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Experimental studies in metal affinity displacement chromatography of proteins

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Abstract

Metal affinity displacement chromatography was employed for the purification of proteins. The mobile phase modifier imidazole was shown to exhibit complex induced gradients in these displacement systems resulting in different imidazole microenvironments in each protein displacement zone. Furthermore, the induced imidazole gradient produced an elevated displacer concentration at the rear of the displacement train. While adsorption isotherms measured under the initial carrier conditions were unable to predict these displacements, isotherms measured under the induced imidazole conditions qualitatively predicted the effluent displacement profiles. It is believed that these induced imidazole gradients speed up the kinetics of the displacement process and are in part responsible for the sharp boundaries seen in these separations. This work demonstrates the ability of this bioseparation technique to effect efficient multicomponent separations and illustrates the importance of mobile phase modifier effects in metal affinity displacement chromatography.

1. Introduction

The concept of ligand-exchange chromatography using stationary phases with immobilized metal chelates was first introduced by Helfferich [1]. In 1975, Porath et al. [2] extended the technique to the separation of proteins and nucleic acids. In immobilized metal affinity chromatographic (IMAC) systems, the exposed electron-donating amino acid residues on the protein surface, such as the imidazole group of histidine, the thiol group of cysteine, and the indoyl group of tryptophan, contribute to the binding of proteins to immobilized metal ions [3]. These unique interactions enable IMAC systems to selectively interact with classes of complementary biopolymers. Furthermore, the relatively inexpensive IMAC adsorbents have distinct economic advantages over biospecific affinity systems [4,5]. Several workers have attempted to elucidate the mechanism of adsorption in IMAC systems [6–16]. However, the physicochemical properties of protein retention in IMAC are not well understood at present. Porath et al.'s pioneering work has catalyzed considerable work on the purification of biomolecules using IMAC systems [17–28].

Displacement chromatography is rapidly emerging as a powerful bioseparation method due to the high throughput and product purity associated with the process [29–31]. The displacement process is based on the competition of

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solutes for adsorption sites on the stationary phase according to their relative binding affinities and mobile phase concentrations. This technique offers distinct advantages in preparative chromatography as compared to the conventional elution mode. The displacement process takes advantages of the non-linearity of the isotherms such that a larger feed can be separated on a given column with the purified components recovered at significantly higher concentrations. Furthermore, the tailing observed in elution chromatography is greatly reduced in displacement chromatography due to self-sharpening boundaries formed in the process. Whereas in elution chromatography the feed components are diluted during the separation, the feed components are often concentrated during displacement chromatography. These advantages are particularly significant for the isolation of biopolymers from dilute solutions such as those encountered in biotechnology processes. Although traditional stationary phase materials such as reversed-phase and ion-exchange have been successfully employed in the displacement mode [29–38], research on displacement chromatography with more specific adsorbent materials is scarce at present [39–43].

Metal affinity displacement chromatography (MADC) is a bioseparation technique which combines the unique selectivity of IMAC systems with the high throughput and purity of displacement chromatography. We have previously demonstrated that MADC can be successfully carried out using ribonuclease A as the displacer [42]. Furthermore, tailing observed in these displacement separations was significantly improved by the appropriate use of imidazole as a mobile phase modifier. While imidazole and histidine have been employed as mobile phase modifiers in elution IMAC systems [3,9], their behavior is quite different from more traditional mobile phase modifiers (e.g. salt, methanol). In this paper, we present experimental results of MADC for multicomponent protein separations. In addition, an investigation of the effects of induced imidazole gradients in these MADC systems is presented.

2. Experimental

2.1. Materials

Bulk chelating Superose (10 μm) containing covalently bound iminodiacetic acid (IDA) and 110 × 5 mm I.D. glass columns were donated by Pharmacia LKB Biotechnology (Uppsala, Sweden). Bulk Bioseries strong cation exchanger (SCX) material was obtained from Rockland Technologies (Newport, DE, USA). POROS R/H reversed-phase chromatographic columns (100 × 4.6 mm I.D.) were purchased from PerSeptive Biosystems (Cambridge, MA, USA). The chelating Superose and SCX materials were slurry packed into 110 × 5 mm and 250 × 4.6 mm I.D. columns, respectively. Acetonitrile and sodium monophosphate were obtained from Fisher Scientific (Fairlawn, NJ, USA). Cytochrome *c* from horse heart, lactoferrin from bovine milk, myoglobin from horse heart, ribonuclease A from bovine pancreas, cupric sulfate, ethylenediaminetetraacetic acid (EDTA), imidazole, sodium chloride and urea were obtained from Sigma (St. Louis, MO, USA).

2.2. Apparatus

An fast protein liquid chromatography (FPLC) system (Pharmacia LKB) was employed for the displacement experiments and analysis of proteins. This system consisted of two Model P-500 pumps connected to the chromatographic column via a Model MV-7 valve. The column effluent was monitored by a Model UV-M detector and a Pharmacia strip-chart recorder. Fractions of the column effluent were collected with a Model Frac-100 fraction collector for further analysis. The system was controlled using a LCC-500-Plus controller.

An HPLC system was employed for the analysis of imidazole. This system consisted of a Model LC 2150 pump (LKB, Bromma, Sweden), a Model 7125 sampling valve with a 20-μl sample loop (Rheodyne, Cotati, CA, USA), a spectroflow 757 UV–Vis detector (Applied Biosystems, Foster City, CA, USA) and a Model C-

R3A Chromatopac integrator (Shimadzu, Kyoto, Japan).

An MI-410 pH microelectrode was purchased from Microelectrodes (Londonderry, NH, USA).

2.3. Procedures

Immobilization of Cu^{2+}

The IDA columns were loaded with Cu^{2+} by sequential perfusion with ten column volumes of 0.3 *M* cupric sulfate aqueous solution, pH 3.9, six column volumes of deionized water, and ten column volumes of the carrier solutions described below.

Adsorption isotherms of proteins and imidazole

Adsorption isotherms were determined by frontal chromatography according to the technique of Jacobson et al. [44] by using a 36 × 2 mm I.D. microbore column packed with Cu^{2+}-charged IMAC material. The column effluents were monitored at 280 nm for proteins and 230 nm for imidazole.

Operation of displacement chromatograph

In all displacement experiments, the columns were initially equilibrated with the carrier and then sequentially perfused with feed, displacer and regenerant solutions. Fractions of the column effluent were collected directly from the column outlet to avoid extra column dispersion of the purified components. Fractions collected throughout the displacement runs were assayed by analytical HPLC. The pH values of the fractions were measured by a microelectrode pH meter.

MADC of proteins

Displacement experiments were carried out using 110 × 5 mm I.D. columns packed with Cu^{2+}-charged IMAC stationary phase materials. The feed mixture contained 3 mg cytochrome *c*, 12 mg lactoferrin, and 30 mg ribonuclease A in 3 ml of a 25 m*M* phosphate buffer carrier, pH 5.0, containing 1.0 *M* sodium chloride and 5 m*M* imidazole. Displacer was 15 mg/ml myoglobin in a 25 m*M* phosphate buffer carrier, pH 8.0, containing 1.0 *M* sodium chloride and 5 m*M* imidazole. The regenerant was 15 column volumes of 25 m*M* phosphate buffer, pH 12.0, containing 2.0 *M* sodium chloride. After each displacement run, Cu^{2+} of the column was removed completely using 0.1 *M* EDTA solution (pH 7.0) and recharged with 0.3 *M* cupric sulfate solution. In all displacements the feed was loaded at a flow-rate of 0.2 ml/min and the displacement was performed at 0.1 ml/min at 22°C. Sodium chloride was added to all carrier solutions in order to quench non-specific ionic adsorption. Imidazole was included in all carrier solutions in order to enhance the displacement kinetics.

Imidazole frontal chromatography with a pH step change

Frontal chromatography was carried out using pH step change from 5.0 to 8.0 in the presence of 5 m*M* imidazole and 1.0 *M* sodium chloride in 25 m*M* phosphate buffer. Flow-rate was 0.2 ml/min.

FPLC analysis of proteins

Fractions collected during the displacement experiments were analyzed by gradient elution reversed-phase chromatography. The analyses were carried out using a 100 × 4.6 mm I.D. POROS reversed-phase column on the FPLC system. A 4-min linear gradient of 10–70% acetonitrile in 50 m*M* phosphate buffer, pH 2.2 was employed. Displacement fractions were diluted 20–100-fold with the first eluent buffer and 25-μl samples were injected. The flow-rate was 1.0 ml/min. The column effluents were monitored at 280 nm and column temperature was maintained at 22°C. Quantitative analyses were carried out and the data was used to construct displacement chromatograms.

HPLC analysis of imidazole

Imidazole analysis was carried out with an HPLC system. A 250 × 4.6 mm SCX column was employed for the separation of imidazole. The carrier consisted of 15 m*M* phosphate, pH 5.0.

Displacement fractions were diluted 20-fold with the carrier buffer and 20-μl samples were injected. Flow-rate was 1.2 ml/min. The column effluents were monitored at 230 nm.

Copper analysis

Copper was analyzed using a Perkin-Elmer Model 3030 atomic absorption spectrophotometer. Effluent fractions were diluted and the atomic absorbance was monitored at 320.4 nm. The copper ion concentration was then calculated using an appropriate calibration plot.

3. Results and discussion

We have previously reported on the use of immobilized metal ion chromatographic systems in the displacement mode for the purification of proteins using ribonuclease A as the displacer [42]. The appropriate use of imidazole as a mobile phase modifier significantly improved tailing observed with certain proteins in MADC. In this manuscript, we present experimental results of MADC for the efficient separation of a multicomponent protein mixture. In addition, an investigation of the effects of induced imidazole gradients in these MADC systems is presented.

3.1. MADC of proteins using imidazole as a mobile phase modifier

A displacement experiment using myoglobin as the displacer was carried out using a model feed mixture of cytochrome *c*, lactoferrin and ribonuclease A. In this separation, imidazole was used as a mobile phase modifier to increase the displacement kinetics [42]. Furthermore, a pH step change was employed between the feed (pH 5.0) and displacer solutions (pH 8.0) in order to concentrate the feed proteins at the front of the column during the loading process. In the presence of imidazole, relatively higher affinity of proteins at lower pH was observed due to the lack of effective imidazole adsorption [45]. As

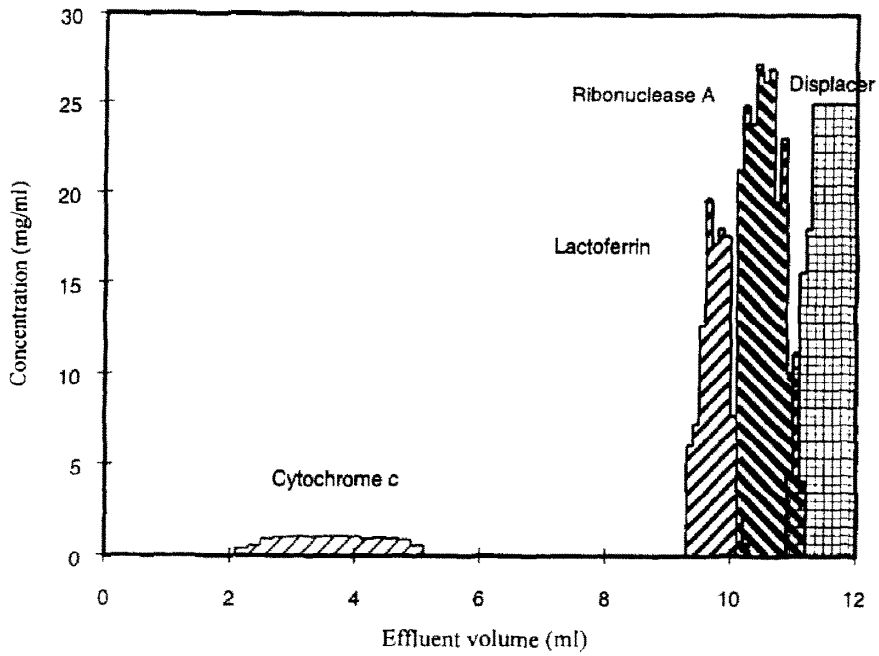

Fig. 1. Displacement chromatogram of a three-protein mixture. Column, 110 × 5 mm I.D. Cu^{2+}-charged metal chelate Superose (10 μm); carrier, 1.0 *M* sodium chloride and 5.0 m*M* imidazole in 25 m*M* phosphate buffer, pH 8.0; displacer, 15 mg/ml myoglobin in carrier; flow-rate 0.1 ml/min; temperature 22°C; feed, 3 mg cytochrome *c*, 12 mg lactoferrin and 30 mg ribonuclease A in 3 ml carrier (pH 5.0); fraction volume, 100 μl.

seen in Fig. 1, cytochrome *c* eluted ahead of the displacement train while lactoferrin and ribonuclease A were well displaced by the myoglobin. This separation resulted in the concentration of lactoferrin and ribonuclease A and produced extremely sharp boundaries between the displacement zones. In fact, this is the first multicomponent displacement separation with sharp boundaries in IMAC systems reported in the literature.

A solute movement analysis of this displacement system was carried out using experimentally determined adsorption isotherms of the feed proteins and myoglobin displacer under the displacement conditions (i.e. 5 m*M* imidazole and pH 8.0). As seen in Fig. 2, the isotherms of lactoferrin and ribonuclease A remained in the linear adsorption region while the myoglobin demonstrated non-linear behavior. Since the operating line lies above the feed protein isotherms, one would expect elution of the feed proteins. However, as shown earlier in Fig. 1, both ribonuclease A and lactoferrin are in fact displaced by the myoglobin front. Clearly, the isotherms under the carrier conditions fail to predict the displacement behavior.

In order to examine this displacement system in the absence of pH effects, the experiment was repeated at constant pH 8.0. In addition, imidazole content and pH was measured from the fractions of the column effluent using HPLC analysis and microelectrode pH probe, respectively. As expected from the solute movement analysis, shown in Fig. 2, this experiment resulted in the elution of the feed proteins ahead of the displacement train (Fig. 3). Furthermore, the desorption of the bound imidazole by the proteins resulted in the elevated imidazole concentrations between each protein zone while pH was maintained at 8.0 throughout the displacement experiment.

3.2. pH and imidazole profiles in MADC

Our laboratory has recently demonstrated that induced mobile phase modifier effects can have a profound effect on the behavior of ion-exchange displacement systems [34,35,38]. In order to

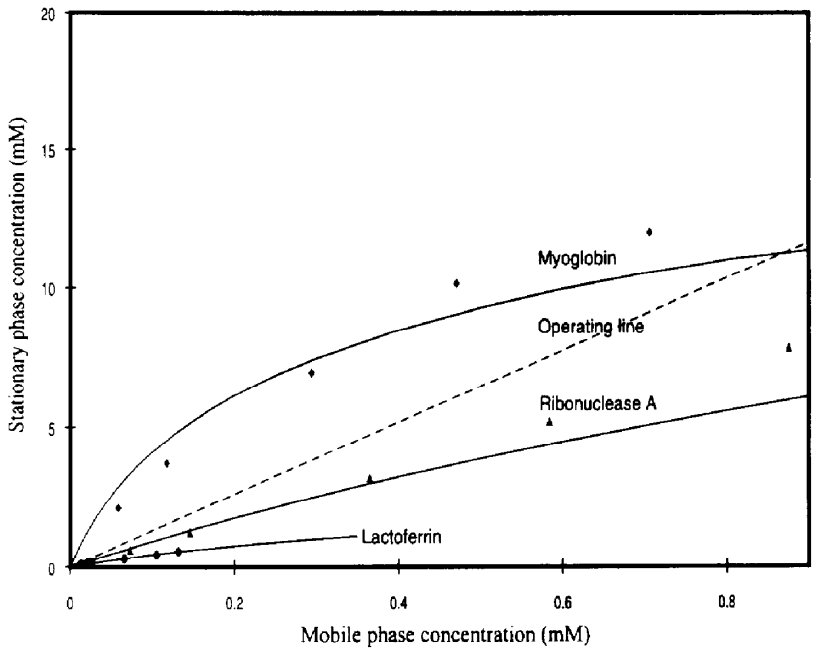

Fig. 2. Adsorption isotherms of proteins at 5 m*M* imidazole concentration and operating line. Column, 36 × 2 mm I.D. Cu^{2+}-charged metal chelate Superose; carrier, 1.0 *M* sodium chloride and 5 m*M* imidazole in 25 m*M* phosphate buffer, pH 8.0.

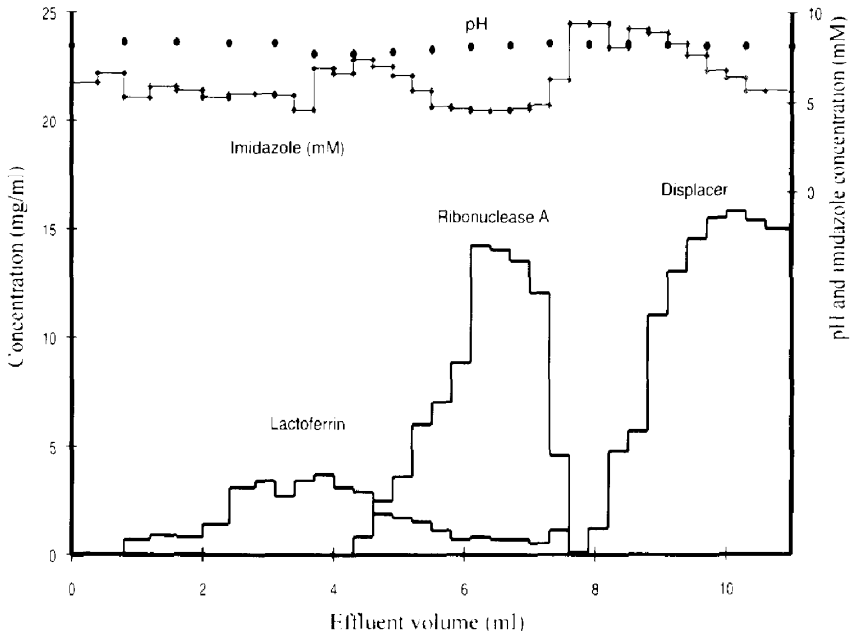

Fig. 3. Displacement chromatogram of a two-protein mixture at constant pH. Chromatographic conditions as in Fig. 1 with the exception of: feed, 12 mg lactoferrin, and 30 mg ribonuclease A in 3 ml carrier (pH 8.0). pH and imidazole concentration were measured.

better understand these metal affinity displacement separations, a detailed study of the effects of induced imidazole gradients in these systems was carried out. The displacement separation presented in Fig. 1 was repeated and the collected fractions were evaluated for their pH and imidazole content as well as their protein compositions. Again, the same pH step change was employed between the feed (pH 5.0) and displacer solutions (pH 8.0). The resulting pH and imidazole profile is shown in the displacement chromatogram presented in Fig. 4. In this separation, the feed volume was 3 ml and the column dead volume (i.e. the total void volume of the system) was 2.2 ml. While the pH change propagated through the column at the void volume of the system, the imidazole concentration profile was quite unusual. During the loading of the feed (0–3.0 ml), the imidazole concentration increased immediately after the column void volume. This initial increase in imidazole concentration can be attributed to the desorption of bound imidazole by the adsorbing feed proteins. At approximately 5.2 ml (corresponding to the pH breakthrough), the imidazole concentration abruptly decreased down to approximately 0.07 m*M*. The concentration of imidazole then increased in each successive protein displacement zone. Local imidazole concentrations of 0.3, 0.8, 1.2 and 5.0 m*M* were observed in the lactoferrin, ribonuclease A, initial myoglobin and final myoglobin displacer zones, respectively. As seen in the figure, the myoglobin concentration profile exhibited two distinct concentrations. The initial myoglobin zone was concentrated to 22 mg/ml while the final displacer emerged at the inlet concentration of 15 mg/ml. The successive step changes in imidazole concentration play an important role in the unique characteristics of this displacement separation and can be expected to increase the kinetics of the displacement process.

3.3. Cu^{2+} leakage during the displacement experiments

In order to determine the extent of Cu^{2+} leakage during the displacement experiment, the Cu^{2+} ion contents of collected fractions were

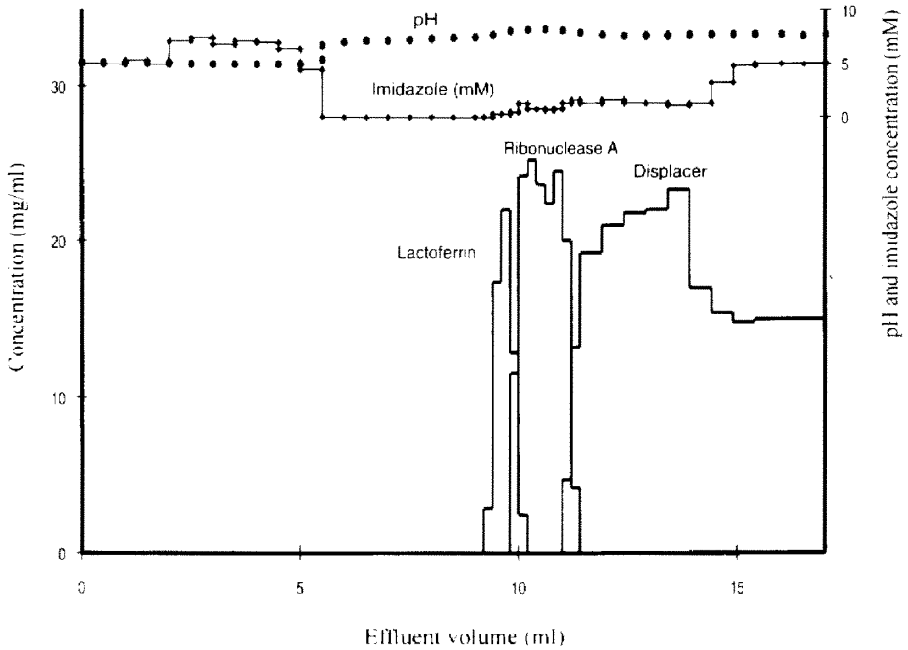

Fig. 4. Displacement chromatogram of a two-protein mixture. Chromatographic conditions as in Fig. 1 with the exception of: feed, 12 mg lactoferrin, and 30 mg ribonuclease A in 3 ml carrier (pH 5.0). pH and imidazole concentration were measured.

analyzed using atomic absorption. The amount of the Cu^{2+} ions leaching during the initial equilibration of the column was less than 0.1 ppm. During the displacement experiment a continuous leaching of 0.5 ppm was detected. The total amount of Cu^{2+} ions co-eluting in the product zones (lactoferrin and ribonuclease A) was 0.001 mg Cu^{2+} as compared to 42 mg of protein. This relatively low level of Cu^{2+} leaching had no observable effect on the reproducibility of the displacement experiments. However, in order to assure reproducibility, the Cu^{2+} ions were removed from the stationary phase after the displacement experiment and the column was reloaded with Cu^{2+} ions prior to the next experiment. Future work with alternative stationary phase ligands (e.g. nitrilotriacetic acid or trimethylethylenediamine) which bind Cu^{2+} more strongly will attempt to minimize Cu^{2+} leakage in displacement systems.

3.4. *Adsorption isotherms and frontal chromatography of imidazole*

In order to gain insight into the effect of pH on imidazole adsorption behavior, several experiments were carried out. The imidazole adsorption isotherms were measured at pH 5, 6 and 8. As seen in Fig. 5, the imidazole isotherm moved upwards with steeper initial slopes as the pH increased. Thus, the sudden step change from pH 5.0 to 8.0, carried out during the displacement experiment, resulted in a shift of the equilibrium, causing the immediate uptake of imidazole by the stationary phase material. This explains the dramatic reduction in imidazole concentration observed following the pH breakthrough in Fig. 4.

A control experiment was carried out to examine the imidazole response to a pH step change. Frontal chromatography was carried out using a pH step change from 5.0 to 8.0 in the presence of 5 m*M* imidazole in carrier. As seen in Fig. 6, the imidazole concentration profile was quite similar to that observed during the displacement experiment (Fig. 4). Again, the pH change produced a dramatic reduction of imidazole concentration (0.07 m*M*) at 5.2 ml of effluent volume and a reappearance of the imidazole front at approximately 15 ml. In fact, it is

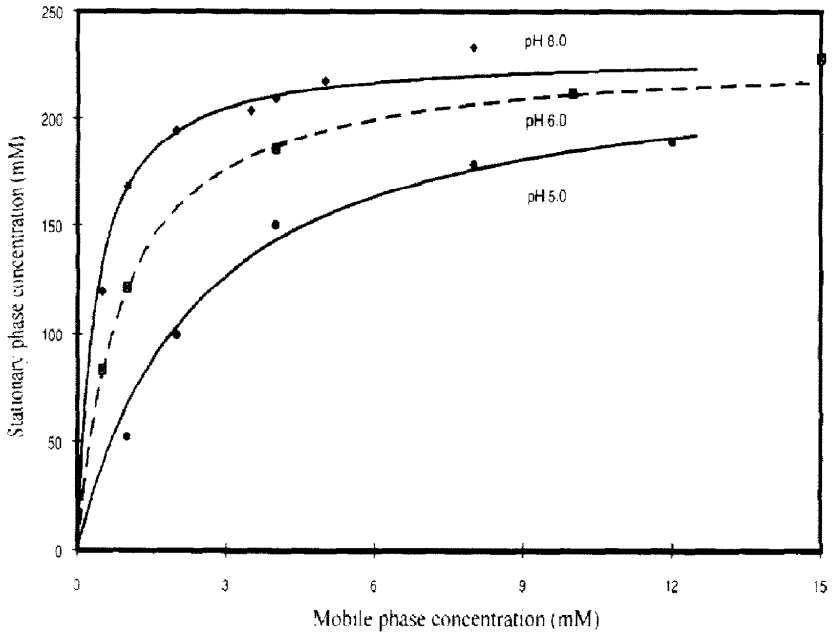

Fig. 5. Adsorption isotherms of imidazole at various pH. Column, 36 × 2 mm I.D. Cu^{2+}-charged metal chelate Superose; carrier, 1.0 *M* sodium chloride in 25 m*M* phosphate buffer, pH 5.0, 6.0 and 8.0.

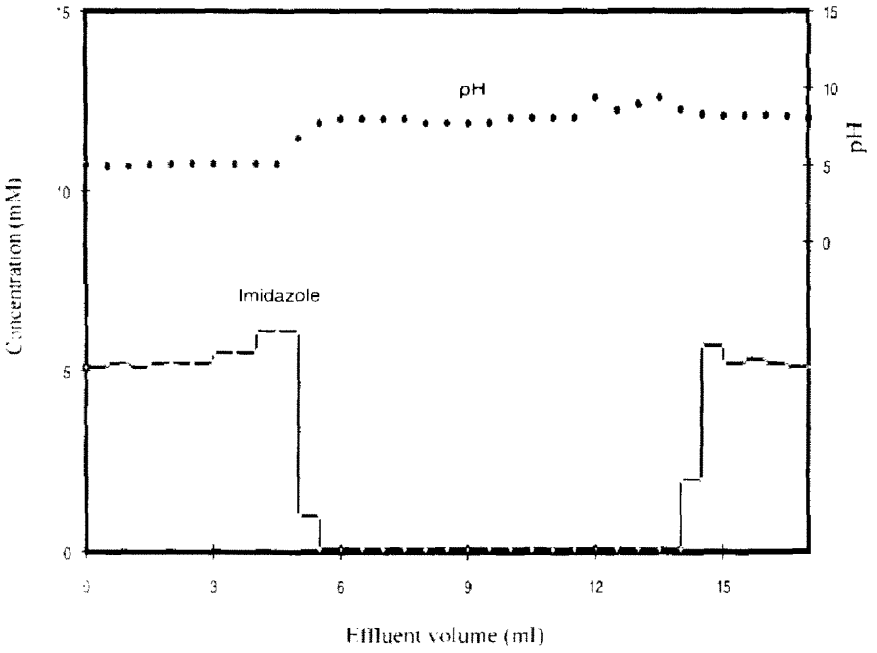

Fig. 6. Imidazole frontal chromatogram with a pH step change. Column, 110 × 5 mm I.D. Cu^{2+}-charged metal chelate Superose (10 μm); carrier, 1.0 *M* sodium chloride and 5.0 m*M* imidazole in 25 m*M* phosphate buffer, pH 5.0; temperature 22°C; feed, carrier with pH adjusted to pH 8.0; fraction volume, 500 μl.

likely that the breakthrough of imidazole at 15 ml is responsible for the initial elevated concentration of myoglobin in the displacement zone. Due to the absence of proteins, the step increase in imidazole concentrations seen in Fig. 4 were not observed in this control experiment shown in Fig. 6.

It is important to note that these complex imidazole profiles can be readily obtained in the absence of pH step change. By equilibrating the column with a large volume (250 column volumes) of 0.07 m*M* imidazole, pH 8.0, and by adding 5 m*M* imidazole to the displacer solution, very similar profiles can be obtained. Clearly, incorporating the pH step change, results in a dramatic reduction in the equilibration time and concomitant increases in product throughput.

3.5. Adsorption isotherms of proteins under induced imidazole concentrations

Fig. 7 shows the adsorption isotherms measured under the induced imidazole gradient conditions of 0.3, 0.8 and 1.2 m*M* for lactoferrin, ribonuclease A and myoglobin, respectively. In contrast to the solute movement analysis presented in Fig. 2, the operating line in this analysis was seen to intersect the effective isotherms of lactoferrin and ribonuclease A. (*Note*: the operating line was constructed using the effective myoglobin concentration at the front of the displacement zone, 22 mg/ml). Furthermore, the intersection of the operating line with these isotherms qualitatively predicted the concentrations and breakthrough volumes observed in the displacement experiment of Fig. 4. Thus, once the local imidazole concentrations in the displacement zones are known, a solute movement analysis can then be carried out using the appropriate adsorption isotherms. Clearly, a quantitative understanding of mobile phase modifier effects would facilitate the design and optimization of efficient MADC separations. We are currently developing a detailed model of the non-linear adsorption of proteins in immobilized metal affinity systems [46].

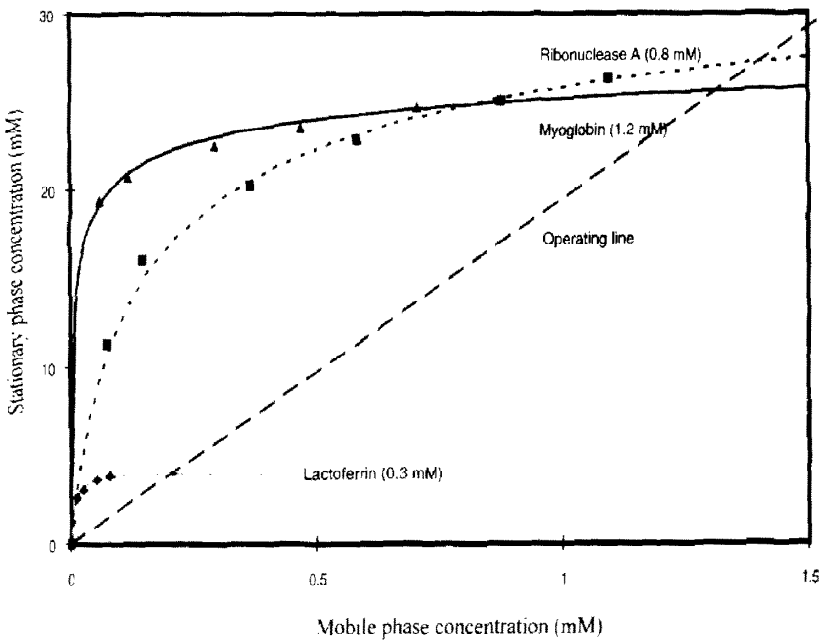

Fig. 7. Adsorption isotherms of proteins under induced imidazole concentrations and operating line. Column, 36 × 2 mm I.D. Cu^{2+}-charged metal chelate Superose; carrier, 1.0 *M* sodium chloride in 25 m*M* phosphate buffer, pH 8.0, containing 0.3, 0.8 and 1.2 m*M* imidazole for lactoferrin, ribonuclease A and myoglobin, respectively.

4. Conclusions

We have demonstrated that MADC resulted in multicomponent separations with very sharp boundaries between the zones. The mobile phase modifier imidazole was shown to exhibit complex induced gradients in these displacement systems resulting in different imidazole concentrations in each protein displacement zone. Furthermore, the induced imidazole gradient produced an elevated displacer concentration at the front of the displacer breakthrough. While adsorption isotherms measured under the initial carrier conditions were unable to predict these displacements, isotherms measured under the induced imidazole conditions qualitatively predicted the effluent displacement profiles. This work demonstrates the ability of this bioseparation technique to effect efficient multicomponent separations and illustrates the importance of mobile phase modifier effects in MADC. Work is currently underway in our laboratory to develop a theoretical framework to explain these complex IMAC displacement systems [46].

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