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Chemically Selective Displacers for High-Resolution Protein Separations in Ion-Exchange Systems: Effect of Displacer−Protein Interactions

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Displacer lead compounds were selected from a commercially available database to identify potential selective displacers for a binary protein mixture in ion exchange chromatography. Parallel batch screening experiments were carried out with these lead compounds to study the effect of displacer concentration on the relative amounts of the proteins displaced. Experiments were conducted with a mixture containing ribonuclease A and α-chymotrypsinogen A which exhibited very similar retention behavior under linear gradient conditions. The batch displacement results indicated that most of these lead compounds were indeed selective for displacing ribonuclease A. In fact, one of these displacers exhibited extremely high selectivity, displacing essentially all of the ribonuclease A while displacing minimal α-chymotrypsinogen A at a displacer concentration of 10 mM. These results were validated under column conditions, with the ribonuclease A being displaced and the α-chymotrypsinogen A remaining on the column after the displacer breakthrough. In order to examine whether this was mass action or chemically selective displacement, an affinity ranking plot based on the Steric Mass Action (SMA) model was generated, and the results confirmed that this was not a mass action displacement. In order to test the hypothesis that displacer protein binding was playing a role in these separations, Surface Plasmon Resonance (SPR) was carried out. The results suggest that while the chemically selective displacer interacted with α-chymotrypsinogen A, it had no interaction with ribonuclease A. The ability to exploit protein displacer binding in concert with appropriate displacer resin affinities opens up new possibilities for creating selective displacement systems.

Displacement chromatography has been successfully employed for the purification of proteins using hydroxyapatite,[1,2] hydrophobic interaction,[3] and ion exchange chromatographic systems.[4−10] Ion exchange displacement chromatography in particular has attracted significant attention as a powerful technique for the purification of biomolecules.[7,8,11] A wide variety of classes of displacers, such as polyelectrolytes,[8] polysaccharides,[12] low-molecular-mass dendrimers,[10] amino acids,[13] antibiotics,[14] and aminoglycoside-polyamine,[15] has been identified for protein separations in ion-exchange systems. The application of low-molecular-mass displacers has attracted attention due to several distinct operational advantages,[7] most significantly the ability of some of these molecules to act as mass action displacers.[6,16]

Selectivity in ion exchange systems can be exploited in various ways. A number of studies have addressed the effect of eluting salt type and concentration on protein selectivity for ion exchange systems. The results indicated that both co-ion and counterion have an effect on protein selectivity.[17−23] Kopaciewicz and coworkers[17] demonstrated that while the cation slightly altered the selectivity, the anion could have a significant effect on the retention

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time as well as the selectivity in anion exchange systems. In addition, changes in the eluting salt type and gradient mode have been shown to significantly enhance the selectivity of closely related variants.[21]

Mass action displacement chromatography has been investigated for protein separations.[16,24] Gallant et al. developed operating regime plots from the Steric Mass Action (SMA) model[25] to determine the operating conditions for displacement chromatography and employed numerical simulations to examine the behavior of mass-action displacement systems. Kundu et al.[16] then applied operating regime plots to develop column displacement separations and demonstrated that under appropriate operating conditions the protein of interest could be displaced, while impurities with lower affinity were eluted in the induced salt gradient and higher retained impurities were desorbed after the breakthrough of the displacer front. Shukla et al. have successfully employed mass action displacement chromatography for the purification of an antigenic vaccine protein.[6]

Chemically selective displacement chromatography is a recently developed displacement separation technique.[26,27] In this process, 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. This new chromatographic mode has the potential to provide high product purity and yield for challenging separation problems without the requirement of carefully choosing and controlling operating conditions. Although it has been found that displacer chemistry can have a significant effect on the selectivity of displacement systems,[26,27] it is not clear what the chemically selective displacement mechanism is and how to improve the selectivity of these systems.

Previous work in our laboratory has demonstrated that parallel batch screening can be applied in concert with quantitative structure efficacy models for the identification of mass action displacers.[28] In the present work, a modified parallel batch screening displacement technique is employed to identify selective displacers for ribonuclease A (RNase A) over α-chymotrypsinogen A (α-chy A) from a group of displacers in a commercially available chemical database (Matrix Scientific, Columbia, SC). The most promising batch results are then validated under column conditions. An affinity ranking plot is then used to examine if these are mass action displacements. Finally, SPR is carried out to test the hypothesis that displacer protein binding is playing an important role in these selective displacement systems.

THEORY

In an ion exchange displacement operation, it has been shown by a stability analysis derived using the Steric Mass Action Model[25] that the order of elution of feed components in a displacement train can be determined from the following expression

$$\left(\frac{K_a}{\Delta}\right)^{1/\nu_a} < \left(\frac{K_i}{\Delta}\right)^{1/\nu_i} \quad (1)$$

where Δ is the partition ratio of the displacer (Q_d/C_d); Q_d and C_d are the concentrations of the displacer on the stationary and mobile phases, respectively; "a" and "i" refer to components in the displacement train, ν is the characteristic charge or the number of interaction sites each molecule has with the stationary phase material, and K is the equilibrium constant of the reaction between the solute and the salt counterions on the surface. The left-hand side of eq 1, defined as the dynamic affinity[29] of the component "a" (λ_a), is the affinity of the solute for the stationary phase under the equilibrium condition that exists in an isotachic displacement train.

Displacer affinity ranking plots[30] originate from the rearrangement of the definition expression of dynamic affinity as follows:

$$\log(\lambda) = \frac{1}{\nu}\log(K) - \frac{1}{\nu}\log(\Delta) \quad (2)$$

Therefore, affinity ranking plots can be obtained by plotting the dynamic affinity (λ) against the operational parameter (Δ) using the linear SMA parameters. On these plots, lower values of Δ correspond to higher values of solute concentrations and result in higher values of λ. In a mass-action selective displacement, the experiment is operated under conditions where the dynamic affinity of the displacer falls between the dynamic affinities of the different solutes, resulting in the displacement of the less strongly bound protein, and the eventual desorption of the more strongly bound protein after the displacer breakthrough. Affinity ranking plots thus provide a means of comparing the affinities of different solutes over a range of operating conditions and give a prediction of when mass-action selective displacement will occur.

EXPERIMENTAL SECTION

Materials. Bulk strong cation exchange stationary phase HP Sepharose SP was donated by GE Healthcare (Uppsala, Sweden). Ribonuclease A (RNase A), α-chymotrypsinogen A (α-chy A), sodium phosphate (monobasic), sodium phosphate (dibasic), sodium chloride, sodium hydroxide, acetonitrile, and trifluoroacetic acid (TFA) were purchased from Sigma-Aldrich (St. Louis, MO). Symmetry 300 3.5 μm C4 RPLC column (100 mm × 4.6 mm) was a gift from Waters (Milford, MA). All potential selective displacer molecules were purchased from Matrix Scientific (Columbia, SC) except chloroquine diphosphate that was from Sigma-Aldrich.

Equipment. Parallel batch screening experiments were carried out on 96-well Multiscreen-HV Durapore membrane-bottomed plates (Millipore, Bedford, MA). The supernatants from the wells after equilibration with the displacer were recovered using a vacuum manifold (Millipore). Displacement experiments were carried out using a Waters 590 HPLC pump (Waters) connected to a chromatography column via a Model C10W 10-port valve (Valco, Houston, TX). The column effluent during displacement experiments was monitored using a model 484 UV−vis absorbance detector (Waters). Fractions of the column effluent were collected using a LKB 2212 Helirac fraction collector (LKB,

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Bromma, Sweden). Analytical chromatographic experiments were carried out using a model 600 multisolvent delivery system, a 712 WISP auto injector, and a 484 UV−vis absorbance detector controlled by a Millennium chromatography software manager (Waters). Biacore 3000, C1 series sensor chips, and coupling reagents (*N*-ethyl-*N*′-(3-dimethylaminopropyl)carbodiimide (EDC), *N*-hydroxysuccinimide (NHS), and ethanolamine-HCl) were purchased from Biacore International AB (Uppsala, Sweden).

Parallel Batch Screening. Potential displacers were tested by a well-established parallel batch screening protocol.[15,28,31] The bulk stationary phase (SP Sepharose HP) was first washed twice with deionized water and then three times with the buffer (50 mM sodium phosphate, pH 6) and was allowed to equilibrate for 2 h. After gravity settling of the stationary phase, the supernatant was removed, and 3.0 mL of the remaining stationary phase slurry was equilibrated with 36 mL of protein solution containing 5 mg/mL of the total protein concentration in equal amounts (RNase A and α-chy A are both 2.5 mg/mL) in a carrier buffer, pH 6, at 20 °C. The protein solution was equilibrated with the resin for 5 h to attain complete equilibration, during which the stationary phase was allowed to gravity settle. Upon settling, the supernatant was removed, and the protein content in the supernatant was determined by RPLC at 280 nm. The mass of the protein adsorbed on the stationary phase was then calculated by mass balance. For the screening experiments, 120 μL of different initial concentrations of a displacer solution was added to 10 μL aliquots of the stationary phase slurry with bound protein. (Note that (1) a different displacer molecule and concentration were employed for each vial to enable parallel screening and that (2) 20 μL total volume of 1:1 stationary phase slurry was used to distribute 10 μL aliquots stationary phase in order to minimize the operating error.) The system was equilibrated for 5 h. After equilibrium was achieved, the supernatant was removed and tested by RPLC for the determination of the "percentage of protein displaced".

Column Displacement Experiment. The self-packed SP Sepharose HP column (100 mm × 5 mm) was initially equilibrated with the carrier buffer (50 mM sodium phosphate, pH 6) and then sequentially perfused with the feed solution containing 35 mg of RNase A and α-chy A followed by the 10 mM displacer solutions. Two hundred microliter fractions were collected for subsequent analysis of the proteins and displacers. Following the infusion of displacer, column regeneration was carried out using a solution of 750 mM NaOH and 25% acetonitrile. The carrier buffer flow rate in the column displacement experiment was 0.2 mL/min, and the effluent was monitored at 280 nm.

Protein and Displacer Analysis. Linear gradient RPLC with a Symmetry 300 C4 3.5 μm (100 mm × 4.6 mm) column was used to evaluate the amount of α-chy A and RNase A in the supernatant of the parallel batch screening experiments as well as the protein and displacer amounts in the column experiment. A linear gradient of 25−100% (v/v) buffer B was carried out in 5 min [buffer A: 0.1% (v/v) trifluoroacetic acid (TFA) in deionized water; buffer B: 90% (v/v) acetonitrile and 0.1% (v/v) TFA in deionized water]. The flow rate was 1 mL/min, and the column effluent was monitored at 280 nm.

Determination of SMA Parameters. The characteristic charge and equilibrium constant for each protein and displacer were estimated using retention data obtained from isocratic elution experiment as described elsewhere.[32] Isocratic experiments for each protein/displacer were carried out at pH 6.0 with 5 salt concentrations chosen between 50 mM sodium phosphate and 50 mM sodium phosphate + 950 mM sodium chloride. The obtained SMA parameters were then used to generate affinity ranking plots as described in the theory section.

Protein Immobilization. RNase A and α-chy A were immobilized onto C1 Sensor chips using standard amine coupling protocol. Sodium phosphate (50 mM), pH 6, was used as a running buffer. The carboxymethyl surface in the flow cell was activated with a 35 μL injection of a 1:1 (v/v) ratio of 0.4 M EDC and 0.1 M NHS mixture at 5 μL/min flow rate and then coupled with 50 μL of 1 mg/mL protein dissolved in running buffer. The protein solution was reapplied until no increase in signal response was observed, indicating that the sensor chip channel was saturated with the protein. The remaining activated groups were then blocked with a 35 μL injection of 1.0 M ethanolamine, pH 8.2.

Analysis of Protein−Displacer Interaction. An *N*′1′-(4-methylquinolin-2-yl)ethane-1,2-diamine dinitrate solution (10 mM) (representing the chemically selective displacer) was prepared in running buffer and diluted to make 6 different concentrations ranging from 10 to 0.3 mM. A spermidine solution (10 mM) (representing the nonselective displacer) was prepared in running buffer and diluted to make 5 different concentrations ranging from 10 to 0.6 mM. A displacer solution (25 μL) of each concentration of each displacer was injected at 25 μL/min flow rate. For cases where the protein−displacer complexes did not dissociate and the signal responses did not come back to baseline within a reasonable time frame, 1 M NaCl was used as a regenerating buffer after each interaction experiment was complete. All resulting sensorgrams were processed by first subtracting the binding response recorded from the control flow cell (prepared by exposure to all immobilization buffers without any protein) to minimize baseline drift as well as systematic noise.

RESULTS AND DISCUSSIONS

In the present work, RNase A and α-chy A were used as the protein pair for this selective displacer study. These two proteins were selected because they exhibit very similar affinities in ion exchange but different affinities in hydrophobic interaction chromatography (HIC), thus they represent proteins with similar ion-exchange retention but different hydrophobicities. As described in the Experimental Section, parallel batch experiments were carried out at various displacer concentrations using resin that had been first equilibrated with a binary mixture containing 5 mg/mL of the total protein concentration in equal amounts. Before discussing the selective displacers, it is instructive to examine the behavior of a typical high-affinity displacer, such as neomycin sulfate. As can be seen in Figure 1, this molecule displaces the same amount of both of these proteins at any given displacer concentration. These results clearly illustrate that this displacer exhibits no selectivity for this protein pair under the batch displacement conditions.

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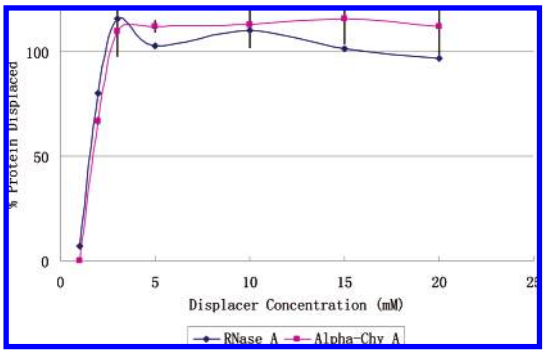

Figure 1. Batch screening result of neomycin sulfate on SP Sepharose HP.

Based on previous data from our lab for this protein pair,[28] an early virtual screening was carried out using an SVM classification model with an in-house support vector regression program. This model successfully resulted in an enrichment of an initial large database from Matrix Scientific containing 15155 molecules. The net result of this classification model was that 7 molecules were predicted to be selective from the database, and their chemical structures are presented in Figure 2.

The 7 chosen molecules were evaluated for their ability to displace the two proteins using the parallel batch screening protocol described in the Experimental Section (Note: *N*′4′-{7-chloro-2[2-(2-phenyl)vinyl]quinolin-4-yl}-*N*′1′,*N*′1′- diethylpentane-1,4-diamine was not evaluated since it was not soluble in the carrier.). The experiments were carried out under a range of displacer concentrations, and the resulting values of the % protein displaced were used to generate the plots shown in Figure 3.

In contrast to the results shown in Figure 1 for the high affinity displacer, most of the displacers evaluated in Figure 3 exhibited some selectivity for this binary protein mixture under these batch conditions. In fact, *N*′1′-(4-methylquinolin-2-yl)ethane-1,2-diamine dinitrate (Figure 3(a)) showed significant selectivity. This displacer also exhibited "exclusivity" since at concentrations less than 10

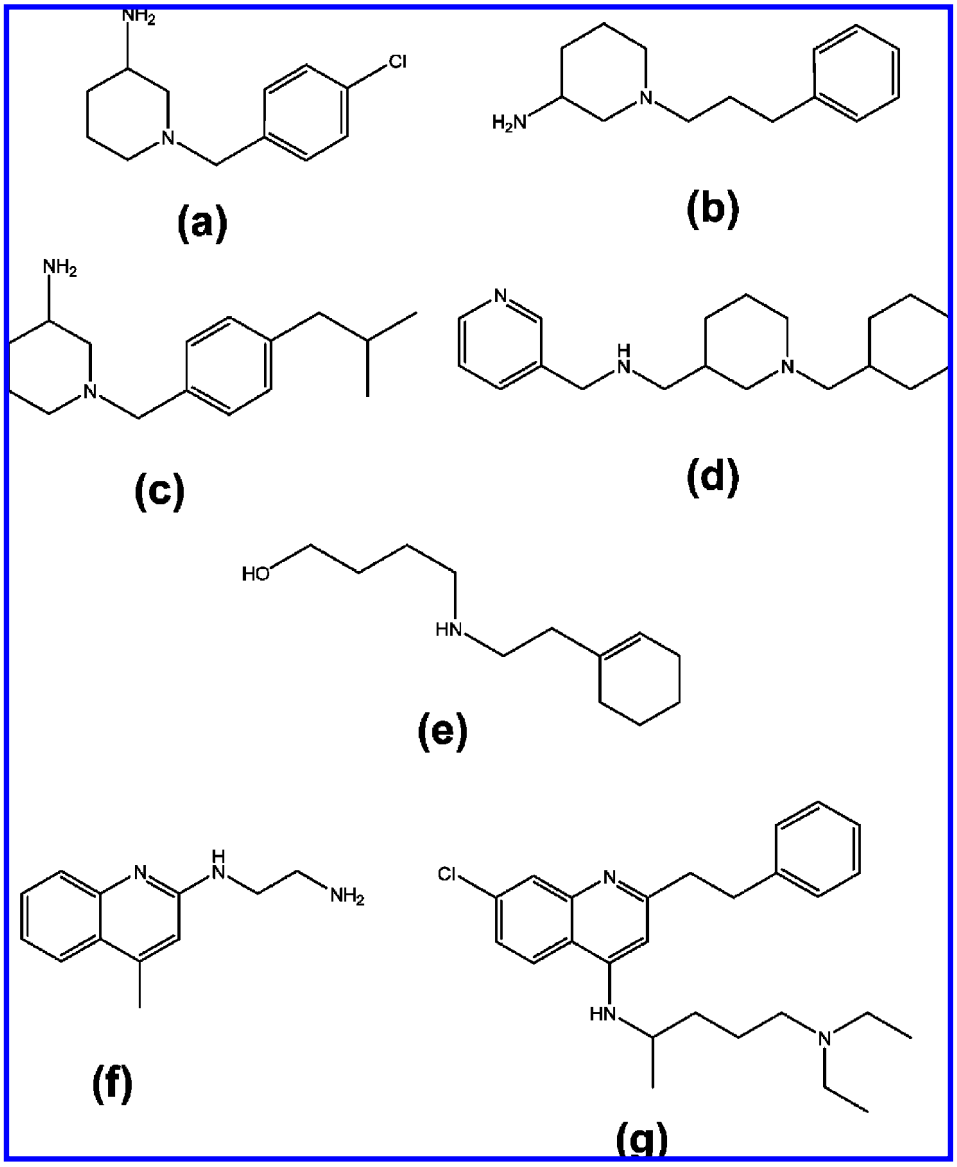

Figure 2. Molecular structures (a) 1-(4-chlorobenzyl)piperidin-3-amine sulfate; (b) 1-(3-phenylpropyl)piperidin-3-amine sulfate; (c) 1-(4-isobutylbenzyl)piperidin-3-amine sulfate; (d) [(1-cyclohexylpiperidin-3-yl)methyl](pyridin-3-ylmethyl)amine sulfate; (e) 4-(2-cyclohex-1-enylethyl-amino)butan-1-ol sulfate; (f) *N*′1′-(4-methylquinolin-2-yl)ethane-1,2-diamine dinitrate; and (g) *N*′4′-{7-chloro-2[2-(2-phenyl)vinyl]quinolin-4-yl}-*N*′1′,*N*′1′-diethylpentane-1,4-diamine.

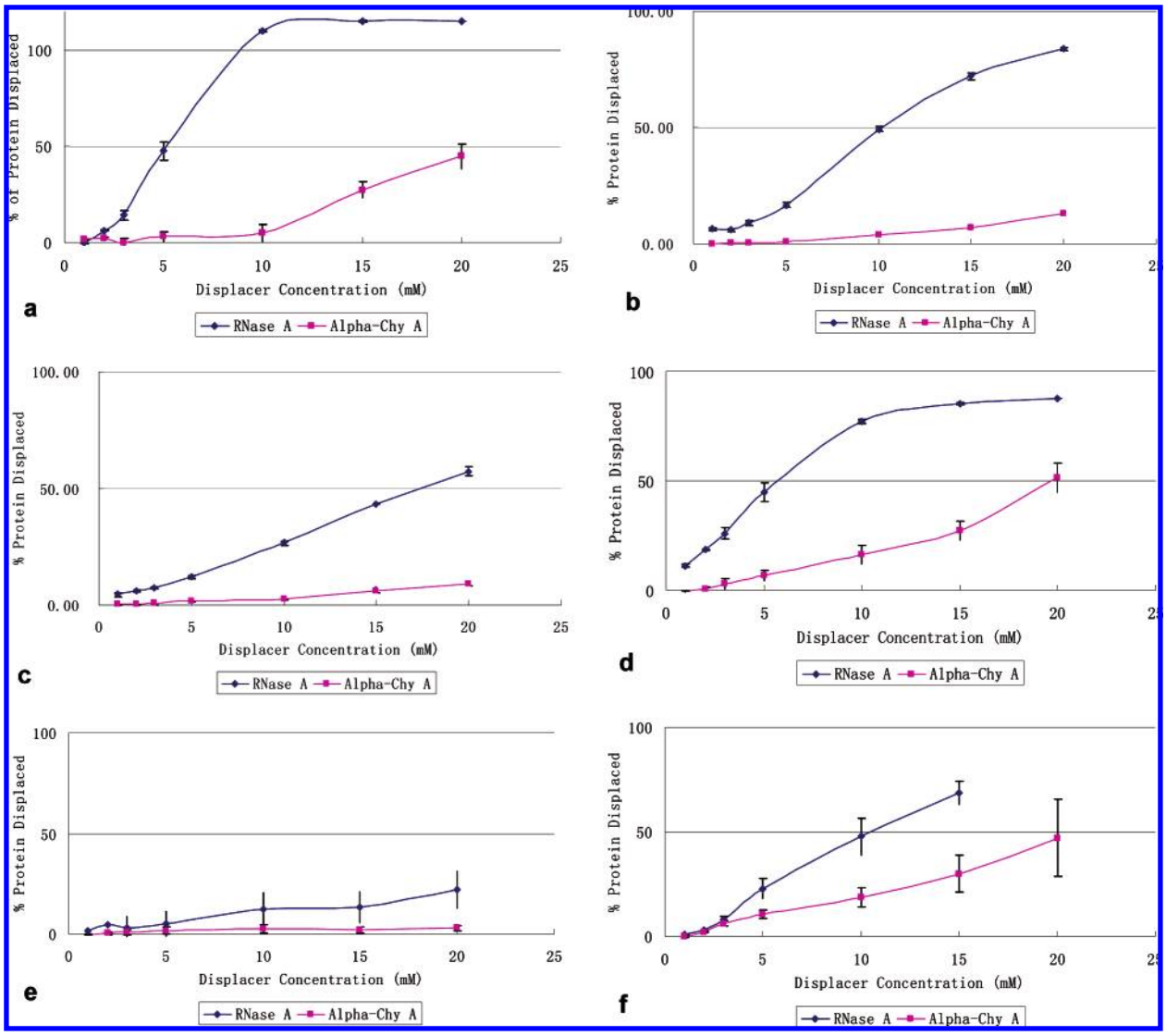

Figure 3. Batch screening result of (a) *N*′1′-(4-methylquinolin-2-yl)ethane-1,2-diamine dinitrate; (b) 1-(4-isobutylbenzyl)piperidin-3-amine sulfate; (c) 1-(4-chlorobenzyl)piperidin-3-amine sulfate; (d) [(1-cyclohexylpiperidin-3-yl)methyl](pyridin-3-ylmethyl)amine sulfate; (e) 4-(2-cyclohex-1-enylethylamino)butan-1-ol sulfate; and (f) 1-(3-phenylpropyl)piperidin-3-amine sulfate.

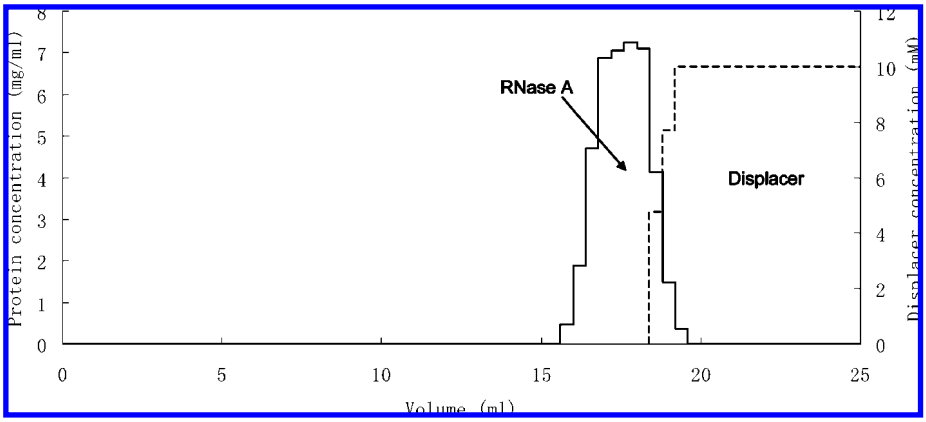

Figure 4. Selective displacement of RNase A on HP Sepharose SP using 10 mM *N*′1′-(4-methylquinolin-2-yl)ethane-1,2-diamine dinitrate. Column: 100 mm × 5 mm i.d. HP Sepharose SP; carrier: 50 mM phosphate, pH 6.0; protein: 35 mg of α-chy A and RNase A; flow rate: 0.2 mL/min.

mM, this molecule exhibited minimal displacement of α-chy while successfully displacing large amounts of RNase A. Although these batch experiments are subject to some experimental error, the qualitative results clearly demonstrate that selectivity was achieved.

In order to examine if the results obtained from the parallel batch displacement experiments were applicable to column conditions, a column displacement was carried out with 10 mM of the displacer lead compound *N*′1′-(4-methylquinolin-2-yl)ethane-

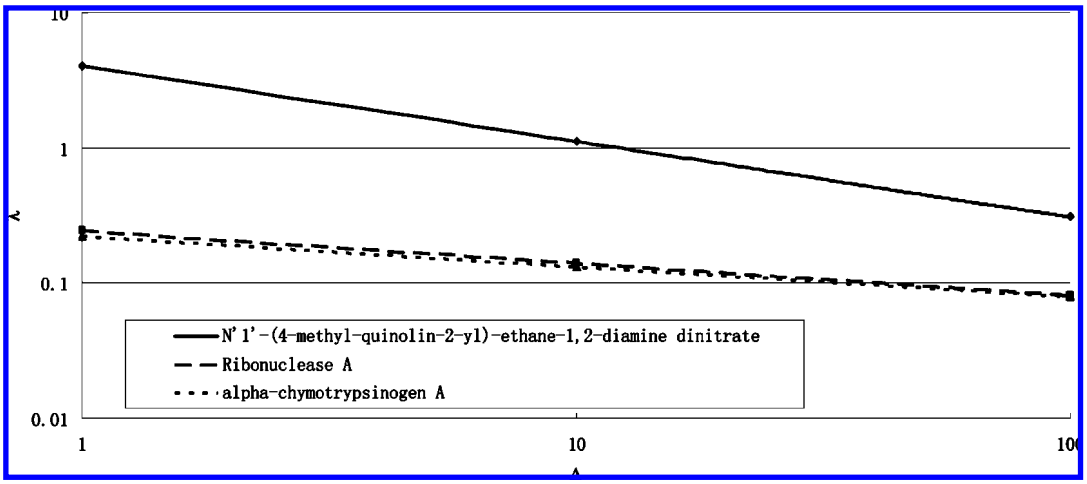

Figure 5. The affinity ranking plot of RNase A, α-chy A, and *N*′1′-(4-methylquinolin-2-yl)ethane-1,2-diamine dinitrate on SP Sepharose HP at pH 6.

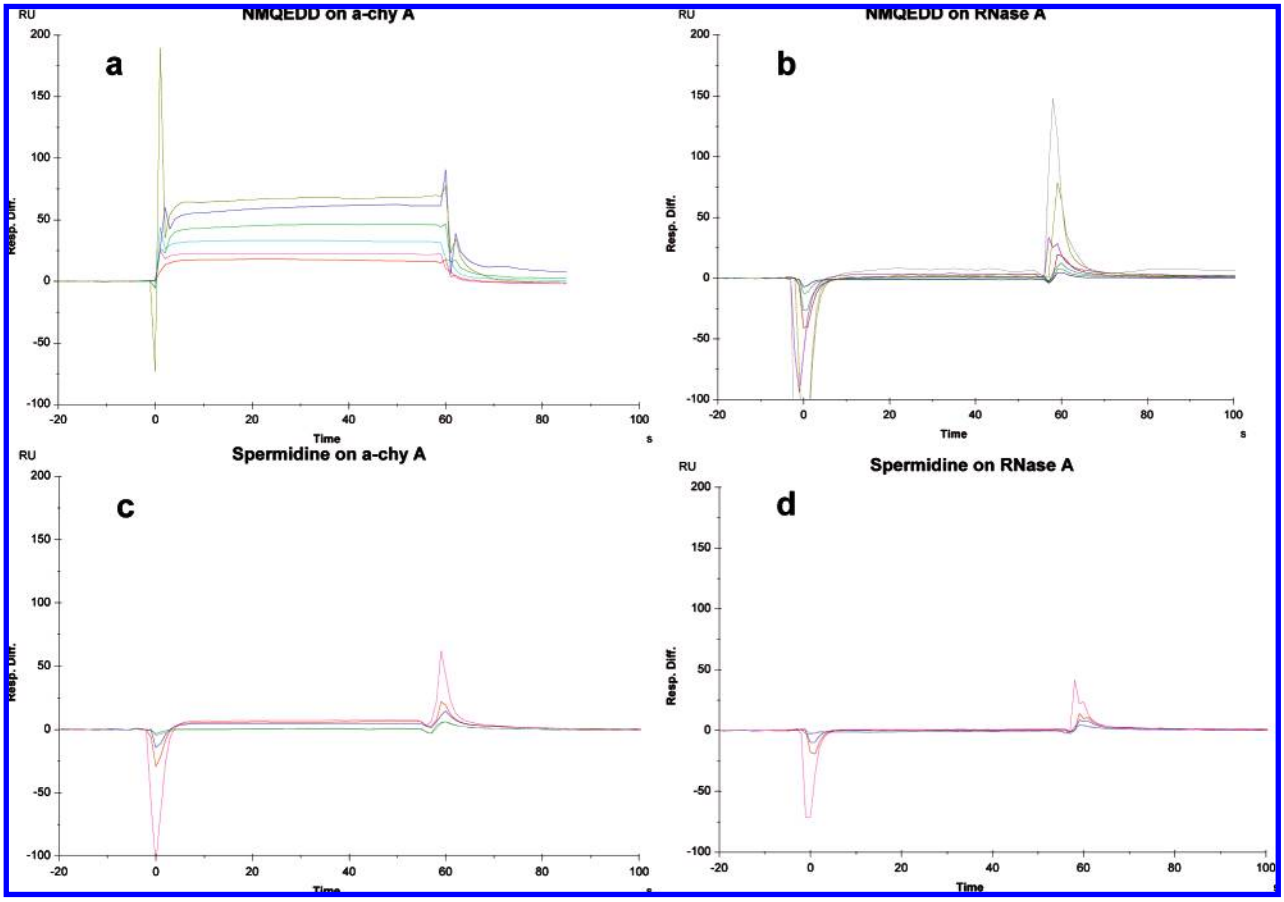

Figure 6. SPR results of protein−displacer interactions: (a) *N*′1′-(4-methylquinolin-2-yl)ethane-1,2-diamine dinitrate (NMQEDD) on α-chy A; (b) *N*′1′-(4-methylquinolin-2-yl)ethane-1,2-diamine dinitrate (NMQEDD) on RNase A; (c) spermidine on α-chy A; and (d) spermidine on RNase A.

1,2-diamine dinitrate. As seen in Figure 4, this column experiment resulted in the selective displacement of RNase A with α-chy A remaining behind the displacer zone, confirming the selectivity of this displacer under column conditions.

An affinity ranking plot (Figure 5) was generated based on the SMA parameters of the two proteins and the selective displacer *N*′1′-(4-methylquinolin-2-yl)ethane-1,2-diamine dinitrate to examine if this selective displacement was due to mass action. The SMA parameters (ν and K) were determined as described in the Experimental Section, and resulting values are listed in Table 1. The affinity ranking plot corresponding to this separation is presented in Figure 5. As seen in the plot, the dynamic affinities for these two proteins were very similar under all possible experimental conditions. It can also be seen in the figure that the displacer's dynamic affinity curve is significantly above the curves of the two proteins at all of the tested experimental operating conditions. These results indicate that according to the conventional SMA model, *N*′1′-(4-methylquinolin-2-yl)ethane-1,2-diamine

Table 1. SMA Parameters of RNase A, α-chy A, and N′1′-(4-Methylquinolin-2-yl)ethane-1,2-diamine Dinitrate

solute	characteristic charges (ν)	equilibrium constant (K)
ribonuclease A	4.2	0.0026
α-chymotrypsinogen A	4.5	0.0011
N′1′-(4-methylquinolin-2-yl)ethane-1,2-diamine dinitrate	1.8	12.144

dinitrate should displace both proteins under all conditions and that there is no possibility of this displacer acting as a mass action displacer. Clearly, this selective displacement is due to some other mechanism which cannot be predicted from the affinity ranking plot which is based of single component isotherm parameters.

The identified selective displacers from the batch displacement experiments all have at least one aromatic group in their molecular structures. In fact, 4-(2-cyclohex-1-enylethylamino)butan-1-ol sulfate was the only tested displacer that contains no aromatic ring in the structure, and it showed no selectivity. In addition, the aromatic moieties in the structures of selective displacers were all located away from the charges and sometimes even at the opposite end of the structures. Since RNase A and α-chy A have different hydrophobicities, it was hypothesized that the aromatic group in these displacers may be contributing to some secondary interactions with α-chy A such as hydrophobic interactions.

As described above, this displacer has higher affinity for the chromatographic resin than either protein for all concentrations evaluated. Thus, according to the affinity ranking plot analysis (which is based on single component SMA isotherm parameters), both proteins should be displaced at any displacer concentration under column conditions. However, if in addition to competition for the resin, binding occurs between the displacer and one of the proteins, this may affect the ability of this displacer to efficiently displace the protein that it binds to. In other words, this displacer will selectively NOT displace the protein that it binds to, while still acting as an efficient displacer for the other protein based on its high resin binding affinity. This is in contrast to the previous work by Torres and Peterson[9] which described the use of a negatively charged carboxymethyldextran to elute a positively charged impurity protein from a cation exchanger. In that work, the carboxymethyl dextran did not bind to the column and did not act as a competitive displacer for binding sites on the stationary phase material.

In order to examine this hypothesis, SPR experiments were carried out as described in the Experimental Section, and the results are presented in Figure 6.

As seen in Figure 6, the chemically selective displacer interacted with immobilized α-chy A but not RNase A. On the other hand, the nonselective displacer spermidine did not interact with either of the proteins. These SPR results support the hypothesis that interaction of this displacer with the more hydrophobic protein α-chy A may be playing a role in its inability to efficiently displace this protein under both batch and column conditions (although its affinity for the resin indicates that it should readily displace this protein). While the SPR results were duplicated on repeated runs on a single SPR chip, the background signal from the unreacted carboxylic acid groups on the chip make this a difficult experiment to repeat on separate chips. We are currently exploring alternative techniques to examine displacer protein binding such as NMR and MD simulations.

Based on these results, two additional potential selective displacer candidates were manually chosen from the chemical data base which also contained aromatic moieties which were located away from charged moieties in the molecules. Parallel batch screening experiments were carried out to analyze their selectivi-

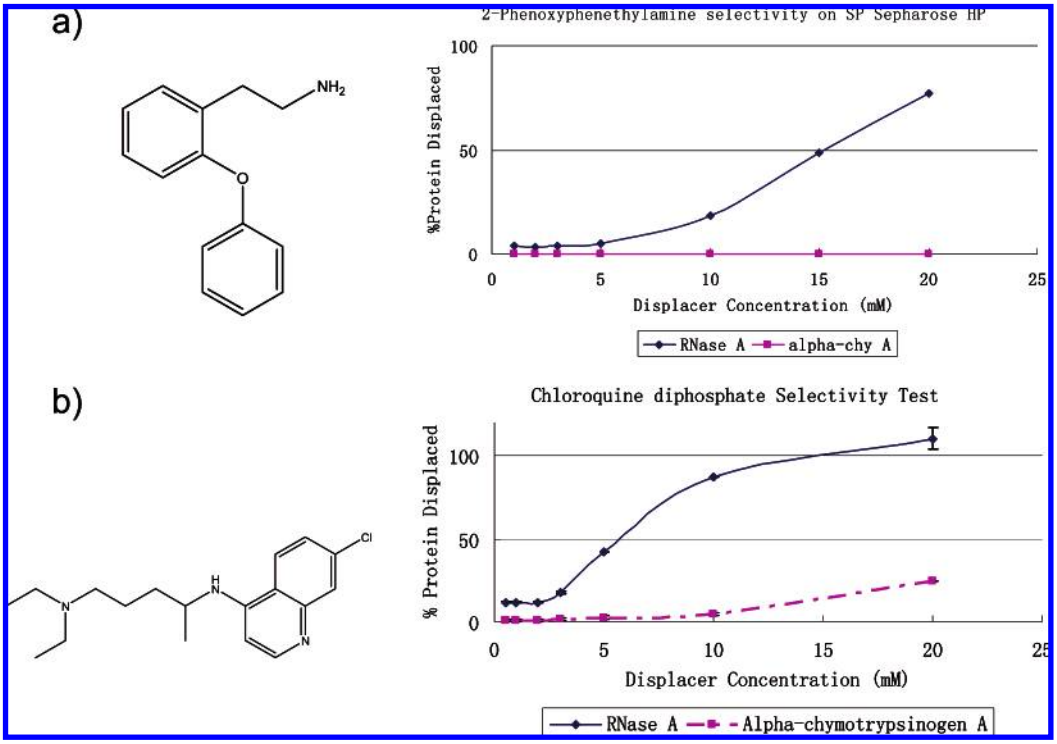

Figure 7. The chemical structures and parallel batch screening results of (a) 2-phenoxyphenethylamine and (b) chloroquine diphosphate.

ties, and the results and the displacer structures are presented in Figure 7.

As seen in Figure 7, these two additional displacers also showed good selectivity and exclusivity for displacing RNase A while not displacing α-chy A. Although all the selective displacers identified by the screening experiments earlier in this work contained at least two charges, 2-phenoxyphenethylamine, containing only one charge. Nevertheless, it was successful in displacing 75% RNase A and no α-chy A at a concentration of 20 mM. These results also support the hypothesis that the aromatic group plays an important role in this particular class of selective displacers.

CONCLUSION

In this work, parallel batch screening experiments were carried out with lead compounds to identify selective displacers for a binary protein mixture which exhibited very similar retention behavior under linear gradient conditions. The results indicated that most of these lead compounds were indeed selective and that one of these displacers exhibited extremely high selectivity. These results were then validated under column conditions. SMA parameters were determined, and an affinity ranking plot was generated to ensure that the type of selective displacement found in this work was not mass-action selective displacement. The mechanism of chemically selective displacement was investigated by SPR experiments, and the results suggest that protein displacer interaction may play an important role. The hydrophobic interaction between the displacer and the protein α-chy apparently makes it more difficult for the displacer to displace this protein from the resin surface. As the displacer front travels through the column, it must efficiently displace proteins ahead of the self-sharpening displacer concentration wave. Any additional reactions such as protein−displacer interactions may complicate this process and make this displacement process less efficient, moving the selected protein behind the displacer front. While the protein displacer interaction for the current set of selective displacers seems to be based on hydrophobicity, other types of interactions may also be used to open up new opportunities for chemically selective separation systems. This new class of displacers not only enables highly efficient column chromatographic applications but also provides an opportunity for the development of new preparative protein purification techniques such as chemically selective batch separations.

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