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The effect of multi-component adsorption on selectivity in ion exchange displacement systems

N. Tugcu[1], S.M. Cramer*

Department of Chemical and Biological Engineering, Rensselaer Polytechnic Institute, 110 8th Street, Troy, NY 12180, USA

Abstract

This paper examines chemically selective displacement chromatography using affinity ranking plots, batch displacer screening experiments, column displacements, multi-component adsorption isotherms and spectroscopy. The affinity ranking plot indicated that the displacers, sucrose octasulfate (SOS) and tatrazine, should possess sufficient affinity to displace the proteins amyloglucosidase and apoferritin over a wide range of operating conditions. In addition, the plots indicated that the separation of these proteins by displacement chromatography would be extremely difficult. Further, the two proteins were shown to have very similar retention times under shallow linear gradient conditions. When batch displacement experiments were carried out, both tartrazine and SOS exhibited significant selectivity differences with respect to their ability to displace these two proteins, in contrast to the affinity ranking plot results. Column displacement experiments carried out with sucrose octasulfate agreed with the predictions of the affinity ranking plots, with both proteins being displaced but poorly resolved under several column displacement conditions. On the other hand, column displacement with tartrazine as the displacer resulted in the selective displacement and partial purification of apoferritin. Single- and multi-component isotherms of the proteins with or without the presence of displacers were determined and were used to help explain the selectivity reversals observed in the column and batch displacement experiments. In addition, fluorescence and CD spectra suggested that the displacers did not induce any structural changes to either of the proteins. The results in this paper indicate that multi-component adsorption behavior can be exploited for creating chemically selective displacement separations.
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1. Introduction

Ion exchange displacement chromatography has been shown to be a promising technique for preparative protein separations [1–3]. In addition, displacement chromatography of proteins has been successfully carried out in hydroxyapatite [4–6] and hydrophobic interaction and reversed phase chromatographic systems [7,8]. Various classes of displacers, such as polyelectrolytes [9–13], polysaccharides [14,15] and low-molecular-mass dendrimers [16], amino acids [17], and antibiotics [18] have been identified for ion exchange systems. In particular, these low molecular weight displacers can be used in selective displacement chromatography [2]. In fact, the ability to use low-molecular-mass displacers has attracted significant attention due to several distinct operational advantages [19].

Selectivity in ion exchange systems can be exploited in various ways. A number of studies have addressed the effect of salt type and concentration on protein selectivity in ion exchange systems. The results indicated that both co-ion and counter-ion can have an effect on protein selectivity [20–26]. Kopaciewicz et al. [20] have demonstrated that while cation type slightly altered the selectivity, the anion type significantly affected the retention time as well as the selectivity

* Corresponding author. Tel.: +1 518 276 2739; fax: +1 518 276 4030.
 E-mail address: crames@rpi.edu (S.M. Cramer).
[1] Present address: Merck Research Labs, RY805S-100, P.O. Box 2000, 126 E. Lincoln Ave., Rahway, NJ 07065, USA.

for anion exchange systems In addition, changes in the displacing salt type and gradient mode have been shown to significantly enhance the selectivity for closely-related variants [24]. We have developed a high throughput screening technique for the rapid identification of potential displacers for both protein [27] and oligonucleotide [28] separations. In addition, recent results indicate that displacer chemistry can have a significant effect on the selectivity of displacement systems [29]. In this paper, we examine this phenomenon in detail by examining single and multi-component adsorption isotherms under a variety of conditions. In addition, batch and column displacement experiments are carried out to probe the nature of this apparent chemically selective displacement phenomenon.

2. Experimental protocol

2.1. Materials

Source 15Q (15 μm) strong anion exchange stationary phase material was donated by Amersham Biosciences (Uppsala, Sweden) and the stationary phase was slurry packed into a 50 mm × 5 mm i.d. column. TSK-Gel G3000SWXL size exclusion column (300 mm × 7.8 mm i.d.) and a TSK-Gel SWXL (40 mm × 6 mm i.d.) guard column were donated by TOSOH BIOSEP (Montgomeryville, PA, USA). Amyloglucosidase and apoferritin were purchased from Sigma (St. Louis, MO, USA) and ICN Biomedicals Inc. (Aurora, OH, USA), respectively. Sodium chloride and sodium sulfate were purchased from Fisher Scientific (Pittsburgh, PA, USA). Tris–HCl and Tris–base were purchased from Sigma. Tartrazine and sucrose octasulfate (SOS) were purchased from Aldrich (Milwaukee, WI, USA) and Toronto Research Chemicals Inc. (Ontario, Canada), respectively.

2.2. Apparatus

Linear gradients were run on a Pharmacia fast protein liquid chromatographic (FPLC) system consisting of two P-500 pumps and a LCC-500 controller (Amersham Biosciences, Uppsala, Sweden). Protein and displacer analysis was carried out using a model 600 multisolvent delivery system, a model 712 WISP autoinjector and a model 996 Photodiode array (PDA) absorbance detector controlled by a Millenium chromatography manager (Waters, Milford, MA, USA). Absorbance measurements for SOS were carried out using a Lambda 6 UV–vis spectrophotometer (Perkin-Elmer, Wilton, CT, USA). Fluorescence spectra were collected using a Shimadzu RF-5000 spectrofluorophotometer (Shimadzu Corporation, Kyoto, Japan). Circular dichroism spectra were obtained using a JASCO 720 spectropolarimeter (Japan Spectroscopic Co. Ltd., Japan). SELCON software (http://www.srs.dl.ac.uk/VUV/CD/cpmsd.html) was used for structure analysis.

2.3. Procedures

2.3.1. Determination of SMA parameters for proteins

The characteristic charge (ν) and equilibrium constant (K) of the proteins were estimated using isocratic elution chromatography. The isocratic experiments were carried out in 20 mM Tris, pH 7.5 with varying salt concentrations. The resulting capacity factor data was used to provide estimates of the K and ν of the proteins as described elsewhere [30]. The linear SMA parameters were then used to generate dynamic affinity lines for the proteins as described in reference [31]. The position of these dynamic affinity lines were used to predict the elution order of the components in a displacement train.

2.4. Determination of SMA parameters for displacers

The linear SMA parameters for displacers were obtained using retention times from linear gradient experiments. Once the retention volumes were obtained, using at least two different gradient conditions, the linear SMA parameters, K and ν were calculated as described elsewhere [32]. Linear gradient experiments were carried out at different total gradient times using buffer A (50 mM Tris, pH 7.5) and buffer B (50 mM Tris, pH 7.5, containing 2.5 M NaCl). Experiments were performed at 0.5 ml/min on Source 15Q. The retention times for tartrazine were obtained by monitoring the UV absorbance at 254 nm. The retention times for SOS were obtained by collecting fractions of the column effluent and assaying for SOS [33].

2.5. Batch displacement experiments

Batch displacement experiments were carried out separately for each protein. The bulk stationary phase (Source 15Q; 2.5 ml) was first washed with deionized water and then the carrier buffer, 20 mM Tris with 30 mM NaCl, pH 7.5 was added, and allowed to equilibrate for 2 h. After gravity settling of the stationary phase, the supernatant was removed and 30 ml of 1.5 mg/ml amyloglucosidase or apoferritin in the carrier buffer was added and then equilibrated in a shaker for 6 h at 23 °C. The supernatant was analyzed by size exclusion chromatography to determine the protein concentration and the amount adsorbed on the stationary phase was calculated through a mass balance. The supernatant was then removed and 25 μl aliquots of the stationary phase with adsorbed protein were added to separate vials. Aliquots (300 μl) of 10 mM solutions of each displacer in the carrier buffer were then added to each vial, and allowed to equilibrate for 6 h. After equilibration, the stationary phase was allowed to gravity-settle and the supernatants were removed and analyzed to determine the percentage of protein displaced by each displacer. These experiments were carried out in duplicate.

2.6. Displacement experiment

For the displacement experiment, a Source 15Q (50 mm × 5 mm i.d.) column was initially equilibrated with the carrier buffer 20 mM Tris with 30 mM NaCl, pH 7.5 and then sequentially perfused with feed, displacer and regenerant solutions. The experimental conditions, such as the feed load, flow rate and displacer concentration can be found in the figure legends. Appropriate fractions (400 μl) of the column effluent were collected during the displacement experiments for subsequent analysis of proteins and displacer. The displacement experiment was carried out at a flow rate of 0.2 ml/min and the effluent was monitored at 235 nm. The column was regenerated sequentially with five column volumes of 2 M NaCl and 1 M NaOH with 25% (v/v) acetonitrile solutions.

2.7. Protein and displacer analysis

Protein and tartrazine analysis of the fractions collected during the displacement experiment was performed by size exclusion chromatography using a TSK-Gel G3000SWXL (300 mm × 7.8 mm i.d.) with a TSK-Gel SWXL (40 mm × 6 mm i.d.) guard column with 50 mM phosphate and 100 mM NaCl, pH 6.0. Five microliter samples were injected at a flow rate of 1.0 ml/min and the effluent was monitored at 235 nm. SOS containing fractions were assayed for SOS as described elsewhere [33].

2.8. Measurement of protein and displacer isotherms

All the isotherm measurements were performed in the batch mode. The bulk stationary phase (Source 15Q) was first washed with deionized water and then the carrier buffer (20 mM Tris with 30 mM NaCl, pH 7.5) was added and allowed to equilibrate for 2 h. After gravity settling of the stationary phase, the supernatant was removed and 25 μl aliquots of the stationary phase were added to separate vials. To measure the single-component isotherms of the proteins, 300 μl of varying concentrations (0.5–15 mg/ml) of each protein in the carrier buffer was added to the individual vials. After equilibration was complete (6 h), the stationary phases were allowed to gravity-settle and the supernatants were removed and analyzed to determine the concentration of protein in the carrier buffer and on the stationary phase. Multi-component isotherms of both proteins were carried out in the same manner, but with both proteins present in the initial solutions (300 μl) in a 1:1 ratio. Initial protein concentrations varied between 0.25 and 7.5 mg/ml for each protein such that total protein concentration covered the same range as the single component experiments. For multi-component isotherm measurements of each protein in the presence of a displacer, varying concentrations (0.5–15 mg/ml) of protein solutions (300 μl) containing a constant concentration (10 mM) of displacer solution were employed. Multi-component isotherms of both proteins in

the presence of 10 mM displacer were measured in the same manner. Again, both proteins were present in the initial solutions (300 μl) in a 1:1 ratio (concentrations varying between 0.25 and 7.5 mg/ml for each protein). For single component isotherm measurement of displacers, varying concentrations (0.25–15 mM for tartrazine and 0.25–10 mM for SOS) of displacer solutions (300 μl) were employed. These experiments were all carried out in triplicate at 23 °C.

2.9. HPLC analysis of proteins in the supernatant

Size exclusion chromatography was employed for the analysis of the supernatants in terms of tartrazine, amyloglucosidase and apoferritin content. In these experiments, the carrier buffer was 50 mM phosphate with 100 mM NaCl at pH 6.0. Five microliters of each supernatant solution obtained from the isotherm experiments was injected and the analyses was carried out in duplicate at a flow rate of 1.0 ml/min. The absorbance was monitored between 215 and 280 nm.

2.10. Fluorescence and CD spectroscopy

Fluorescence spectra of each protein alone (0.5 mg/ml) and in the presence of SOS (10 mM) were obtained. For this purpose, excitation was carried out at 280 nm and emission data were collected between 200 and 600 nm for all cases. CD spectra were collected for the proteins alone and in the presence of tartrazine in the wavelength range of 180–260 nm. A band width of 1 nm, cuvettes with 0.02 cm path length and scan speed of 10 nm/min, were employed during these experiments. Three scans were carried out for each solution.

3. Results and discussion

Dynamic affinity ranking plots provide a graphical tool for predicting the relative affinities of displacers and proteins under isotachic displacement conditions [31]. The linear SMA parameters (ν and K) of the proteins and displacers were determined as described in the experimental section. Table 1 shows the resulting linear SMA parameters for the proteins and displacers. Fig. 1 presents the affinity ranking plot where the dynamic affinity lines of tartrazine, SOS, amyloglucosidase and apoferritin are shown.

As seen in this figure, the dynamic affinity lines for the two proteins are very similar, indicating that their separation by displacement chromatography will be quite difficult. Further, as shown in Fig. 2, retention data obtained with a

Table 1
Linear SMA parameters of proteins and displacers

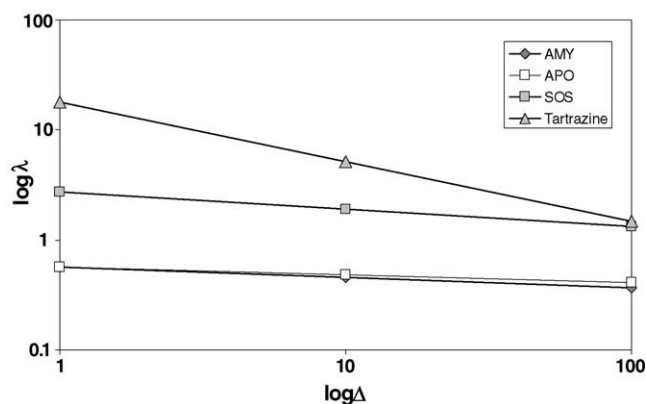|	ν	K
SOS	6.35	597
Tartrazine	1.85	206
Amyloglucosidase	10.36	3.04E−3
Apoferritin	13.69	4.47E−4

Fig. 1. Affinity ranking plot for tartrazine, SOS, amyloglucosidase and apo-ferritin.

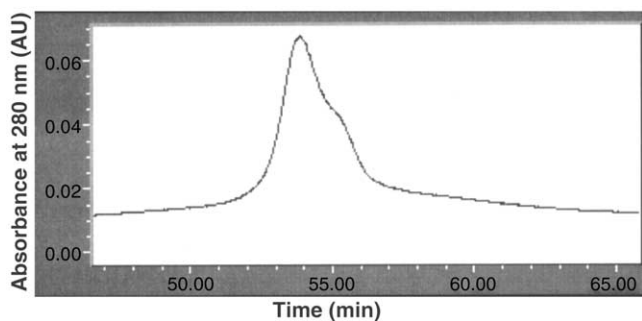
Fig. 2. A mixture of apoferritin and amyloglucosidase under linear gradient condition. Column: 50 mm × 5 mm i.d. Source 15Q; buffer A: 20 mM Tris, pH 7.5 and buffer B: A + 600 mM NaCl; gradient slope: 12 mM NaCl/column volume; flow rate: 0.2 ml/min.

linear salt gradient suggested that the two proteins also have very similar retention times under gradient conditions. On this chromatogram, the initial peak represents amyloglucosidase and the shoulder represents apoferritin. Thus, even with a very shallow gradient, the resolution of these two proteins is quite poor.

It can also be seen in Fig. 1 that the dynamic affinity lines for both displacers lie well above the dynamic affinity lines of the proteins, indicating that both displacers should possess sufficient affinity to displace these proteins. In addition, as shown in Fig. 1, the affinity lines do not cross, indicating that the Steric Mass Action model does not anticipate any selectivity changes or any possibility of selective displacement. In contrast to these results, when batch displacement experiments were carried out (Table 2), both tartrazine and

Table 2
Batch displacement results for amyloglucosidase and apoferritin by using tartrazine and SOS

Proteins	%Protein displaced	
	SOS	Tartrazine
Amyloglucosidase	40.10	59.80
Apoferritin	83.44	31.95

SOS exhibited significant selectivity differences with respect to their ability to displace these two proteins.

One significant difference between the affinity ranking plot and batch displacement approaches is that the ranking plots are generated under single-component adsorption conditions, while the batch displacement experiments were run under multi-component conditions (one protein–one displacer). In order to examine this behavior from a different perspective, column displacement experiments were carried out.

3.1. Column displacement experiments

The initial column displacement was run using SOS as the displacer. For this experiment, 10 mM SOS concentration was employed, the same as in the batch displacement experiments. This displacer concentration corresponds to a partition ratio, Δ of 24. This quantity is the ratio of displacer concentration on the stationary phase to that in the mobile phase [31]. As seen in Fig. 3, while both proteins could be effectively displaced, amyloglucosidase and apoferritin were not well separated in this column displacement.

This result agrees qualitatively with the prediction of the dynamic affinity lines (Fig. 1) that both proteins should be readily displaced by SOS, however, the elution order of the proteins were not the same as predicted by the plot. In addition, while the batch experiments indicated that significantly more apoferritin should be displaced when SOS was used as the displacer, the column displacement experiment shows that SOS, at 10 mM, could readily displace both proteins. Finally, due to the difficulty of the separation as evidenced by the proximity of the affinity lines of the two proteins, there was minimal separation of the two proteins in this "traditional" displacement experiment. To evaluate the effect of displacer concentration, experiments with 5 ($\Delta = 38$) and 3 mM SOS ($\Delta = 64$) were carried out and the results are shown in Figs. 4 and 5, respectively.
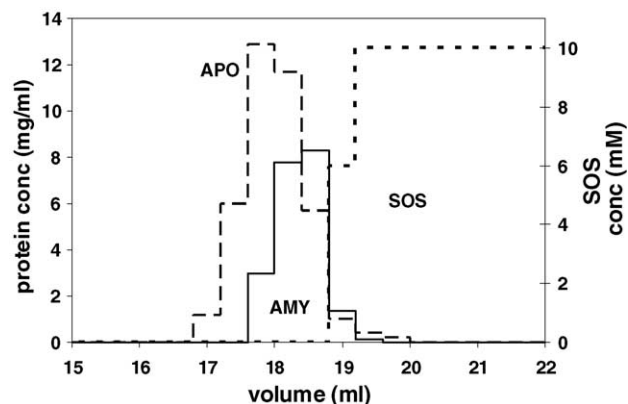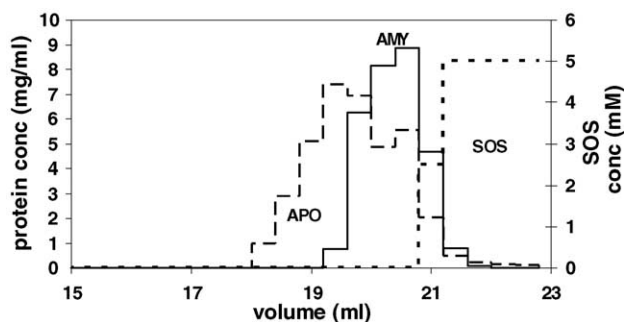

Fig. 3. Displacement separation of 24 mg of apoferritin (0.98 mg/ml) and amyloglucosidase (0.75 mg/ml) mixture using SOS as a displacer. Column: 50 mm × 5 mm i.d. Source 15Q; carrier: 20 mM Tris–HCl + 30 mM NaCl, pH 7.5; displacer: 10 mM; flow rate: 0.2 ml/min.

Fig. 4. Displacement separation of 24 mg of apoferritin (0.98 mg/ml) and amyloglucosidase (0.75 mg/ml) mixture using SOS as a displacer. Column: 50 mm × 5 mm i.d. Source 15Q; carrier: 20 mM Tris–HCl + 30 mM NaCl, pH 7.5; displacer: 5 mM; flow rate: 0.2 ml/min.

While a slight improvement in the resolution can be observed in both displacements due to broadening of the protein zones, the yields of these experiments are still low. In addition, even at these relatively low SOS concentrations, a selective displacement profile could not be obtained.

A column displacement experiment was then carried out using 10 mM tartrazine ($\Delta = 49$) as the displacer (Fig. 6). As was shown in Table 2, batch displacement experiments indicated that tartrazine can effectively displace amyloglucosidase but not apoferritin.

Fig. 6 shows that this experiment resulted in the selective displacement of apoferritin with amyloglucosidase emerging in the displacer zone. Clearly, selective displacement using tartrazine was not predicted by the SMA model and the dynamic affinity plot. In addition, as seen in the displacements with SOS, apoferritin still elutes from the column first when tartrazine is used as the displacer, in contrast with the batch displacement experiments where significantly more amyloglucosidase was displaced than apoferritin (see Table 2).

These results suggest that there is some discrepancy between the SMA model predictions (Fig. 2) and the ob-
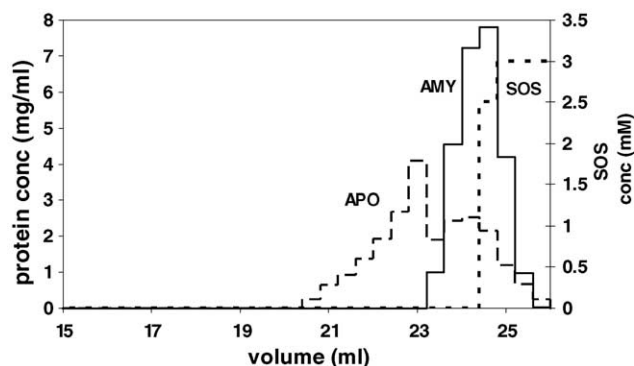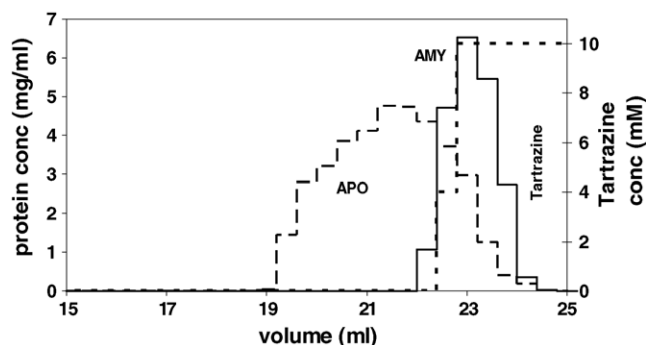

Fig. 6. Displacement separation of 24 mg of apoferritin (0.98 mg/ml) and amyloglucosidase (0.75 mg/ml) mixture using tartrazine as a displacer. Column: 50 mm × 5 mm i.d. Source 15Q; carrier: 20 mM Tris–HCl + 30 mM NaCl, pH 7.5; displacer: 10 mM; flow rate: 0.2 ml/min.

served column displacement experiments. One major difference is the early elution of apoferritin, which could not be predicted with single-component linear SMA parameters. In addition, even though batch displacement experiments had identified both SOS and tartrazine as candidates for selective displacements, only tartrazine actually resulted in a selective displacement of apoferritin over amyloglucosidase.

The remainder of this paper addresses several possible explanations for these discrepancies. One hypothesis is that these differences are related to the isotherm changes of these proteins in the presence of displacers. In an attempt to address this hypothesis, single- and multi-component isotherms were determined and batch displacement experiments were revisited under different conditions. These discrepancies may also be the result of secondary and/or tertiary structural changes experienced by the proteins due to some interaction with the displacers. Accordingly, fluorescence and circular dichroism spectra of these proteins were measured in the presence of displacers.

3.2. Single-component isotherms of proteins and displacers

To discuss variations due to multi-component adsorption effects, single-component isotherms of the proteins (amyloglucosidase and apoferritin) and displacers (tartrazine and SOS) were first obtained as controls. Fig. 7 presents the single-component isotherms of amyloglucosidase and apoferritin. (*Note*: All stationary phase concentrations are given per ml of solid stationary phase which does not include intra or inter-particular volumes.) As seen in the figure, apoferritin and amyloglucosidase exhibit similar adsorption behavior under these conditions. In addition, both single component isotherms are Langmuirian in shape. Fig. 8 shows the single component isotherms of tartrazine and SOS. Under these conditions, both displacers exhibit strong binding with tartrazine having a square shaped isotherm.

Fig. 5. Displacement separation of 24 mg of apoferritin (0.98 mg/ml) and amyloglucosidase (0.75 mg/ml) mixture using SOS as a displacer. Column: 50 mm × 5 mm i.d. Source 15Q; carrier: 20 mM Tris–HCl + 30 mM NaCl, pH 7.5; displacer: 3 mM; flow rate: 0.2 ml/min.
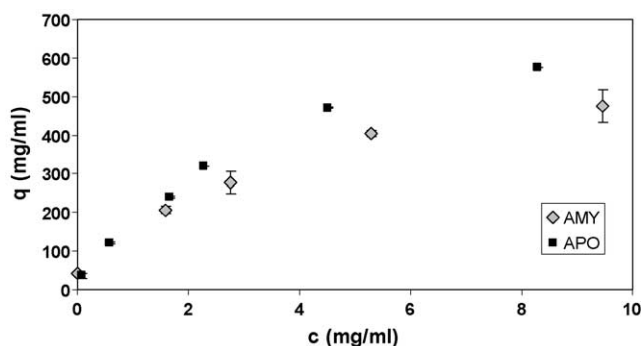
Fig. 7. Single-component isotherms for apoferritin and amyloglucosidase. Buffer: 20 mM Tris + 30 mM NaCl, pH 7.5. Initial protein concentrations varied between 0.5 and 15 mg/ml.
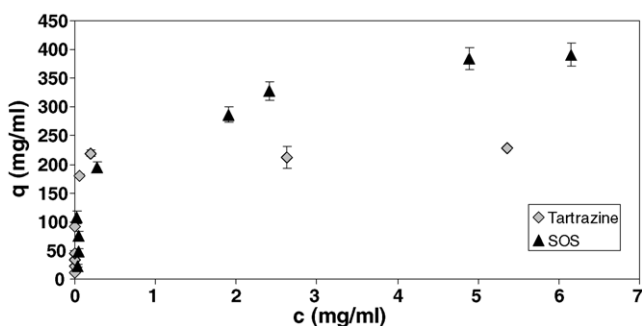

Fig. 10. Multi-component isotherms of a protein in the presence of tartrazine (10 mM). Buffer: 20 mM Tris + 30 mM NaCl, pH 7.5. Initial protein concentrations varied between 0.25 and 7.5 mg/ml.

component adsorption of apoferritin was significantly altered. Under these competitive binding conditions, amyloglucosidase is effective at displacing/desorbing apoferritin. These results indicate that during column loading, apoferritin is likely to be sample displaced [34] by amyloglucosidase. This is in agreement with the protein elution order observed in all column displacements (Figs. 3–6).

3.4. Multi-component isotherms: one protein and one displacer

In order to shed light on the batch displacement results shown in Table 2, multi-component isotherms for apoferritin and SOS, apoferritin and tartrazine, amyloglucosidase and SOS, and amyloglucosidase and tartazine were measured. The effects were evaluated separately for tartrazine and SOS as shown in Figs. 10 and 11, respectively. As seen in Fig. 10, tartrazine has a significant impact on the adsorption of amyloglucosidase where the shape changed from Lamgmuirian to a sigmoidal shaped isotherm. On the other hand, the shape of the apoferritin isotherm remained Langmuirian. Sigmoidal isotherms are often associated with lateral interactions between adsorbed proteins which are enhanced at high protein loadings [35]. There are several observations that can be made about these isotherms. In the linear region (low mobile phase concentrations) apoferritin is more highly ad-

Fig. 8. Single-component isotherms of tartrazine and SOS. Buffer: 20 mM Tris + 30 mM NaCl, pH 7.5. Initial displacer concentrations varied between 0.25 and 15 mM for tartrazine and 0.25 and 10 mM for SOS.

3.3. Multi-component isotherms: apoferritin and amyloglucosidase

To investigate the adsorption behavior of apoferritin and amyloglucosidase under multi-component conditions, the isotherms were measured in the presence of both proteins (Fig. 9). While the adsorption of amyloglucosidase experienced a slight decrease under multi-component conditions (as compared to its single component behavior), the multi-
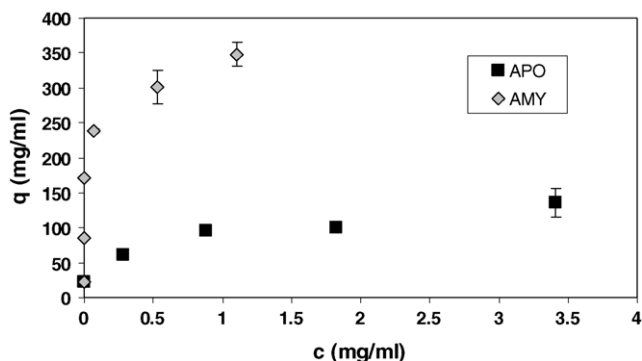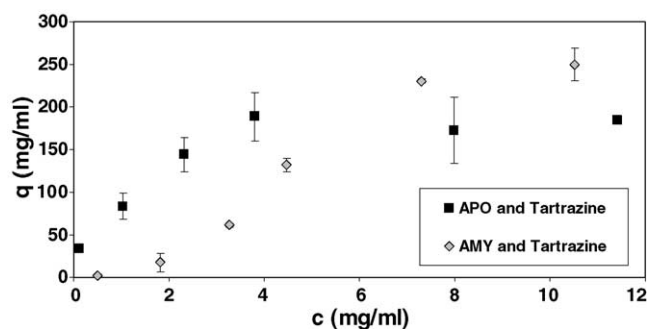

Fig. 9. Multi-component isotherms for apoferritin and amyloglucosidase. Buffer: 20 mM Tris + 30 mM NaCl, pH 7.5. Proteins were present in 1:1 ratio with initial concentrations varying between 0.25 and 7.5 mg/ml.
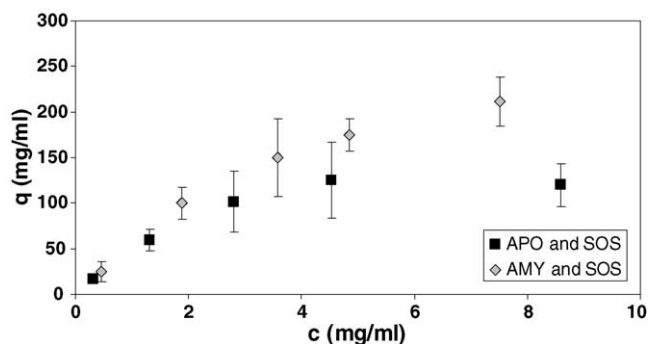

Fig. 11. Multi-component isotherms of a protein in the presence of SOS (10 mM). Buffer: 20 mM Tris + 30 mM NaCl, pH 7.5. Initial protein concentrations varied between 0.25 and 7.5 mg/ml.

sorbed than amyloglucosidase. However, in the non-linear region (high mobile phase concentrations), amyloglucosidase is more highly adsorbed than apoferritin in the presence of tartrazine. These results explain the batch displacement results obtained for amyloglucosidase and apoferritin. Batch displacements performed at relatively low protein concentrations showed more amyloglucosidase displaced than apoferritin when tartrazine was used as the displacer, in qualitative agreement with the isotherm data in Fig. 10.

Fig. 11 shows the multi-component isotherms of apoferritin and amyloglucosidase in the presence of SOS. In contrast to the case with tartrazine, amyloglucosidase no longer displays a sigmoidal isotherm. While the shapes are similar to the single-component isotherm, changes in the linear and non-linear regions were observed in the presence of SOS. At low concentrations, apoferritin and amyloglucosidase were similarly adsorbed (with amyloglucosidase exhibiting slightly higher adsorption). However, in the nonlinear region amyloglucosidase is more highly adsorbed than apoferritin. These results are in qualitative agreement with the batch displacement experiments performed using SOS as the displacer.

The results shown in Figs. 10 and 11 indicate that while the apoferritin and amyloglucosidase isotherms remain Langmuirian in the presence of SOS, the amyloglucosidase isotherm exhibits a sigmoidally shaped isotherm in the presence of tartrazine. There may be several reasons for this to occur: tartrazine may form a bridge between amyloglucosidase molecules in the adsorbed state leading to an increase in the concentration of protein on the stationary phase at high loadings, and/or tartrazine may induce some structural changes in amyloglucosidase which can lead to surface aggregation phenomena of the protein.

3.5. Multi-component isotherms: both proteins and one displacer

In order to examine the adsorption behavior of these systems in more detail, isotherms in the presence of both proteins and a displacer were obtained as shown in Figs. 12 and 13. In Fig. 12, the behavior of amyloglucosidase and apoferritin in the presence of tartrazine is shown. Under these

Fig. 13. Multi-component isotherms of both proteins in the presence of SOS (10 mM). Buffer: 20 mM Tris + 30 mM NaCl, pH 7.5. Proteins were present in 1:1 ratio with initial concentrations varying between 0.25 and 7.5 mg/ml.

conditions, amyloglucosidase still displays a sigmoidal behavior, whereas apoferritin still has a Langmuirian shaped isotherm. In the linear region (low mobile phase protein concentrations), apoferritin is more highly adsorbed than amyloglucosidase. On the other hand, amyloglucosidase is more strongly adsorbed at high protein mobile phase concentrations. This isotherm is helpful in explaining why apoferritin elutes first in the displacement train when tartrazine is used as the displacer. During the displacement experiment with tartrazine (Fig. 6), apoferritin reaches ∼5 mg/ml and amyloglucosidase reaches ∼7 mg/ml isotachic concentrations. As seen in Fig. 12, these concentrations lie in the non-linear region, where amyloglucosidase is more highly adsorbed than apoferritin. Thus, under these multi-component adsorption conditions it is expected that apoferritin should be the less retained protein as was observed in the displacement experiments.

Multi-component isotherms also were obtained for amyloglucosidase and apoferritin in the presence of SOS as the displacer as shown in Fig. 13. Again, amyloglucosidase exhibits a sigmoidal adsorption profile whereas the apoferritin isotherm has a Langmuirian shape. In addition, there is a significant change in the adsorption profile from the linear to the non-linear regions. Whereas amyloglucosidase is less adsorbed in the linear region, it becomes more highly adsorbed in the non-linear region. During the displacement experiments carried out with SOS (Figs. 3–5), the plateau concentration of apoferritin ranges from 5 to 12 mg/ml while the amyloglucosidase concentration varies between 8 and 10 mg/ml. Clearly, these concentrations are in the non-linear region of the multi-component isotherm where apoferritin is less strongly adsorbed than amyloglucosidase (Fig. 13). Although these concentrations lie beyond the range of the figure, the q values have reached their saturation concentrations and will not change substantially at higher mobile phase concentrations. The results shown in Fig. 13 indicate that apoferritin is less strongly bound at high concentrations and is thus expected to elute first from the column as was the observed behavior during displacement experiments with SOS.

In contrast to the isotherms with one protein and one displacer shown in Figs. 10 and 11, in the presence of both proteins and either displacer (Figs. 12 and 13), amyloglucosidase
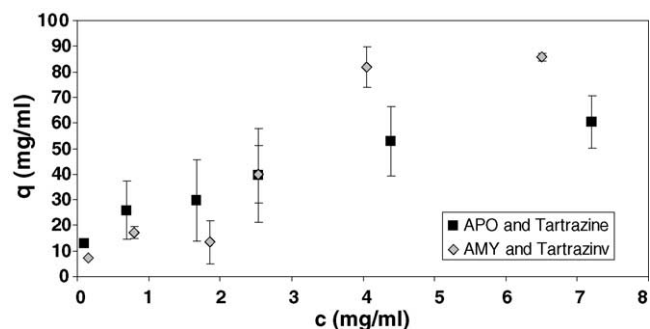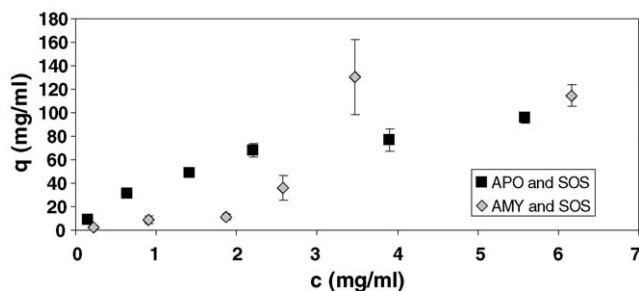

Fig. 12. Multi-component isotherms of both proteins in the presence of tartrazine (10 mM). Buffer: 20 mM Tris + 30 mM NaCl, pH 7.5. Proteins were present in 1:1 ratio with concentrations varying between 0.25 and 7.5 mg/ml.

always exhibits a sigmoidal shape. This clearly indicates that apoferritin and SOS have a combined effect on the adsorption behavior of amyloglucosidase. Thus, even though we did not observe sigmoidal isotherms when only two proteins were present, the presence of the displacer changes the multi-component equilibria resulting in enhanced competition between the proteins for the available sites on the stationary phase (Fig. 9). Even though apoferritin has a net negative charge at this pH, patches of positive charge on absorbed apoferritin may provide an additional positive surface for amyloglucosidase to adsorb, thus increasing its affinity at high concentrations. There are several other possible explanations for this multi-component sigmoidal behavior, such as tartrazine bridging between adsorbed protein and/or partial unfolding of the adsorbed amyloglucosidase in the presence of tartrazine. These multi-component isotherms with both proteins and displacers help to explain why apoferritin was the first protein to elute from the column during the displacement experiments. In order to examine whether possible structural changes induced in the proteins by the presence of the displacer played a role, spectral studies were carried out.

3.6. Fluorescence and CD spectra to check for structural changes

Since SOS has no UV-absorbance or fluorescence, fluorescence and CD spectra in the presence of SOS could be readily obtained. In addition to each protein alone, spectra was obtained after incubating each protein with SOS for 30 min. The resulting spectra indicated that the λ_{max} value for each protein did not shift when SOS was present in the mixture. This indicates that no changes can be detected in the tertiary structures of these proteins due to interaction with SOS.

Fluorescence spectra of the proteins could not be collected in the presence of tartrazine due to its strong emission in the yellow region of the spectra. Accordingly, in order to examine any secondary structural changes of the proteins, CD spectroscopy was carried out. CD spectra of each protein alone, as well as in the presence of tartrazine were obtained. As indicated by the data, there were no significant changes in the secondary structure of these proteins in the presence of tartrazine. Table 3 shows the estimated fractions of α-helix, β-sheet and turns for the proteins alone and in the presence of the displacer. The data in the table indicates that the percentage

of α-helix and β-sheet for each protein do not change by the addition of tartrazine.

As indicated by both fluorescence and CD spectral analysis, apoferritin and amyloglucosidase do not experience any significant structural changes in the presence of either displacer. Thus, the question of why tartrazine acts as a selective displacer for apoferritin still remains.

4. Conclusions

The work in this paper has attempted to shed light on chemically selective displacement results observed with batch displacement experiments. Affinity ranking plots were constructed and the results indicated that under a wide range of operating conditions, both SOS and tartrazine should displace both proteins, amyloglucosidase and apoferritin under column displacement conditions.

While the displacement experiments carried out with SOS agreed with the predictions of the affinity ranking plots, the two proteins could not be resolved under several column displacement conditions. On the other hand, the displacement experiment using tartrazine as the displacer resulted in the selective displacement of apoferritin, which was not predicted by the affinity ranking plot. Further, this separation resulted in significant purification of the proteins which was not possible under isotachic displacement conditions.

After examining the single- and multi-component isotherms of proteins alone and in the presence of displacers, several issues were resolved. The results of multi-component isotherm analysis of each protein with tartrazine indicated that at low protein concentrations, amyloglucosidase would be less adsorbed than apoferritin, which was the trend observed during batch displacement experiments. On the other hand, at low protein concentrations, amyloglucosidase was slightly more adsorbed than apoferritin in the presence of SOS, also in agreement with the batch displacement results.

Different adsorption behavior was observed in the linear and non-linear regions of the multi-component isotherms of both proteins with displacers. Because of its sigmoidal adsorption behavior, amyloglucosidase was always less adsorbed than apoferritin at low protein concentrations. However, at high protein concentrations, amyloglucosidase became more strongly adsorbed than apoferritin. This non-linear region of the isotherm also represents the isotachic concentrations experienced by the proteins during column experiments. This helps to explain the results that apoferritin was the first protein to elute in the column displacement experiments.

Fluorescence and CD spectra results for proteins alone and in the presence of tartrazine and SOS suggests that the displacers do not induce significant secondary or tertiary structural changes in the proteins under conditions where the batch and column experiments were carried out.

The results presented in this paper demonstrate that multi-component adsorption effects can have a significant impact

Table 3
Fractions of secondary structure components of proteins alone and in the presence of tartrazine

	α-Helix	β-Sheet	Turns	Others	F-sum
Amyloglucosidase	0.271	0.264	0.246	0.227	1.009
Amyloglucosidase and tartrazine	0.268	0.27	0.244	0.225	1.007
Apoferritin	0.355	0.17	0.249	0.228	1.002
Apoferritin and tartrazine	0.338	0.165	0.249	0.221	0.973

on protein selectivity. While the importance of batch displacement experiments to screen for and identify displacers is well established [27–29], the conditions experienced during column displacement experiments may not be reflected by batch displacement experiments. For those cases where a discrepancy occurs, changes in the multi-component adsorption behavior may be the cause and should be investigated. The precise reason for these changes in multi-component adsorption behavior is under active investigation in our laboratory and will be the subject of a future report.

Acknowledgements

References

[1] K.A. Barnthouse, W. Trompeter, R. Jones, P. Inampudi, R. Rupp, S.M. Cramer, J. Biotechnol. 66 (1998) 125.
[2] A.A. Shukla, R.L. Hopfer, D.N. Chakravarti, E. Bortell, S.M. Cramer, Biotechol. Prog. 14 (1998) 92.
[3] J.A. Gerstner, J. Morris, T. Hunt, R. Hamilton, N.B. Afeyan, J. Chromatogr. A 695 (1995) 195.
[4] R. Freitag, J. Breier, J. Chromatogr. A 691 (1995) 101.
[5] S. Vogt, R. Freitag, J. Chromatogr. A 760 (1997) 125.
[6] C. Kasper, J. Breier, S. Vogt, R. Freitag, Bioseparations 6 (1996) 247.
[7] C. Horvath, A. Nahum, J. Frenz, J. Chromatogr. 218 (1981) 365.
[8] A.A. Shukla, K.M. Sunasara, R.G. Rupp, S.M. Cramer, Biotechnol. Bioeng. 68 (2000) 672.
[9] J.A. Gerstner, S.M. Cramer, BioPharm 5 (9) (1992) 42.
[10] G. Jayaraman, S.D. Gadam, S.M. Cramer, J. Chromatogr. 630 (1993) 53.
[11] A.R. Torres, E.A. Peterson, J. Chromatogr. 499 (1990) 47.
[12] S.-C.D. Jen, N.G. Pinto, J. Chromatogr. 519 (1990) 87.
[13] S.-C.D. Jen, N.G. Pinto, J. Chromatogr. Sci. 29 (1991) 478.
[14] A.L. Lee, A.W. Liao, C. Horvath, J. Chromatogr. 443 (1988) 31.
[15] A.W. Liao, C. Horvath, Ann. N. Y. Acad. Sci. 589; Biochem. Eng. 6 (1990) 182.
[16] G. Jayaraman, Y.-F. Li, J.A. Moore, S.M. Cramer, J. Chromatogr. A 702 (1995) 143.
[17] A. Kundu, S. Vunnum, G. Jayaraman, S.M. Cramer, Biotechnol. Bioeng. 48 (1995) 452.
[18] A. Kundu, S. Vunnum, S.M. Cramer, J. Chromatogr. A 707 (1995) 57.
[19] A. Kundu, S.M. Cramer, Anal. Biochem. 248 (1997) 111.
[20] W. Kopaciewicz, M.A. Rounds, J. Fausnaugh, F.E. Regnier, J. Chromatogr. 266 (1983) 3.
[21] W. Kopaciewicz, F.E. Regnier, Anal. Chem. 133 (1983) 251.
[22] R.E. Barron, J.S. Fritz, J. Chromatogr. 284 (1984) 13.
[23] A.N. Hodder, M.I. Aguilar, M.T.W. Hearn, J. Chromatogr. 476 (1989) 391.
[24] A.N. Hodder, M.I. Aguilar, M.T.W. Hearn, J. Chromatogr. 506 (1990) 17.
[25] M.A. Rounds, F.E. Regnier, J. Chromatogr. 283 (1984) 37.
[26] G. Malmquist, N. Lundell, J. Chromatogr. 627 (1992) 107.
[27] C.B. Mazza, K. Rege, C.M. Breneman, N. Sukumar, J.S. Dordick, S.M. Cramer, Biotechnol. Bioeng. 80 (2002) 60.
[28] N. Tugcu, C.B. Mazza, C.M. Breneman, Y. Sanghvi, J. Moore, S.M. Cramer, Sep. Sci. Technol. 37 (2002) 1667.
[29] N. Tugcu, A.S. Ladiwala, C.M. Breneman, S.M. Cramer, Anal. Chem. 75 (2003) 5806.
[30] S.D. Gadam, G. Jayaraman, S.M. Cramer, J. Chromatogr. 630 (1993) 37.
[31] C.A. Brooks, S.M. Cramer, AIChE J. 38 (1992) 1969.
[32] A.A. Shukla, K.A. Barnthouse, S.S. Bae, J.A. Moore, S.M. Cramer, Ind. Eng. Chem. Res. 37 (1998) 4090.
[33] A. Kundu, A.A. Shukla, K.A. Barnthouse, J.A. Moore, S.M. Cramer, BioPharm 10 (1997) 64.
[34] G. Guiochon, S. Golshan-Shirazi, A.M. Katti, Fundamentals of Preparative and Non-Linear Chromatography, Academic Press, San Diego, 1994.
[35] S.-C.D. Jen, N.G. Pinto, Ind. Eng. Chem. Res. 34 (1995) 2685.