of Fig. 5 illustrates the behavior of β-lactoglobulins A and B when a 0.5-ml feed slug containing 1 mg of these proteins was injected into a TSK-Gel Q-5PW column which was presaturated with 0.05 *M* NaOH titrated with MES to pH 6.5, and eluted at a flow-rate of 1.0 ml/min with 0.05 *M* NaOH titrated with acetic acid to pH 4.3. As was the case for the feed slugs containing 1 mg of BSA in Figs. 3 and 4, the small amount of protein used to obtain the results shown in the top part of Fig. 5 insures that the two proteins elute from the column at dilute concentrations. The bottom part of Fig. 5 illustrates results from the analytical chromatography of an effluent fraction which contained the entire, single absorbance peak shown in the top part of Fig. 5. The results indicate that both forms of β-lactoglobulin have co-eluted from the column as a single peak in the experiment shown in the top part of Fig. 5, which in turn indicates that the band velocity curves for both proteins at low concentrations intersect the retained pH front when the band velocity curves and the pH gradient are plotted as in Fig. 2.

To investigate the chromatographic behavior of β-lactoglobulins A and B under concentration overloaded conditions when a retained pH front is employed, a 10-ml feed slug containing 100 mg of these proteins was injected into the same column as before. To perform the experiments, the column was presaturated with 0.05 *M* NaOH titrated with MOPS to pH 7.5 and eluted at 0.2 ml/min with 0.05 *M* NaOH titrated with acetic acid to pH 4.3. Results are given in Fig. 6, which shows the effluent pH and protein concentration profiles for the two proteins, with the latter quantity determined from the analysis of individually collected effluent fractions. As illustrated, two rectangular bands, each containing one of the β-lactoglobulin variants, were formed on the intermediate plateau. As also illustrated, the maximum in protein concentration in the vicinity of the retained pH front that was observed for BSA occurred to a much smaller degree for the case

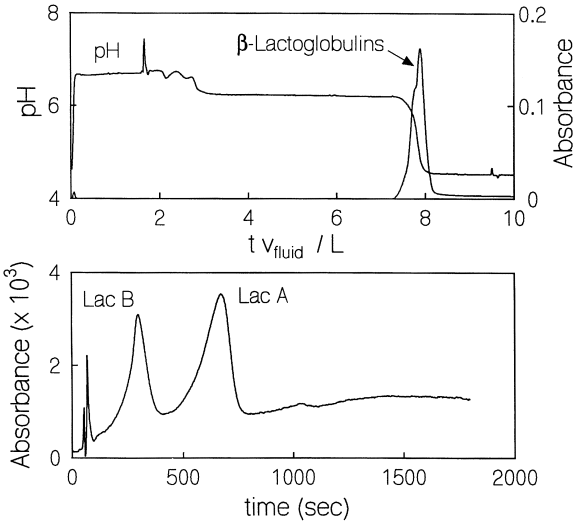

Fig. 5. Top: UV absorbance and pH effluent profiles for the chromatography of 1 mg of β-lactoglobulins A and B in a 0.5-ml feed slug using a TSK-Gel Q-5PW column packing. The column was presaturated with 0.05 *M* NaOH titrated with MES to pH 6.5 and eluted with 0.05 *M* NaOH titrated to pH 4.3 with acetic acid. The flow-rate was 1.0 ml/min and the absorbance was measured at 305 nm. Bottom: Chromatographic analysis of the protein containing fraction from the top part of the figure with the UV absorbance measured at 278 nm.

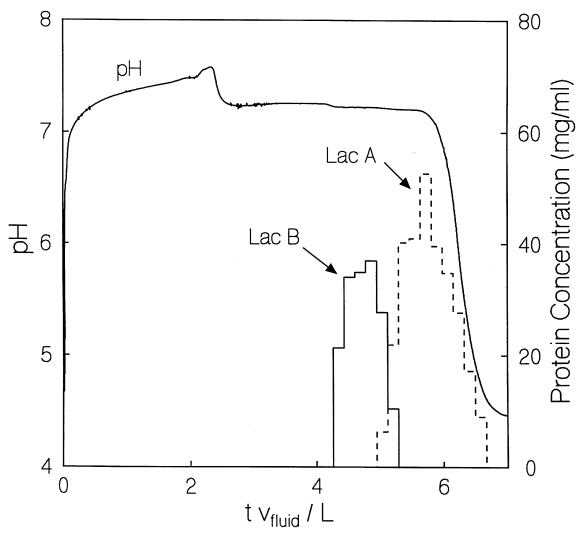

Fig. 6. Effluent pH and protein concentration profiles for the chromatography of 100 mg of β-lactoglobulins A and B in a 10-ml feed slug using a TSK-Gel Q-5PW column packing. The column was presaturated with 0.05 *M* NaOH titrated with MOPS to pH 7.5 and eluted with 0.05 *M* NaOH titrated to pH 4.3 with acetic acid. The flow-rate was 0.2 ml/min, and the protein concentrations were determined by collecting and analyzing 1-ml column fractions to produce the histogram shown.

β-lactoglobulins A and B, evidently because β-lactoglobulins A and B are more strongly adsorbed onto the column packing at a given pH as compared to BSA.

The recovery of β-lactoglobulins A and B in the column effluent determined by summing the amounts of these two proteins in the collected fractions ranged from 82 to 95% of the total protein present in the feed slug for all of the experiments shown in this section. Since the purity of the β-lactoglobulins A and B used to constitute the feed slug was reported by the supplier to be approximately 90%, this indicates that the actual recovery of β-lactoglobulins A and B ranged approximately from 90 to 100% as far as could be determined within the experimental accuracy.

Fig. 7 shows results obtained with the same presaturation and elution buffers used in Fig. 6, but where 150 mg of protein were injected into the column in a 15-ml feed slug. As shown, two rectangular bands, each containing one of the β-lactoglobulin variants, were again formed on the intermediate plateau. Furthermore, the results in Figs. 6 and 7 indicate that the effect of increasing the amount of protein injected into the column is to increase the width of the protein bands when they exit the column, but to leave the protein concentrations in the bands unchanged. This is the expected behavior when displacement is the mechanism causing the formation of the protein bands, as discussed by numerous workers [3].

In order to investigate further the separation of β-lactoglobulins A and B, an experiment was performed in which the same elution buffer was used as in Figs. 6 and 7, but the presaturation buffer was produced by titrating 0.05 *M* NaOH with MES. Results are shown in Fig. 8, which illustrates the effluent pH and protein concentration profiles obtained. As shown, the major effect of replacing MOPS with MES in the presaturation buffer is to decrease both the pH of the intermediate plateau and the protein concentrations on the intermediate plateau. According to Fig. 2, this behavior is expected on the basis that, if the pH of the intermediate

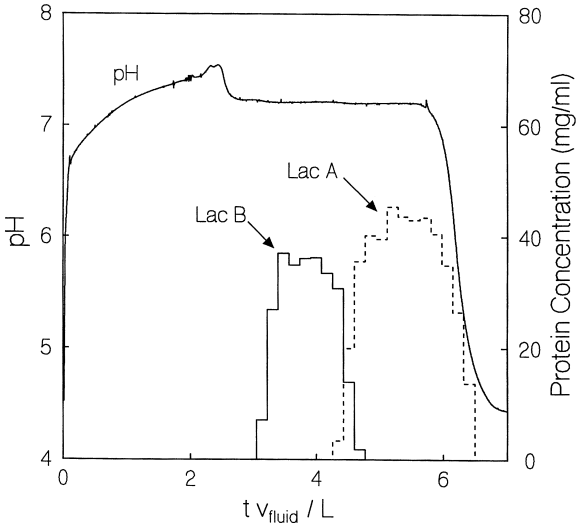

Fig. 7. Effluent pH and protein concentration profiles for the chromatography of 150 mg of β-lactoglobulins A and B in a 15-ml feed slug using a TSK-Gel Q-5PW column packing. The column was presaturated with 0.05 *M* NaOH titrated with MOPS to pH 7.5 and eluted with 0.05 *M* NaOH titrated to pH 4.3 with acetic acid. The flow-rate was 0.2 ml/min, and the protein concentrations were determined by collecting and analyzing 1-ml column fractions to produce the histogram shown.

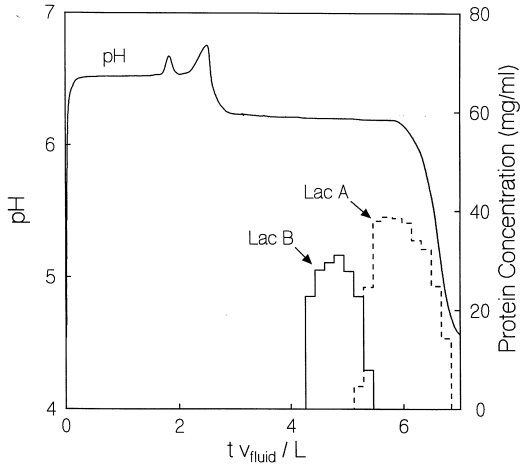

Fig. 8. Effluent pH and protein concentration profiles for the chromatography of 100 mg of β-lactoglobulins A and B in a 10-ml feed slug using a TSK-Gel Q-5PW column packing. The column was presaturated with 0.05 *M* NaOH titrated with MES to pH 6.6 and eluted with 0.05 *M* NaOH titrated to pH 4.3 with acetic acid. The flow-rate was 0.2 ml/min, and the protein concentrations were determined by collecting and analyzing 1-ml column fractions to produce the histogram shown.

plateau is reduced, the protein concentration corresponding to the maximum concentration step change that intersects the retained pH front is also reduced.

Fig. 9 illustrates results from an experiment where all of the conditions were the same as in Fig. 6, except that the elution and presaturation buffers were produced by titrating 0.025 *M* NaOH with the appropriate buffering species, rather than 0.05 *M* NaOH, so that the elution buffer has a lower ionic strength and buffering capacity, and the retained pH front has a lower velocity [12]. The figure shows the UV absorbance profiles for experiments where the feed slug contained 100 mg of β-lactoglobulins A and B.

Since the retained pH front and the protein band always travel at the same velocity, Eq. (1) leads to the conclusion that if v_{band}/v_{fluid} is changed by a certain factor under conditions where $v_{fluid}/v_{band} >> 1$, then the liquid-phase protein concentrations in the displacement train will change by an identical factor provided that the amount of protein adsorbed is near its saturation value so that it is relatively unaffected by changes in the buffering species concentration. Furthermore, for the case where the column is presaturated with one buffering species and eluted with another buffering species, Eq. (1) indicates that when the buffering species concentration in the elution buffer is changed by a certain ratio, the velocity of the pH front and therefore the velocity of the protein band will change by the same ratio provided again that $v_{fluid}/v_{band} >> 1$. Results in Fig. 9 are in agreement with this approximate analysis, and indicate that the buffering species concentration in the elution buffer, the value of v_{band}/v_{fluid}, and the protein concentration in the band as determined by dividing the total amount of protein in the feed slug by the volume of the rectangular band in the column effluent, all change by factors that range from between 1.5 and 2.0.

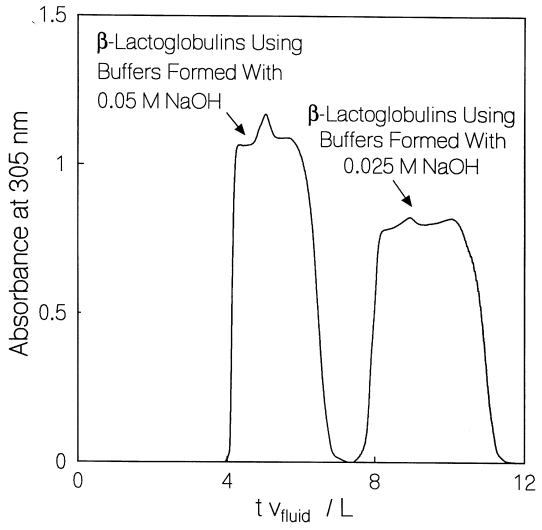

Fig. 9. Comparison of effluent UV absorbance profiles for the chromatography of 100 mg of β-lactoglobulins A and B in a 10-ml feed slug. The presaturation and elution buffers had pH values of 7.5 and 4.3, and were produced in one case by titrating 0.025 *M* NaOH with MOPS and acetic acid, respectively, and in the other case by titrating 0.05 *M* NaOH with MOPS and acetic acid, respectively. The flow-rate was 0.2 ml/min and the UV absorbance was measured at 305 nm.

3.3. Additional process considerations

A variety of additional modifications to the chromatographic technique described here may be employed if needed. For example, if the proteins being separated exhibit low solubilities within the pH range employed, and if changes in the ionic strength of the elution buffer are not sufficient to prevent protein precipitation, then mobile-phase-modifying reagents such as urea or glycerol which enhance protein solubility and reduce aggregation can be added to the elution buffer [25]. In addition, pH gradients are often avoided in protein chromatography not only because proteins tend to precipitate at pH values near their isoelectric point, but also because proteins may denature when they are exposed to pH extremes. To address this, the pH of the intermediate plateau can be optimally selected to simultaneously enhance protein solubility while also avoiding protein denaturation. Finally, as illustrated in Figs. 7 and 8, when a retained pH front is employed, only the most strongly adsorbed protein in the feed mixture will directly contact the pH gradient. It may therefore be appropriate in certain cases to add a spacer component to the mixture to be separated which would possess a higher affinity for the column packing than any of the proteins in the feed mixture, yet would desorb at the eluent pH. This would insure that all the proteins in the original feed mixture would elute from the

column in a uniform pH environment on the intermediate pH plateau, so that any deleterious effects of the pH gradient would be avoided.

4. Conclusions

It is demonstrated in this study that a retained, self-sharpening pH front formed using adsorbed buffering species can be used to produce a sequence of isotachic, rectangular (or quasi-rectangular) bands each containing a single protein similar to the displacement train formed in displacement chromatography. The utility of the method is demonstrated by using it for the preparative-scale separation of two closely related protein variants having isoelectric points differing by only 0.13 pH units.

5. Symbols

C	Concentration in liquid phase (mol/l)
$C_{\text{Elution buffer}}$	Liquid phase concentration of buffering species in elution buffer (mol/l)
L	Column length (cm)
q^*	Adsorbed concentration at equilibrium per unit volume of solid adsorbent phase (mol/l)
Q	Ion-exchange capacity of adsorbent (mol/l)
t	Time (s)
v_{fluid}	Interstitial fluid velocity (cm/s)
v_{band}	Protein band velocity (cm/s)
$v_{\Delta C}$	Velocity of an adsorbate concentration front (cm/s)

5.1. Greek

α	Interstitial void volume in bed
ϵ	Internal porosity of particle

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