Metal affinity displacement chromatography of proteins

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ABSTRACT

Immobilized metal ion affinity chromatographic (IMAC) systems were employed in the displacement mode for the simultaneous concentration and purification of proteins. This work demonstrates that proteins can be employed as efficient displacers of other proteins under appropriate conditions in IMAC systems and that the displacement behavior is due to the specific interactions of the proteins with the immobilized Cu^{2+} support. The adsorption isotherms of a variety of proteins were measured and the effect of crossing adsorption isotherms on the displacement chromatographic process was investigated. Tailing observed with certain proteins in metal affinity displacement chromatography was significantly improved by the appropriate use of imidazole as a mobile phase modifier. This hybrid bioseparation technique combines the unique selectivity of IMAC with the high throughput and purity obtained in displacement chromatography.

INTRODUCTION

There is presently considerable interest in the use of group specific affinity adsorbents for the concentration and purification of biomolecules [1]. These relatively inexpensive adsorbent materials are capable of selectively interacting with classes of complementary biopolymers and have significant economic advantages over biospecific affinity systems.

While immobilized metal adsorbents have been used for the separation of small molecules since 1961 [2], it was not until the mid seventies that Porath *et al.* [3] extended the technique to the separation of proteins and nucleic acids. This powerful bioseparation tool employs the specific interaction of heavy metal ions with the amino acids histidine, cysteine, and tryptophan to effect a variety of bioseparations. Several workers have attempted to elucidate the mechanism of adsorption in immobilized metal ion affinity chromatographic (IMAC) systems [4–8]. However, the physicochemical properties of protein retention in IMAC are not well understood at present. While good correlations between surface histidine content and protein retention in IMAC have been established [9–12], differences in molecular size, amino acid composition, and net charge between various proteins can also affect retention in IMAC [13]. Porath's work has generated considerable work in the purification of biomolecules using IMAC systems in the elution and gradient mode [10,13–21].

Displacement chromatography is rapidly emerging as a powerful bioseparation method due to the high throughput and product purity associated with the process [22–24]. 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 [22–27], research on displacement chromatography with novel adsorbent materials is scarce at present [28,29].

The use of IMAC for preparative chromatographic separations requires appropriate modes of operation to enable high throughput and purity in these systems. In this report, we present experimental results on the metal affinity displacement chromatography (MADC) of proteins. This hybrid bioseparation technique combines the unique selectivity of IMAC with the high throughput and purity obtained in displacement chromatography.

EXPERIMENTAL

Materials

Bulk chelating Superose (10 μm) containing covalently bound iminodiacetic acid (IDA) and 50 × 5 mm I.D. glass columns were donated by Pharmacia LKB Biotechnology (Piscataway, NJ, USA). Bulk Bioseries strong cation exchanger (SCX) material was a gift from Rockland Technologies (Newport, DE, USA). The IDA and SCX materials were slurry packed into 50 × 5 mm and 100 × 4.6 mm I.D. columns, respectively. Tris–HCl and sodium monophosphate were purchased from Fisher Scientific (Rochester, NY, USA). Sodium chloride, ammonium sulfate, cupric sulfate, cytochrome *c* from horse heart, lysozyme, α-chymotrypsinogen A, ribonuclease A, lactoferrin (bovine), α-chymotrypsin, and imidazole were obtained from Sigma (St. Louis, MO, USA).

Apparatus

A fast protein liquid chromatography apparatus (FPLC) (Pharmacia LKB) was employed for the protein displacement experiments. This system consisted of 2 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. The system was controlled using a LCC-500-Plus controller.

Procedures

Immobilization of Cu^{2+}. The IDA columns were loaded with Cu^{2+} by sequential perfusion with 10 column volumes of 0.3 M cupric sulfate aqueous solution, pH 3.9, six column volumes of distilled water, and five column volumes of the carrier solutions described below.

Protein adsorpton isotherms. Protein adsorption isotherms were determined by frontal chromatography according to the technique of Jacobson *et al.* [30] by using a 50 × 1 mm I.D. microbore column packed with Cu^{2+} charged IMAC material.

Operation of displacement chromatograph. A schematic of the displacement chromatograph system employed in this work is illustrated elsewhere [25]. In all displacement experiments, the columns were sequentially perfused with carrier, feed, displacer, and regenerant solutions. Fractions of the column effluent were collected throughout the displacement runs and were assayed by analytical chromatography.

MADC of proteins. Displacement experiments were carried out using 50 × 5 mm I.D. columns packed with Cu^{2+} charged IMAC stationary phase materials. Unless stated otherwise, the displacer was 30 mg/ml ribonuclease A (RNase A) in the mobile phase carriers described below. The regenerant was 15 column volumes of 25 mM phosphate buffer, pH 4.0, containing 1.0 M sodium chloride. All protein displacements were carried out at a flow-rate of 0.1 ml/min at 22°C.

MADC of α-chymotrypsinogen A and cytochrome c. Displacement experiments were carried out using a feed mixture containing 2 mg α-chymotrypsinogen A and 4 mg cytochrome *c* in 2 ml of a 25 mM phosphate buffer carrier, pH 6.0, containing 1.0 M sodium chloride. Sodium chloride was added to all carrier solutions in order to quench non-specific ionic adsorption. The following experiments were carried out: displacement chromatography with RNase A displacer on a Cu^{2+} charged IMAC column; displacement chromatography with RNase A displacer on a "naked" IDA column; preparative elution chromatography on a Cu^{2+} charged IMAC column.

MADC of α-chymotrypsinogen A, cytochrome c, and lysozyme. Displacement experiments with RNase A as the displacer were carried out using a feed mixture containing either 2 or 4 mg of α-chymotrypsinogen A, 4 mg of cytochrome *c*, and 4 mg of lysozyme in 2 ml of a 25 mM phosphate buffer carrier containing 1.0 M sodium chloride at pH 5.0, 6.0, and 7.0.

MADC of α-chymotrypsinogen A, cytochrome c, and lactoferrin. MADC using RNase A as the displacer was carried out on a feed mixture containing 2 mg α-chymotrypsinogen A, 4 mg cytochrome c, and 16 mg lactoferrin in 2 ml of a 25 mM phosphate buffer carrier, pH 6.0, containing 1.0 M sodium chloride.

Effect of imidazole containing carriers on MADC. Displacement experiments with RNase A as the displacer were carried out using a feed mixture containing 4 mg of cytochrome *c* and 16 mg of lactoferrin in 2 ml of a 25 mM phosphate buffer carrier containing 1.0 M sodium chloride. The following MADC experiments were carried out: pH 6.0 carrier containing 0.2, 0.5, and 3.0 mM imidazole; pH 7.0 carrier containing 0.2 and 0.5 mM imidazole.

High-performance liquid chromatographic (HPLC) analysis. Fractions collected during the displacement chromatographic runs were analyzed by gradient elution cation-exchange chromatography. A Waters Delta Prep 3000 chromatography system with Waters 600E system controller (Millipore, Milford, MA, USA), a Waters 712 WISP auto-injector, a Waters Lambda-Max Model 481 LC spectrophotometer,

and a Waters 745B integrator were assembled to carry out HPLC analysis. Protein analysis were carried out using a 100 × 4.6 I.D. SCX column (Rockland Technologies). A 6-min linear gradient of 0–1.0 M sodium chloride in 50 mM TRIS–HCl buffer, pH 8.0, was employed. Displacement fractions were diluted 10–50 fold with the first eluent buffer and 25-μl samples were injected. The flow was 2.0 ml/min. The column effluents were monitored at 280 nm. Quantitative analyses were carried out and the data was used to construct displacement chromatograms.

RESULTS AND DISCUSSION

Preliminary results from our laboratory had indicated that IMAC systems with immobilized Ni^{2+} were able to produce displacement like behavior of proteins [31]. However, relatively high levels of Ni leakage during the displacement experiments made this system impractical for protein separations. In this manuscript, we will report on MADC using a Cu^{2+} charged IMAC system which exhibits strong interactions with the proteins and is extremely stable under the displacement conditions.

MADC of two component protein mixtures
The separation of the proteins α-chymotrypsinogen A and cytochrome c by displacement chromatography on the IMAC system was investigated using RNase A as the displacer. The carrier contained 1 M sodium chloride to suppress any residual IDA functionalities on the stationary phase material. The resulting displacement chromatogram is shown in Fig. 1. Under these conditions, α-chymotrypsinogen A eluted ahead of the displacement train while the cytochrome c was well displaced by
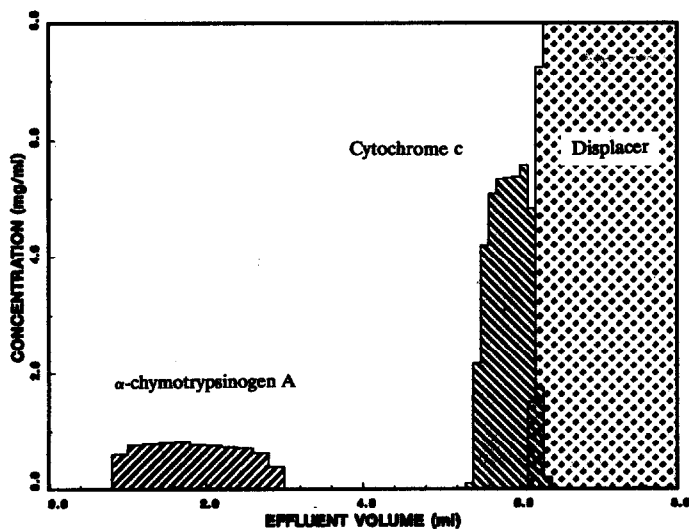

Fig. 1. Displacement chromatogram of a two-component protein mixture. Column, 50 × 5 mm I.D. Cu^{2+} charged metal chelate Superose (10 μm); carrier, 1.0 M sodium chloride in 25 mM phosphate buffer, pH 6.0; displacer, 30 mg/ml RNase A in carrier; flow-rate 0.1 ml/min; temperature 22°C; feed, 2 mg α-chymotrypsinogen A and 4 mg cytochrome c in 2 ml carrier; fraction volume, 100 μl.
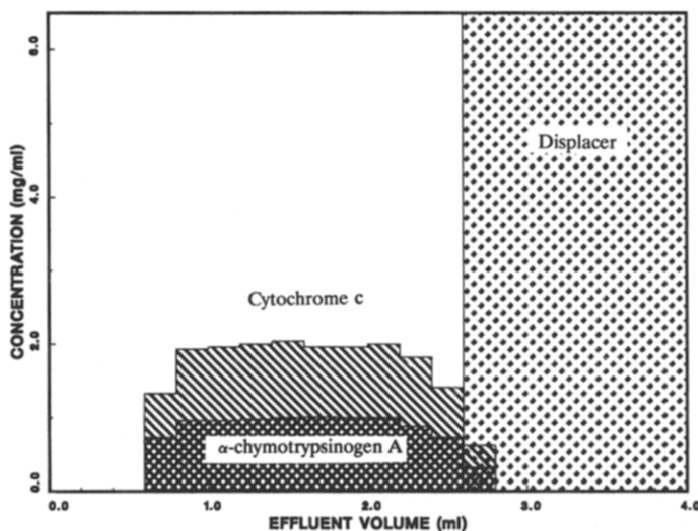
Fig. 2. Displacement chromatogram of a protein mixture on IDA support. Chromatographic conditions as stated in Fig. 1 with the exception of: column, naked IDA; fraction volume, 200 µl.

the RNase A displacer. This separation resulted in a three-fold concentration of the cytochrome c and produced a very sharp boundary between the displacement zones. A 95% recovery of the cytochrome c was achieved at a purity of 96% in this separation. The separation presented in Fig. 1 demonstrates that proteins can be employed as efficient displacers of other proteins under appropriate conditions in IMAC systems.

In order to determine whether this separation was indeed metal affinity displacement chromatography, two control experiments were carried out. The first control consisted of the same displacement separation described for Fig. 1 with the exception of using a "naked" IDA column. In the absence of the metal, the IDA column acts as a weak cation exchanger. As shown in Fig. 2, in the absence of the metal, there was complete mixing of the two feed proteins. In fact, both proteins emerged from the column at the column dead time, due to the action of the high salt mobile phase carrier. Thus, the displacement behavior demonstrated in Fig. 1 was indeed due to the specific interactions of the proteins with the immobilized Cu^{2+}.

In order to dramatize the action of the displacer in these systems, the MADC experiment was repeated in the absence of the displacer. Fig. 3 demonstrates that while the α-chymotrypsinogen A peak was unaffected by the lack of displacer, the cytochrome c zone emerged in the classic overloaded elution state. In contrast to the displacement shown in Fig. 1, the preparative elution presented in Fig. 3 is characterized by significant tailing and dilution of the cytochrome c. These results demonstrate the ability of the displacer to both concentrate and reduce the tailing of solutes in preparative metal chelate chromatography.

MADC of three component protein mixtures
In order to increase the complexity of the feed mixture, we examined the linear
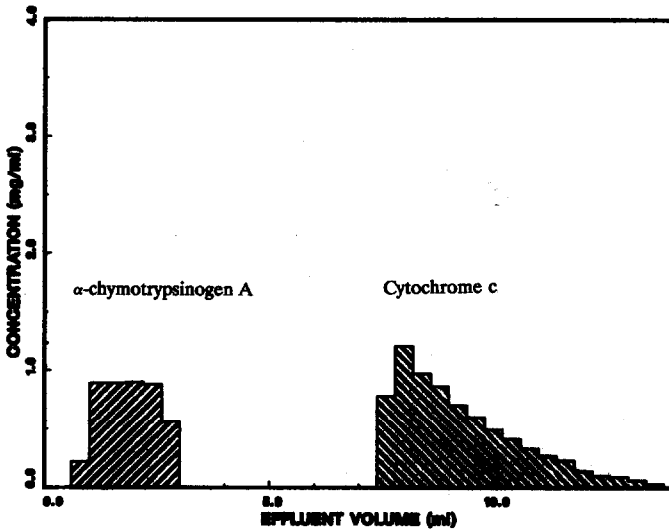
Fig. 3. Preparative elution chromatogram of a two-component protein mixture. Chromatographic conditions as stated in Fig. 1 with the absence of the displacer; fraction volume, 400 μl.

elution chromatographic behavior of a variety of proteins known to exhibit affinity for IMAC systems [9,10,13]. Affinities ranged from very strongly retained proteins such as myoglobin and concanavalin A; to intermediate affinity proteins such as RNase A, lactoferrin, and lysozyme; to relatively low affinity proteins such as cyto-
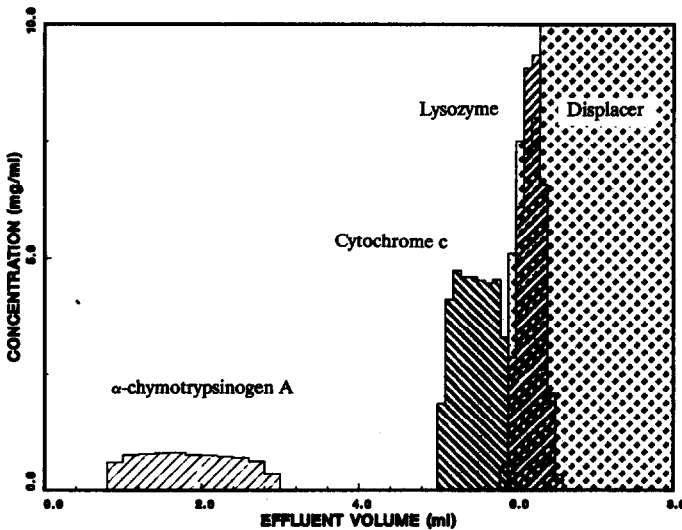
Fig. 4. Displacement chromatogram of a three-component protein mixture. Chromatographic conditions as stated in Fig. 1 with the exception of feed, 2 mg α-chymotrypsinogen A, 4 mg cytochrome c, and 4 mg lysozyme in 2 ml carrier.

chrome c and α-chymotrypsinogen A. Preliminary displacement experiments were carried out using myoglobin as the displacer. However, the limited solubility of the myoglobin (15 mg/ml) as well as the difficulty of regenerating the IMAC column following perfusion with this protein made it impractical as a displacer.

Under linear elution conditions, RNase A had a consistently stronger retention than the proteins lysozyme, cytochrome c and α-chymotrypsinogen A. Accordingly, MADC experiments were carried out using RNase A to displace a feed mixture containing these three proteins. The resulting chromatogram shown in Fig. 4, indicates that α-chymotrypsinogen A eluted ahead of the displacement train while cytochrome c was well displaced during the separation. On the other hand lysozyme emerged from the column immediately following the breakthrough of the displacer. Since RNase A exhibited a stronger retention than lysozyme under linear elution conditions this displacement behavior was indicative of the presence of crossing adsorption isotherms for these two proteins [32].

In an attempt to achieve a successful displacement, the experiment was repeated at pH 5.0 and 7.0. In both cases, the lysozyme emerged from the system after the breakthrough of the displacer. The displacement chromatogram for the pH 5.0 experiment is shown in Fig. 5. Under these conditions, the cytochrome c displacement zone was not fully developed and the emergence of the lysozyme in the displacer zone produced a distortion of the front.

Clearly, in order to develop appropriate displacement conditions, more detailed information on the non-linear adsorption behavior of these proteins was required. Accordingly, the adsorption isotherms of various proteins was measured using microbore frontal chromatography. The resulting adsorption isotherms are presented in Fig. 6. As expected from the displacement results shown in Figs. 4 and 5, the ad-
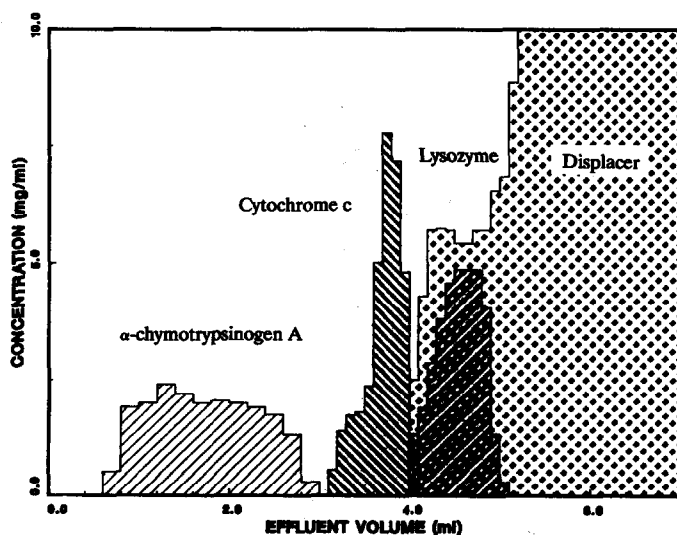

Fig. 5. Displacement chromatogram of a three-component protein mixture. Chromatographic conditions as stated in Fig. 1 with the exception of: feed, 4 mg each of α-chymotrypsinogen A, cytochrome c, and lysozyme in 2 ml carrier; carrier, 1.0 M sodium chloride in 25 mM phosphate buffer, pH 5.0.
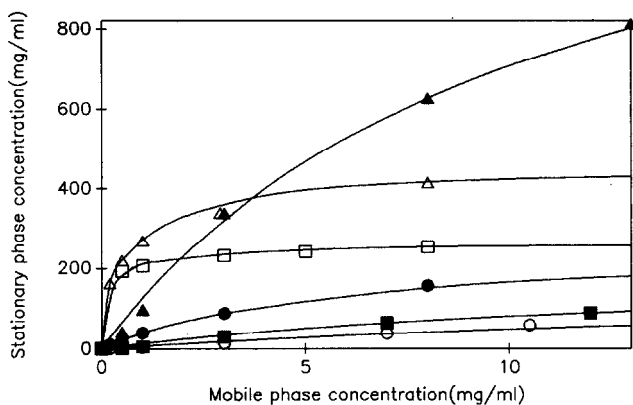
Fig. 6. Protein adsorption isotherms. Column, 50 × 1 mm I.D. Cu^{2+} charged metal chelate superose; carrier, 1.0 M sodium chloride in 25 mM phosphate buffer, pH 6.0. ▲ = Lysozyme; △ = RNase A; □ = lactoferrin; ● = cytochrome c; ■ = α-chymotrypsin; ○ = α-chymotrypsinogen A.

sorption isotherms of lysozyme and RNase A crossed under these carrier conditions.

All protein adsorption isotherms measured on the IMAC system satisfied the displacement requirement for concave downward isotherms [22]. The characteristic high loading capacity of IMAC systems is clearly evident in this data. These adsorption capacities are comparable to those of Belew et al. [33] when one takes into account differences in definitions of stationary phase volume. The relatively high capacity of IMAC systems as compared to ion-exchange and reversed-phase systems may be in part responsible for the ability to carry out displacements on relatively
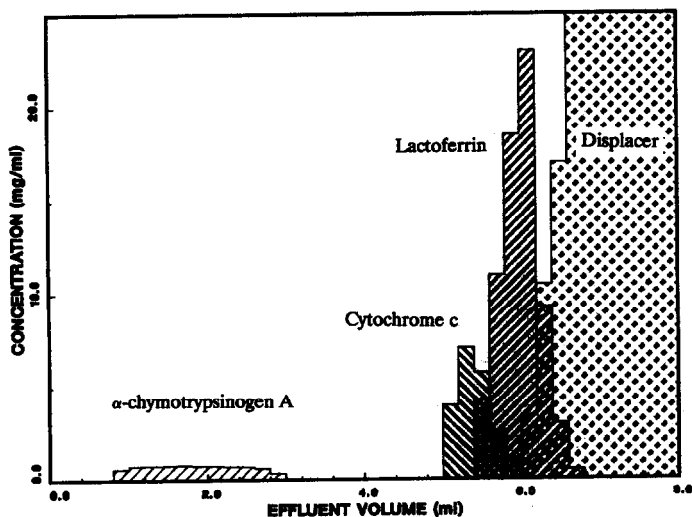

Fig. 7. Displacement chromatogram of a three-component protein mixture. Chromatographic conditions as stated in Fig. 1 with the exception of: feed, 2 mg α-chymotrypsinogen A, 4 mg cytochrome c, and 16 mg lactoferrin in 2 ml carrier; fraction volume, 200 μl.

short IMAC columns. In fact, the combination of high binding capacity and corresponding shorter column lengths may produce additional economic advantages for MADC systems.

As seen in Fig. 6, the adsorption isotherms of RNase A and lactoferrin are well separated at pH 6.0. Accordingly, we investigated the displacement separation of α-chymotrypsinogen A, cytochrome c, and lactoferrin using 30 mg/ml RNase A as the displacer. The resulting chromatogram shown in Fig. 7 indicates that the α-chymotrypsinogen A eluted from the system ahead of the displacement zones of cytochrome c and lactoferrin. While this separation was able to produce a three-fold concentration of the displaced proteins, the boundaries separating the displacement zones were quite diffuse. This is in contrast to the results presented in Fig. 1 where the system produced sharp boundaries. Clearly, the lactoferrin is responsible for the non-ideal behavior in this system. In fact, this type of tailing behavior has been observed in previous research in our laboratory on reversed-phase displacement systems [34].

Imidazole mobile phase modifier effects

While the exact mechanism for this tailing is not known, it is postulated that the tailing in this system is due to either slow desorption kinetics and/or steric effects due to the difference in the molecular weight of the lactoferrin (76 kilodalton) and the other proteins (12–24 kilodalton). In order to minimize the deleterious effect of slow desorption kinetics on the displacement behavior of these systems we employed imidazole as a mobile phase modifier while retaining RNase A as the displacer. Experiments were carried out with 0.2, 0.5, and 3.0 mM imidazole in the carrier. While 0.2 mM imidazole had no apparent effect on the system, 3.0 mM imidazole resulted in the
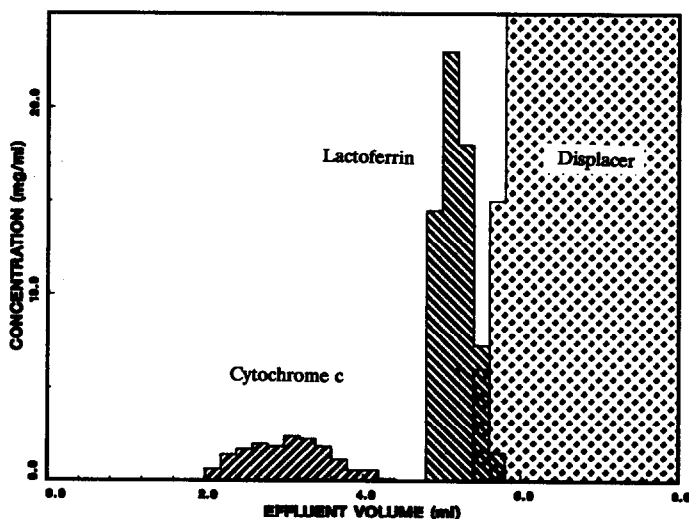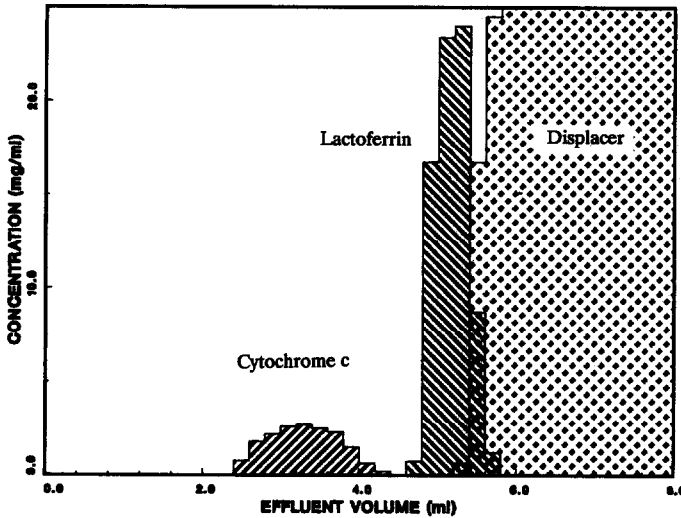

Fig. 8. Displacement chromatogram of a two-component protein mixture. Chromatographic conditions as stated in Fig. 1 with the exception of: carrier, 0.5 mM imidazole and 1.0 M sodium chloride in 25 mM phosphate buffer, pH 6.0; feed, 4 mg cytochrome c and 16 mg lactoferrin in 2 ml carrier; fraction volume, 200 μl.

Fig. 9. Displacement chromatogram of a two-component protein mixture. Chromatographic conditions as stated in Fig. 1 with the exception of: carrier, 0.2 mM imidazole and 1.0 M sodium chloride in 25 mM phosphate buffer, pH 7.0; feed, 4 mg cytochrome c and 16 mg lactoferrin in 2 ml carrier; fraction volume, 200 μl.

elution of both cytochrome c and lactoferrin ahead of the RNase A displacer front. In contrast, the addition of 0.5 mM imidazole to the carrier resulted in significant sharpening of both the lactoferrin tail and the displacer front as shown in Fig. 8. Under these conditions, cytochrome c eluted from the system ahead of the displaced lactoferrin.

In an effort to produce a successful displacement of both cytochrome c and lactoferrin, the experiment was carried out at pH 7.0 with various imidazole concentrations. Fig. 9 shows the separation achieved at pH 7.0 using a 0.2 mM imidazole carrier. Again, the displacement experiment resulted in elution of the cytochrome c and successful displacement of the lactoferrin. Interestingly, a lower imidazole concentration was required to achieve sharp displacement of the lactoferrin at pH 7.0 as compared to pH 6.0. While these results do not establish the exact mechanism for the tailing observed in this system, they indicate that dispersion in MADC systems can be minimized by the appropriate use of imidazole as a mobile phase modifier.

CONCLUSIONS

In this report we have demonstrated that IMAC systems can be employed in the displacement mode for the simultaneous concentration and purification of proteins. This work demonstrates that proteins can be employed as efficient displacers of other proteins under appropriate conditions in IMAC systems and that the displacement behavior is due to the specific interactions of the proteins with the immobilized Cu^{2+} support. The adsorption isotherms of a variety of proteins were measured and the effect of crossing adsorption isotherms on the displacement chromatographic process was investigated. Finally, tailing observed with certain displaced proteins was signif-

icantly improved by the addition of appropriate amounts of imidazole to the carrier solution. We are currently developing alternative displacer for MADC and applying this powerful hybrid bioseparation technique for the direct purification of biopharmaceuticals from complex fermentation broths.

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