Journal of Chromatography A, 1217 (2010) 1249–1254

Contents lists available at ScienceDirect

Journal of Chromatography A

journal homepage: www.elsevier.com/locate/chroma

The effect of feed composition on the behavior of chemically selective displacement systems

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ARTICLE INFO

Article history:
Received 29 July 2009
Received in revised form
30 November 2009
Accepted 3 December 2009
Available online 4 January 2010

Keywords:
Selective displacement chromatography
Protein purification
Ion exchange
Feed composition effect

ABSTRACT

In this paper we examine whether adding a more retained protein to the feed will mitigate displacer–protein interactions in the column, thus affecting the displacement modality that occurs (chemically selective vs. traditional displacement chromatography). STD-NMR experiments were carried out to probe displacer–protein interactions for the chemically selective displacer chloroquine diphosphate and the results indicated that this displacer only had measurable interactions with the protein α-chymotrypsinogen A. For a two component feed mixture containing ribonuclease A and α-chymotrypsinogen A, the separation resulted in the displacement of ribonuclease A, with the more hydrophobic α-chymotrypsinogen A remaining on the column. On the other hand, when the experiment was repeated with cytochrome *c* added to the feed, all three feed proteins were displaced. Column simulations indicated that the combination of sample self-displacement occurring during the introduction of the feed, along with the dynamics of the initial displacement process at the column inlet was responsible for this behavior. These results indicate that for this class of hydrophobic-based selective displacers, in order for the protein to be selectively retained, the protein should be the most strongly retained feed component.

1. Introduction

Displacement chromatography is a powerful separation technique that enables the simultaneous concentration and purification of biomolecules from complex mixtures in a single step [1]. Displacement chromatography has been successfully employed for the purification of proteins using hydroxyapatite [2,3], hydrophobic interaction [4], and ion-exchange chromatographic systems [5–15]. In particular, ion-exchange displacement chromatography has attracted significant attention as a powerful technique for the purification of biomolecules [8,9,16]. While previous work has employed high molecular weight displacers for protein purification in ion-exchange systems [1–3,10,17–19], our laboratory has been actively involved in the development of low molecular mass displacers [8,12–16,20].

Selective displacement chromatography utilizes the design of the displacer to move a selected solute or solutes back into the displacement zone, separating them from the displacement train. There are two classes of selective displacers, mass action and chemically selective displacers. The behavior of mass action selective displacers can be predicted from dynamic affinity plots [20] derived from the steric mass action (SMA) isotherm [21]. These displacers typically have an affinity for the resin that is less than that of the selected protein that is pulled back into the displacement zone [20]. In contrast, in chemically selective displacement chromatography [22,23], desired biomolecules will be selectively displaced even though the operating conditions (displacer concentration, salt concentration, etc.) do not meet the requirements of mass-action displacement. For these chemically selective displacers the SMA isotherm would predict that both proteins would be displaced ahead of the displacement zone, even though experimentally one protein is pulled back into the displacement zone.

Recent developments in selective displacement chromatography include high-throughput studies aimed at identifying novel selective displacers [22,23], development of fluorescent displacers for ease of online separation process monitoring [24], and utilizing surface plasmon resonance [25] to investigate displacer–protein interactions [26]. In addition, a recent study employed saturation transfer difference (STD) NMR [27] to investigate binding events between chemically selective displacers and proteins [26]. This technique takes advantage of intra-protein spin diffusion, inter-molecular dipolar coupling mechanisms and ligand exchange between the bound and free state to characterize binding

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doi:10.1016/j.chroma.2009.12.020

interactions. In that study it was directly verified (by NMR and MD simulations) that chemically selective displacement occurs by a selective binding between the displacer and protein, producing a protein–displacer complex which has a higher affinity for the resin than the protein or displacer alone. This introduces an orthogonal dimension of selectivity into the displacement separation.

In this paper we examine whether adding a more retained protein to the feed will mitigate displacer–protein interactions in the column, thus affecting the displacement modality that occurs (chemically selective vs. traditional displacement chromatography). STD-NMR experiments are carried out to probe displacer–protein interactions and column experiments are conducted using two and three component protein feed mixtures. Finally, column simulations are carried out to examine the state of the column at various stages of the displacement process.

2. Experimental

2.1. Materials

Bulk HP Sepharose SP cation-exchange resin was donated by GE Healthcare (Uppsala, Sweden). Resin was packed into a GE Healthcare Tricorn column (100 mm × 5 mm i.d.). A reversed-phase column (C4) Jupiter (5 μm, 300 Å, 250 mm × 4.6 mm i.d.) were purchased from Phenomenex (Torrance, CA, USA). Sodium phosphate monobasic, sodium phosphate dibasic, horse heart cytochrome *c* (H-CytC), ribonuclease A (RNaseA), α-chymotrypsinogen A (α-chyA), trifluoroacetic acid (TFA), hydrochloric acid, glacial acetic acid, sodium hydroxide, and chloroquine diphosphate salt were purchased from Sigma–Aldrich (St. Louis, MO, USA). Sodium chloride was purchased from Fisher Scientific (Pittsburgh, PA, USA). Acetonitrile was purchased from Acros Organic (Geel, Belgium). NMR sample tubes (5 mm thin wall, 600 MHz) were purchased from Wilmad Lab Glass (Buena, NJ). Deuterium oxide (99.96% purity) was purchased from Cambridge Isotope Laboratories Inc. (Andover, MA, USA). All water used in the experiments was de-ionized and filtered using the Millipore (Billerica, MA, USA) Milli-Q Ultrapure Water Purification system.

2.2. Apparatus

Freeze drying of NMR samples was done on a Labconco Freeze Dry System from Labconco (Kansas City, MO, USA). NMR spectra were obtained using a Bruker 600 MHz spectrometer (Billerica, MA, USA) equipped with a cryogenically cooled 5 mm triple-resonance probe head with *z*-axis gradients. NMR data acquisition and analysis was carried out using Topsin 2.1 software package also from Bruker.

All displacement experiments were carried out using a Waters 590 HPLC pump (Waters Corporation, Milford, MA, USA) connected to a chromatography column via a model C10W 10-port valve (Valco, Houston, TX, USA). The data was acquired using a QuickLog (Version 1.4) chromatography workstation (Strawberry Tree Inc., Sunnyvale, CA, USA). The column effluent was monitored using a Waters 484 UV–Vis absorbance detector set to 214 nm. Fractions of the column effluent were collected using RediFrac Fraction Collector (Pfizer-Phamacia, New York, NY, USA).

Protein and displacer analysis of the displacement separation fractions collected was carried out using a Waters 600 multisolvent delivery system controlled by a Millenium chromatography manager. Samples were introduced using a Waters 717 WISP autoinjector and detected using a Waters 996 photodiode array detector. PEEK tubing was used for connecting the different components of the instruments used for the displacement experiment and fraction analysis.

2.3. Procedures

2.3.1. STD-NMR sample preparation and protocol

Protein and displacer stock solutions for NMR analysis were first suspended in 50 mM sodium phosphate buffer at pH 6 or 50 mM sodium acetate buffer at pH 5. Solutions were pH adjusted and then lyophilized. The resulting pellets were suspended in deuterium oxide and lyophilized two times for two cycles before a final suspension was made in deuterium oxide. NMR samples were prepared by diluting the stock solutions to their final analysis concentrations (10 mM for displacers and 0.2 mM for proteins) in D_2O-based buffer and then loading them into the NMR sample tubes.

The saturation transfer difference nuclear magnetic resonance (STD-NMR) protocol is described in detail elsewhere [27]. Briefly, methyl protons within the protein were directly irradiated using a train of highly selective RF pulses, such that ligand (displacer) resonances were not directly perturbed. The extended saturation period provided by the RF pulse train allowed for spin diffusion to spread the saturation of methyl proton magnetization to all protons throughout the entire protein. However, ligand protons within close proximity (<5 Å) to the protein surface also experienced the saturation effect due to inter-molecular dipolar coupling. Transfer of magnetization from the protein to the ligand resulted in an increase in signal intensity for ligand protons at the protein interaction surface relative to those distant. A control spectrum was used where the RF irradiation is applied to a spectral region devoid of ligand and protein resonances. The final STD spectrum was obtained after subtracting the control from the experimental spectra. Ligand exchange between the bound and free states during this saturation period is also a key element of this technique and allowed for measurement of the STD perturbations in the free ligand. Ligand concentrations were present in excess relative to the protein to ensure that saturated ligand remained in the unbound state for detection. A filter was also applied to remove the protein signal, leaving the spectrum of ligand resonances that underwent an STD effect.

Mixtures of 50:1 (10–0.2 mM) of displacer and protein, respectively, were analyzed utilizing the experimental parameters stated below. Additional control STD spectra were acquired on individual displacer and protein samples of the same concentration analyzed in mixtures. A saturation period of 2.4 s was applied as a train of 8 ms gaussian pulses applied at 40 ms intervals with a 50 Hz B_1 field. The experimental and control spectra were acquired in an interleaved fashion with the saturating frequency centered at −0.25 ppm and −10 ppm, respectively. Spectra were acquired using a 2 s acquisition time with a total of 4736 scans. An 80 ms T1rho filter was used to remove residual signal from the protein.

2.3.2. Displacement chromatography

All displacement experiments were conducted at room temperature (25 °C) at a flow rate of 0.2 ml/min. The HP Sepharose SP column was initially equilibrated with the carrier solution (50 mM NaH_2PO_4 at pH 6). The column was then sequentially perfused with feed, displacer and regenerant solutions. Chloroquine diphosphate (5 mM) was used as the displacer. Appropriate fractions (100–200 μl) of column effluent were collected during the displacement experiments and diluted (1–20-fold) for subsequent analysis of protein and displacer as described below. The column was regenerated by sequentially perfusing with 10 column volumes of 2.0 M KCl + 0.1 M KOH, 4 column volumes of 15% glacial acetic acid, and 5 column volumes of 2.0 M NaCl. The column was then equilibrated with the carrier solution for 10 column volumes.

2.3.3. Protein and displacer analysis by RPLC

Protein and displacer analyses from the displacement separations were performed on a reversed-phase (C4) Jupiter column (250 mm × 4.6 mm i.d. column). Fractions were diluted 2–20-fold with the carrier solution, and 20 μl were injected. Linear gradients were carried out with an A buffer of deionized water with 0.1% TFA and a B buffer of 90% ACN, 9.9% deionized water and 0.1% TFA (all v/v). A linear gradient was carried out from 20 to 50% B in 25 min. The flow rate was 1 ml/min, the column effluent was monitored at 280 nm.

3. Results and discussion

Recent work from our lab has demonstrated that chemically selective displacement occurs when the displacer selectively interacts with one of the proteins in the feed, resulting in its elution from the column after the breakthrough of the displacer [25,26]. In this paper we examine whether adding a more retained protein to the feed will mitigate displacer–protein interactions, thus affecting what displacement modality occurs in the column, chemically selective or traditional displacement chromatography. The displacer (chloroquine diphosphate) and proteins used in this study (RNaseA, α-chyA and H-CytC) were selected from previous parallel high-throughput screening of large chemical libraries [25,28,29]. While RNaseA and α-chyA have similar retention times in cation-exchange, H-CytC is more strongly retained. In addition, both RNaseA and H-CytC have significantly lower surface hydrophobicity than α-chyA, as indicated by their relative elution times in HIC [25]. Before column experiments were carried out, it was first necessary to examine the interactions of the displacer chloroquine diphosphate with the proteins.

3.1. NMR for the identification of displacer–protein interactions

Saturation transfer difference nuclear magnetic resonance (STD-NMR) experiments were employed to investigate whether the chemically selective displacer chloroquine diphosphate binds selectively to any of the model proteins and to provide insight into which displacer atoms were directly involved in the binding event.

The proton peak assignments for the displacer were obtained using the 2D NMR experiments described in Section 2 using standard methodologies [30]. A given protein was mixed with the displacer at a 1:50 ratio prior to STD analysis. Single component solutions at the same concentrations used for the displacer–protein mixture were also analyzed as controls to verify the method and results. The STD-NMR spectra along with proton peak assignments for the selective displacer chloroquine diphosphate are shown in Fig. 1.

As can be seen in the figure, the proton peak assignments for chloroquine diphosphate (A) were made as follows: 1 and 2 on the diethyl groups off the amine tail, 3 through 6 on the amine tail, 7 on the methyl group off the amine tail, 8 on the quinoline meta to the chlorine, 9 and 10 on the quinoline ortho to the chlorine, 11 on the quinoline meta to the amine tail and 12 on the quinoline ortho to the amine tail. As can be seen from the STD spectra given in B, C and D, a binding signal was clearly observed for the α-chyA/chloroquine mixture while the RNaseA/chloroquine and H-CytC/chloroquine mixtures produced a relatively weak signal. The protein control experiments (F, G and H) showed no signal aside from a small signal seen for H-CytC (H), which coincides with sodium acetate at 1.91 ppm. However, a small amount of signal was seen for the displacer control experiment (E). This small residual STD signal is attributed to the direct saturation of the displacer, resulting in a slight amount of STD signal in the mixtures (B, C and D) as well as the control (E). If one compares the spectra from C and E, it

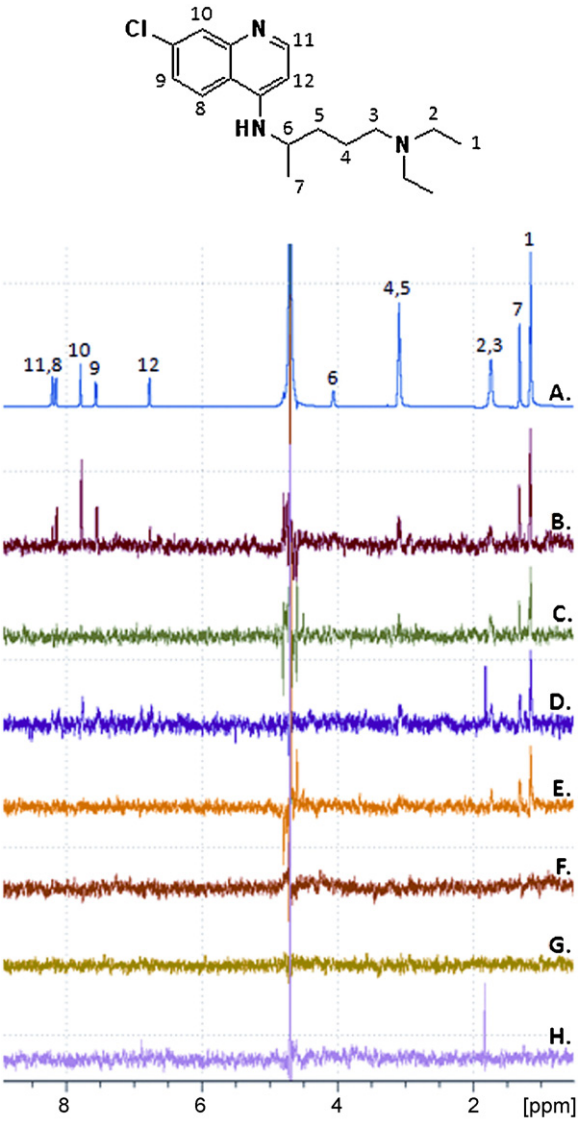

Fig. 1. STD-NMR results for the protein/displacer experiments with proton peak assignments shown for chloroquine diphosphate: (A) chloroquine; (B) α-chyA/chloroquine mixture; (C) RNaseA/chloroquine mixture; (D) H-CytC/chloroquine mixture; (E) chloroquine NMR control; (F) α-chyA NMR control; (G) RNaseA NMR control; (H) H-CytC NMR control.

is clear that the signal obtained for the RNaseA/chloroquine mixture can be attributed primarily to the direct saturation of the displacer. Comparing the spectra from D, E and H, it can be seen that the signal obtained in the aliphatic ppm range for the HCytC/chloroquine mixture can be attributed primarily to the direct saturation of the displacer and the presence of sodium acetate. However, a small binding signal was seen in the aromatic ppm range for the H-CytC/chloroquine mixture. In contrast, when comparing the spectra from B and E, the signal obtained for the α-ChyA/chloroquine mixture is significantly greater than that detected for the displacer control, confirming displacer–protein binding. It should also be noted that the signal seen in the aromatic ppm range for the α-chyA/chloroquine mixture (B) was significantly larger than that from the H-CytC/chloroquine mixture (D), suggesting the signal seen in the later mixture is from a relatively weak or insignificant binding event.

Upon closer analysis of the α-chyA/chloroquine mixture (B) signal it can be seen that the binding is centered on the aromatic portion of the quinoline group, with only a small amount of

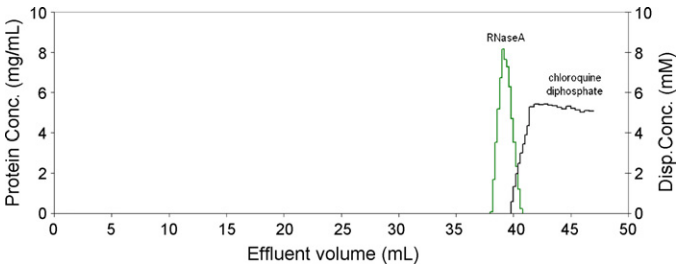

Fig. 2. Displacement of RNaseA and α-chyA on HP Sepharose SP using 5 mM chloroquine diphosphate. Column: 100 mm × 4.6 mm i.d.; carrier: 50 mM sodium phosphate buffer, pH 6.0; flow rate: 0.2 ml/min; 100 μl fractions; protein: 10 mg of each α-chyA and RNaseA in carrier.

signal detected for protons within the amine tail. This would suggest that the binding event is occurring primarily due to hydrophobic interactions, as has been shown for other molecules from this class of selective displacers [17,19,26]. Thus, the STD-NMR experiments demonstrate that chloroquine diphosphate only binds to the protein α-chyA.

3.2. Column selective displacement chromatographic separations (two-component feed mixture)

A column displacement separation was carried out using the displacer chloroquine diphosphate with a two component feed mixture containing RNaseA and α-chyA (note: displacements of this binary protein mixture using high affinity displacers, resulted in displacement of both proteins for a wide range of displacer concentrations [25]). As can be seen in Fig. 2, this separation resulted in the displacement of RNaseA, with the more hydrophobic α-chyA remaining on the column. This is expected since the STD-NMR results indicated that the hydrophobic moiety of the displacer interacts with α-chyA and not with RNaseA.

These results indicate that column selective displacement separations can indeed be successfully carried out using chloroquine diphosphate, which is a relatively inexpensive and commercially available compound.

3.3. Column selective displacement chromatographic separations (three-component feed mixture)

It was hypothesized that adding a more retained protein to the feed would create a scenario in the column that could mitigate the binding of chloroquine diphosphate to α-chyA and reduce the chemically selective displacement effect. In order to check this hypothesis, the displacement was repeated now adding H-CytC to the feed. This experiment (Fig. 3) resulted in the displacement of all three feed proteins. Thus, while chloroquine diphosphate was able to act as a selective displacer for the two protein feed experiment, it

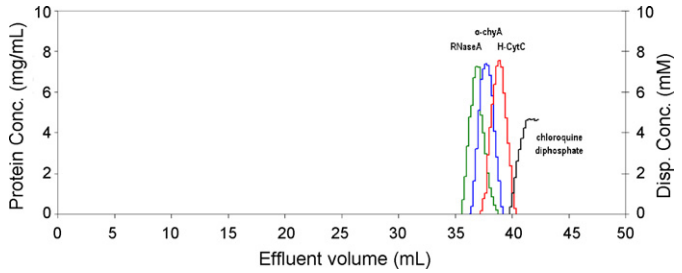

Fig. 3. Displacement of RNaseA, α-chyA, and H-CytC on HP Sepharose SP using 5 mM chloroquine diphosphate. Column: 100 mm × 4.6 mm i.d.; carrier: 50 mM sodium phosphate buffer, pH 6.0; flow rate: 0.2 ml/min; 100 μl fractions; protein: 10 mg of each α-chyA, RNaseA, and H-CytC in carrier.

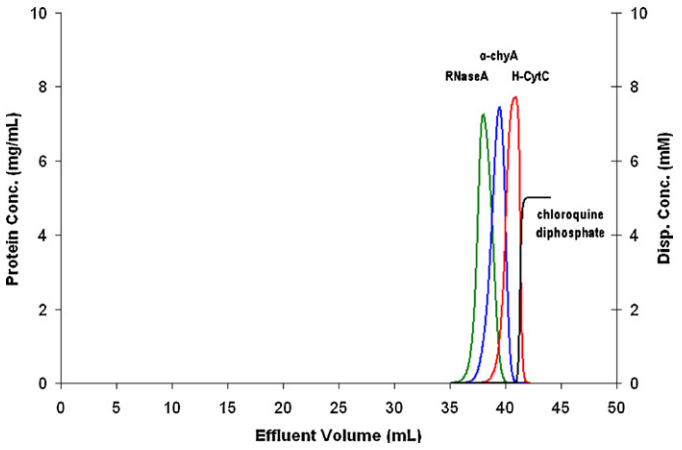

Fig. 4. Simulated column outlet profile.

was not an effective selective displacer of α-chyA when three proteins were in the feed. As indicated by the STD-NMR experiments (Fig. 1), chloroquine diphosphate had minimal interactions with H-CytC. Thus, during the three protein displacement experiment (Fig. 3), chloroquine diphosphate acted as an effective displacer of the most strongly retained H-CytC which in turn, displaced the other two proteins.

In order to better understand the behavior of this three protein feed displacement, a series of column simulations were carried out. The chromatographic model employed in these simulations used the steric mass action (SMA) isotherm [21] in concert with a solid film linear driving force and an equilibrium dispersive mass transport model [31]. The chromatographic model employed along with the resin and protein parameters has been previously described [21,25,32–36]. A simulation was carried out to examine the final effluent profile of this three protein displacement and the results (Fig. 4) were in good agreement with the experimental profile (Fig. 3).

Simulations were also carried out to examine the state of the column at various stages of the displacement process. Figs. 5 and 6 shows the simulated concentration profiles for the three proteins in the column at the end of the feed loading stage and after the introduction of 0.1 column volumes of displacer solution, respectively. In these figures the column mobile phase concentrations are indicated by the dashed curves and the stationary phase concentrations are given by the solid curves. As can be seen in these figures, the combination of sample self-displacement which occurred during the loading of the feed (Fig. 5) along with the initial displacement

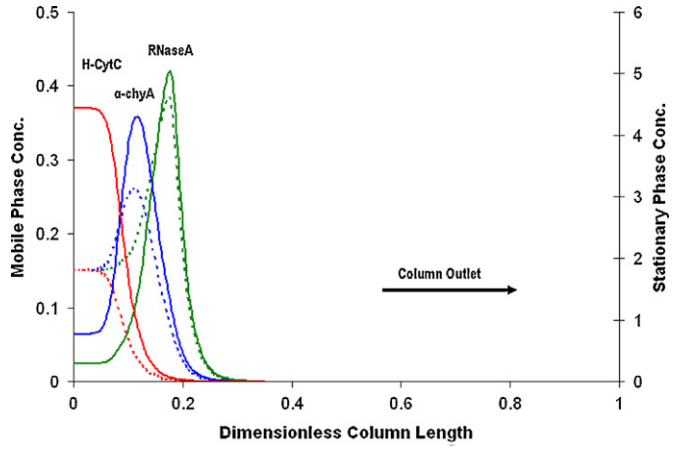

Fig. 5. Simulation of the column at the end of the feed loading.

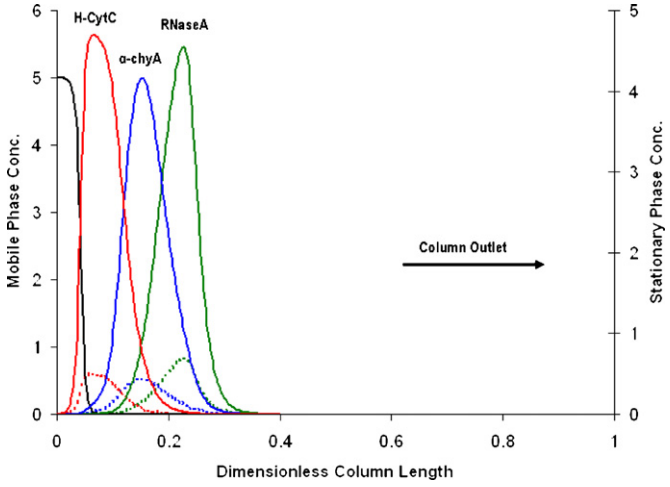

Fig. 6. Simulation after the introduction of 0.1 CV of displacer.

process at the column inlet (Fig. 6) resulted in the placement of H-CytC between the α-chyA and the displacer. This would make it difficult for the selective displacer to come into direct contact with α-chyA due to the presence of H-CytC in the column, thus mitigating the selective displacement effect.

Column experiments were also performed where varying amounts of displacer were added to the feed mixture prior to the column experiment to determine if a selective displacement could be established with this three protein feed. The hypothesis here being that if the chloroquine diphosphate/α-chyA complex was sufficiently strong, this would result in the movement of α-chyA back into the displacer zone during the column experiment. However, the results (not shown) indicated that this did not occur for any of the concentrations of displacer added to the feed and that all three proteins were displaced ahead of the displacer breakthrough. This indicates that the chloroquine diphosphate/α-chyA interaction was not stable enough to selectively retain the α-chyA when a more strongly retained protein (H-CytC) was present in the feed.

These results indicate that adding a more retained protein to the feed can mitigate displacer–protein interactions, thus affecting the displacement modality occurring in the column. Further these results indicate that the elution order of the feed components should be considered in addition to any displacer–protein interactions when designing chemically selective displacements.

4. Conclusion

In this paper we examine whether adding a more retained protein to the feed will mitigate displacer–protein interactions in the column, thus affecting the displacement modality that occurs (chemically selective vs. traditional displacement chromatography). STD-NMR experiments were carried out to probe displacer–protein interactions for the chemically selective displacer chloroquine diphosphate and the results indicated that this displacer only had measurable interactions with the protein α-chyA. For a two component feed mixture containing RNaseA and α-chyA, the separation resulted in the displacement of RNaseA, with the more hydrophobic α-chyA remaining on the column. On the other hand, when the experiment was repeated with H-CytC added to the feed mixture, all three of the feed proteins were displaced. Column simulations indicated that the combination of sample self-displacement occurring during the introduction of the feed, along with the dynamics of the initial displacement process at the column inlet, resulted in the placement of H-CytC between α-chyA and the displacer, eliminating the binding between the selective displacer and α-chyA. When varying amounts of displacer were added to the feed prior to the column experiment, all three proteins were displaced ahead of the displacer breakthrough. This indicates that the chloroquine diphosphate/α-chyA interaction was not sufficiently stable to selectively retain the α-chyA in the presence of the more strongly retained H-CytC in the feed. Thus, for this class of hydrophobic-based selective displacers, in order for the protein to be selectively retained, the protein should be the most strongly retained feed component. However, it should be possible to selectively retain a protein in the displacer zone when it is not the most strongly bound feed component, provided that the displacer–protein complex is sufficiently stable. Clearly the binding kinetics associated with chemically selective displacement (displacer–protein, protein–resin, displacer–resin) play a crucial role in optimizing chemically selective displacements in a column setting. Active work is currently underway in our laboratory to enable the selective retention of any protein in the feed mixture, regardless of elution order. In order to accomplish this, we are defining optimal kinetic regimes for effective chemically selective displacement.

Acknowledgements

This work was partially supported by NIH Grant 5R01 GM047372 and NSF Grant CBET-0730830. The assistance of Andrew Levine and Blaine Trafton in some of the experiments is also gratefully acknowledged. The authors would also like to acknowledge Prof. Scott A. McCallum of the Biology Department at Rensselaer Polytechnic Institute for his assistance in the STD-NMR experiments.

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