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High-performance ion-exchange chromatography and adsorption of plasma proteins

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Abstract

Resolution and recovery are primary concerns in protein chromatography. Separations are often by size, ion exchange, or hydrophobic–hydrophilic properties of the support, eluent and protein. Adsorption of a protein to a synthetic surface plays an essential role in this complex process. In this study, we examined the adsorption properties of three representative plasma proteins (albumin, fibrinogen, and immunoglobulin G) on nonporous column materials containing either quaternary amine or sulfopropyl functional groups. The adsorption properties were studied at 37°C and pH 7.4. Salt gradients were used to examine the adsorption/desorption properties of each of the proteins on each type of surface. The salt concentrations at desorption were measured and compared to the protein isoelectric points. In addition, we examined protein recoveries as a function of desorption time. Our results suggest that protein recoveries depend not only on the protein, eluent and surface, but also the residence time and overall charge concentration during the initial adsorption process. Finally, we correlated the number of charge sites on a molecule with the width of a chromatographic band at half height. The data produced as a result of this study may be used to determine the actual unfolding time of a protein, given a certain set of conditions. The data may also help in understanding the chemistry and dynamics of the protein adsorption processes in ion-exchange chromatography as well as provide key structural information about the proteins.

Keywords: Proteins; Albumin; Immunoglobulins; Fibrinogen

1. Introduction

Protein adsorption onto solid surfaces is the fundamental process that determines protein chromatographic behavior. Ion-exchange chromatography has been used for several years in high-performance liquid chromatography (HPLC) to separate proteins and is assumed to be one of the most gentle separation processes. A number of researchers have studied the effects of various charge groups associated with the solute, stationary phase, and surrounding medium in chromatographic retention and resolution. In 1983, Kopaciewicz et al. [1] published an equilibrium expression that correlated the displacement of counter ions with protein, using the Z number. The Z number is known as the number of charges associated in the interfacial interactions. A correlation has since been made between the Z number and the retention of the biomolecule [2]. It was concluded that only the interaction at the solute–surface contact area would result in changes recognizable by chromatographic behavior [3]. Using this information, Chicz and Regnier [4,5] reported that variations in the primary structure of a protein could be identified using a strong cation-exchange support. This model has been recently refined to predict gradient elution times for proteins [6].

In 1985, Kopaciewicz et al. [7] reported strong binding of hemoglobin to densely charged ion-exchange supports when the protein was left on the

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support for prolonged periods. The hemoglobin could not be desorbed from the support material using a solvent with high ionic strength. Desorption occurred only with the use of a 1:1000 trifluoroacetic acid in isopropanol solution. From this observation, it was suggested that the seemingly irreversible (strong) binding between the support and the protein was hydrophobic, not ionic in nature. In addition, Kopaciewicz showed that the retention and resolution between protein components improved as the density of ion-exchange groups was increased. However, as the ligand density increased, the peaks became broader [7]. For soybean trypsin inhibitor, the half width doubled as the charge density increased from 250 to 750 μmol/g support [7]. Other peaks increased in width as well. This apparently contrasts with the general observation for small molecules that resolution increases with retention time. It is not clear whether this difference is due to the size of the analyte or another factor. Snyder and Kirkland have also suggested that for small molecules and isocratic separations, resolution improves with retention [8]. However, this is not necessarily the case when gradients are used, as will be discussed.

The flow rate or residence time of a protein on a sorbent material has been shown to influence chromatographic peak shape [9] and protein unfolding [10]. In the present study, we examined peak broadening and adsorption behavior of proteins on ion-exchange matrices.

As the pH of the solution around the protein is varied, the number of charge groups can be increased or decreased. At pH 2, approximately 100 hydrogen ions will neutralize the overall charge of albumin, whereas at pH 4.8 [the isoelectric point (p*I*)], the protein is neutrally charged [11]. If the pH of the protein is adjusted, the number of sites available for ion exchange should also be influenced.

In most cases, protein chromatography is carried out using a porous support to maximize surface area and column capacity. It is most often the quality of the separation that the protein chromatographer is interested in. However, porous materials contribute a gel permeation effect and sometimes can influence the chromatography of larger proteins [12]. In the present study, nonporous packings were selected to eliminate porosity effects. It is our goal to understand the adsorption/desorption processes at the protein–surface interface without interference from matrix porosity.

2. Experimental

A Bio-Rad Labs. (Hercules, CA, USA) high-resolution liquid chromatography (HRLC) dual-piston, dual-pump gradient system was used for all separations. We used ultraviolet absorbance in a 9-μl flow cell at 280 nm to detect proteins. Columns were obtained from TosoHaas (Montgomeryville, PA, USA) and included nonporous spherical beads that were 3 μm in diameter. The columns have a protein capacity of approximately 5 mg bovine serum albumin (BSA)/ml for the quaternary amine and 5 mg hemoglobin/ml for the sulfopropyl column. Protein analyses were typically of 100 μg for BSA and immunoglobulin G (IgG) and 20 μg fibrinogen. This is equivalent to $1 \cdot 10^{-9}$, $6 \cdot 10^{-10}$ and $6 \cdot 10^{-11}$ mol/analysis for albumin, IgG and fibrinogen, respectively. Both the anion-exchange and the cation-exchange columns (TosoHaas models 13075 and 13076, respectively) were employed in this study. The column temperature was kept constant at 37°C using a Bio-Rad column heater.

Chemicals and reagents were of the highest quality and were obtained from Sigma (St. Louis, MO, USA). Proteins included fibrinogen (fraction 1) from bovine plasma; BSA (fraction V); and bovine IgG. All proteins were solubilized in tris(hydroxymethyl)aminomethane (Tris)–HCl buffer (pH 7.4) before use, unless otherwise noted. For the pH vs. retention and peak-width study, 5 m*M* 2-(*N*-morpholino)ethanesulfonic acid (MES) buffer was used at pH 4, 5 and 7 while the Tris buffer was used for pH 9 and 10. All solutions were stored at 2 to 8°C when not in use.

To clean the columns, a solution of 0.1% sodium dodecyl sulfate (SDS), 0.1 *M* NaCl and 5 m*M* Tris buffer was passed through for 1 h; 20% methanol in water was then washed through to clear the column of remaining SDS. A solution containing 0.1 *M* NaOH was regularly flushed through each column for approximately 2 h as a cleaning technique, followed by a water rinse. As a conclusion to the clean-up process, a solution of 5 m*M* Tris–HCl, pH

7.4, was washed through until equilibrium between mobile and stationary phase was reached, usually using a flow rate of 1.0 ml/min overnight.

Calibration curves were generated so that protein recovery could be determined. Complete (100%) recovery was assumed when the proteins were individually injected through a low-dead volume connector. Recoveries were calculated from the area under the peaks detected at 280 nm. The proteins were allowed to interact with the surface for variable amounts of time. Residence times were varied by keeping the flow rate constant and adjusting the time of the linear salt gradient. Recoveries were then correlated to residence time of the protein on the surface. Identical results were obtained for a few of the residence times by keeping the salt gradient constant and varying the duration of the initial isocratic (low salt) conditions. These results are discussed in relation to the process, the strength, and the dynamics of protein adsorption.

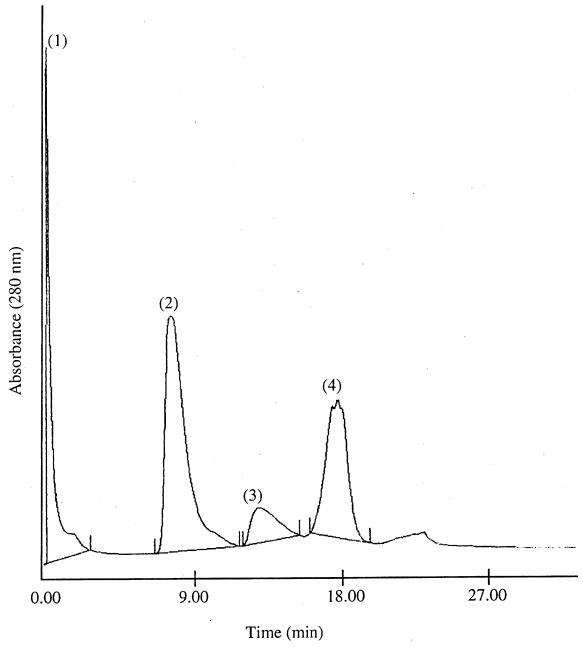

Fig. 1. The separation of the three plasma proteins, IgG (1) and (2), albumin (3), and fibrinogen (4). Conditions are described in the text. The buffer used was 5 m*M* Tris, pH 7.4. Upon injection, the gradient was held at 0 *M* NaCl for 5 min. Salt concentration was then raised to 0.15 *M* NaCl over a period of 5 min. The gradient was held constant at 0.15 *M* NaCl for 5 min. The salt concentration was then raised to 1.0 *M* NaCl, where it was held constant for 5 min. This was followed by a sharp decrease in salt concentration over a period of 1 min to 0 *M* NaCl.

3. Results and discussion

The order of elution in high-performance ion-exchange chromatography (HPIEC) has been described as depending on the number of charged components on the protein that interact with the oppositely charged surface [1–6]. As the salt concentration increases, the most weakly bound proteins desorb, as determined by the number of charged particles that are displaced by the protein [1,2]. The number of displaced ionic groups is known as the *Z* number and has been studied in some detail [1–5]. At least one study has shown that rearranging the charged groups on a protein can drastically alter its retention behavior [13]. In addition, efforts to correlate retention behavior to three-dimensional protein structure [14] have been reported.

The three plasma proteins (albumin, IgG and fibrinogen) were separated by HPIEC using a non-linear gradient (Fig. 1). Of the three proteins, IgG is a classification or mixture of proteins. Some of the IgGs eluted at different salt concentrations. When the anion-exchange column was used, the IgGs appeared to separate into at least two fractions. One fraction eluted with the void peak; the second eluted with 0.023 *M* NaCl. Although the three plasma proteins could be separated, only one protein at a time was injected and analyzed in subsequent investigations. This helped simplify data analysis.

In our recovery experiments, the concentration of sodium chloride was increased from 0.0 to 1.0 *M* using a linear gradient. On the quaternary amine surface, when all three proteins were examined together, the order of elution was IgG, albumin and fibrinogen. The quaternary amine surface is cationic and acts as an anion-exchange substrate under these conditions.

It was expected that the most strongly adsorbed protein in the quaternary amine column would have had the lowest p*I* and be the most electronegative in solution at pH 7.4. However, there was little correlation between p*I* and retention behavior. Fibrinogen (p*I*=5.5) was always the most strongly bound, yet its p*I* was intermediate between albumin (p*I*=4.8) and

the IgGs (pI=6.4 to 7.2). Therefore, the strength of binding between an ion-exchange surface and a protein (represented by retention behavior) cannot be accurately predicted from the pI of the protein and the surface charge. This is likely because ion-exchange binding is due to the local array of externally charged groups on the various proteins, not the net charge.

Recoveries of the three plasma proteins on the anion-exchange column are shown in Fig. 2. For very short (0.3 min) residence times, the recoveries of the three proteins were 100, 65 and 90% for albumin, IgG and fibrinogen, respectively, on the quaternary amine surface. The flow rate used in all experiments was 1.0 ml/min. Therefore, the elution time (in min) and residence volume (in ml) were nearly equivalent. It is anticipated that increasing the flow rate would have shifted the curves in Fig. 2 slightly to the right. That is, greater recoveries would have been obtained at the same residence times with higher flow rates. The magnitude of this effect will be the subject of future work.

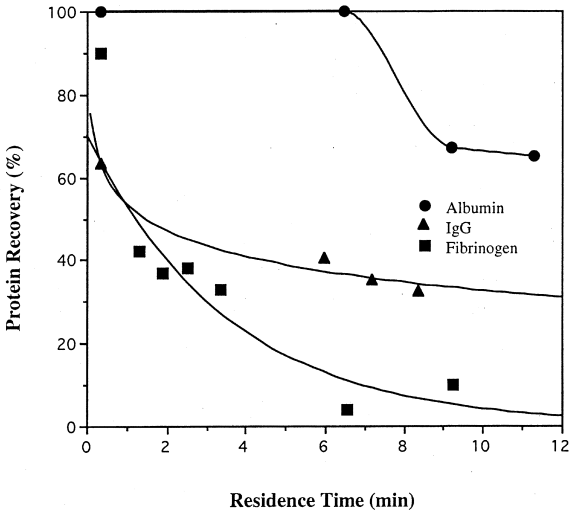

Fig. 2. Protein recovery with time on a quaternary amine support. Proteins were introduced individually to the column under the conditions of pH 7.4, 37°C, and a flow rate of 1.0 ml/min. Recoveries were determined from peak areas by their absorbance measured at 280 nm. Residence times were varied by either prolonging the gradient time (from 0 to 1 *M* NaCl in 5 m*M* Tris) or maintaining the [NaCl]=0 condition for various periods of time. Both methods produced similar results. The average percentage error for all the data points shown was 14% of their mean value.

As the elution time increased, recoveries of IgG decreased in a near-linear manner. This bimodal sorption behavior of IgG was related to the dual elution pattern of the IgG components. The early eluting (void volume) fraction was not retained and was fully recovered for all elution conditions. The late eluting fraction was quickly lost to the support material. If these two fractions had been separated before these experiments, the late eluting fraction would have lost recovery in a manner similar to that of fibrinogen. The aggressive binding properties of this IgG fraction may be related to its need to participate quickly in an immunological response. Perhaps the quaternary amine surface resembles that of a receptor site for which these IgGs target. Fibrinogen recoveries were rapidly diminished under these conditions. This rapid, strong binding of fibrinogen may be related to or explain the observation of a fibrinogen monolayer that typically forms on biological implant surfaces [15–20]. As was suggested in related studies [7,10], these losses of protein with time must be the result of protein unfolding on the chromatographic support. Albumin, for example, resisted unfolding for several minutes. This behavior, together with the strong and rapid binding of fibrinogen, helps explain the phenomenon known as the Vroman effect [21]. In the Vroman effect, when plasma or blood flows over a surface, albumin binds weakly to the surfaces and is replaced by the less abundant fibrinogen. For a quaternary amine surface like that studied here, albumin would be expected to bind weakly and be displaced by fibrinogen. After less than a minute, the fibrinogen would be so strongly bound to the surface that it could not be displaced by other proteins or salts in the blood.

The bimodal adsorption of fibrinogen to polymeric surfaces has been reported [16]. However, the kinetics of the strong binding process have previously not been well characterized. Green et al. [16] suggested that the adsorption kinetics for strong binding may be related to molecular mass. Rousch et al. [17] observed biomodal binding on ion-exchange supports, but did not examine the kinetics of this binding. We plotted protein molecular mass against the time it took for half of the protein to bind to the

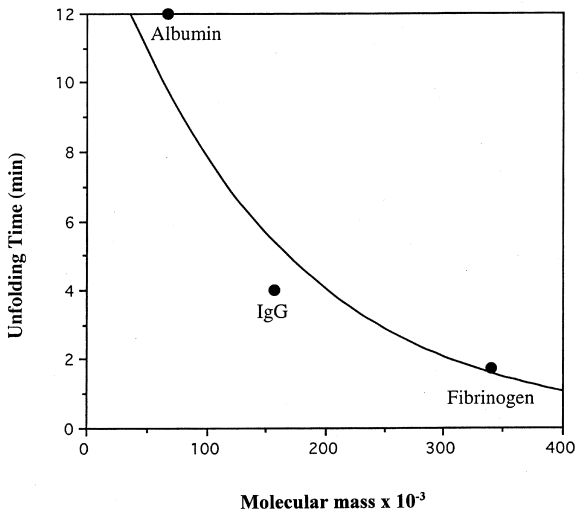

Fig. 3. Correlation between protein unfolding time on a quaternary amine surface and protein molecular mass. Unfolding times are from Table 1. Although the unfolding time for albumin was >12 min, for simplicity the value of 12 min was used. The observed trend for these main plasma proteins was that unfolding time increased as molecular mass decreased.

Table 1
Protein binding to the quaternary amine column

Protein	[NaCl] at desorption[a] (*M*)	Unfolding time (min)[b]
Albumin	0.17	>12
IgG	0.0 and 0.023	4.00[c]
Fibrinogen	0.18	1.75

Both weak and strong interactions are represented. Weak binding is represented by the [NaCl] required to desorb each protein fraction. Strong binding is represented as the unfolding time, or the time required for half of the protein to bind strongly to the surface. Strong binding or unfolding was determined by the protein fraction that would not desorb, even when exposed to a 1 *M* NaCl eluent. Unfolding time was measured in minutes (in this case equivalent to ml). Conditions were as indicated in the footnotes, or otherwise as described in Fig. 1.

[a] Using a 15 min linear gradient from 0 to 1 *M* NaCl.

[b] Time for half of the protein to unfold on the cationic (quaternary amine) surface.

[c] Unfolding time for that fraction of IgG that eluted in the included volume. The excluded fraction of IgG was fully recovered from this column.

support (Fig. 3). While only three data points are shown, the trend is similar to that reported by Green et al. [16].

The correlations between protein recovery and residence time depict important structural features of the three plasma proteins. The adsorption/elution properties of the three proteins on the quaternary amine column are summarized in Table 1. Note the lack of correlation between unfolding time and [NaCl] required to desorb the proteins in Table 1. This can be explained by two processes: initial binding and unfolding. The initial binding is most likely due to the local charge distribution of the protein. The rate of unfolding of a protein should be related to its flexibility, not its charge.

The sulfopropyl surface is anionic and therefore acts as a cation-exchanger under the elution conditions used for the quaternary amine column. The elution order on this surface using a linear gradient appeared to be albumin together with fibrinogen (in the void volume) and the IgGs. Interestingly, IgG appeared as the only retained protein on this surface. The recovery of fibrinogen was 90% when it was isocratically eluted using 1 *M* NaCl in 5 m*M* Tris buffer. However, only 10 to 20% of fibrinogen recovery was obtained when gradient elution from 0 to 1 *M* NaCl in 5 m*M* Tris was used. This indicates that fibrinogen bound strongly and rigidly to the anionic surface similar to its behavior on the cationic (quaternary amine) surface. Once bound, fibrinogen quickly and easily unfolded onto the surface faster and more aggressively on the sulfopropyl than quaternary amine surface. Thus, the void volume actually contained very little fibrinogen under gradient elution conditions.

The recovery results suggest that these three proteins differ greatly in their adsorption behavior. Their adsorption properties can be divided into two categories: strong and weak binding. Weak binding is represented quantitatively by the concentration of NaCl at desorption. Strong binding is indicated by the unfolding time. Tables 1 and 2 together indicate the strong and weak binding behavior of the plasma proteins on oppositely charged surfaces. Since the surface properties were the only variables between the two sets of experiments, the comparison provides an opportunity to determine not only the relative affinity of each surface for the proteins, but also the unfolding rates. Unfolding rates were much faster for fibrinogen on the sulfopropyl than the quaternary amine surface. This difference is possibly related to

Table 2
Protein binding to the sulfopropyl column

Protein	[NaCl] at desorption[a] (*M*)	Unfolding time (min)[b]
Albumin	0.0	Does not unfold
IgG	0.0 and 0.09	1.7[c]
Fibrinogen	0.0	<0.5[d]

Conditions were the same as described either in the footnotes or in Table 1.
[a] Using a 15 min linear gradient from 0 to 1 *M* NaCl.
[b] Time for half of the protein to unfold on the anionic (sulfopropyl) surface.
[c] Unfolding time for that fraction of IgG that eluted in the included volume. The excluded fraction of IgG was fully recovered from this column.
[d] Fibrinogen unfolded too quickly to be measured accurately by this technique.

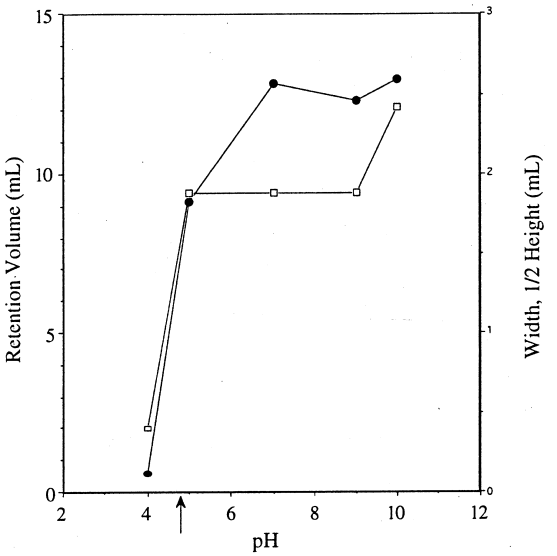

Fig. 4. Effects of pH on the retention volume (●) and peak width of albumin. Peak width (□) was measured at half the peak height. The flow rate was kept at 1.0 ml/min. Other conditions were similar to those described in Fig. 2 except the pH of the eluent and sample solution were adjusted using buffers as described in the text.

the tendency of the protein to unfold or the flexibility of the protein in the binding region. If this is true, then the portion of fibrinogen near the cationic binding region was less stable (more likely to unfold) than the anionic binding region.

An alternative explanation may be that the density of ligands differs between the two columns. Effects of changing the ligand density on retention behavior have been studied [7]. Increasing the ligand density in ion-exchange chromatography caused peak broadening and greater retention. We examined the chromatographic behavior of the plasma protein albumin with pH and studied the broadening of the albumin peak as pH increased. As the pH around albumin shifted to higher values, the number of anionic charges available to bind to the cationic surface increased. Fig. 4 demonstrates how the retention time of albumin increased with pH under conditions of a linear gradient from 0 to 1 *M* NaCl in buffer on a cationic surface. Fig. 4 also shows the p*I* of albumin (arrow). While albumin was weakly retained at pH 4.0, it experienced much stronger retention above its p*I*, at pH 5.0. Retention increased slightly when the pH was shifted further to pH 7.0. However, little change was observed in retention between pH 7.0 and 10.0.

The net positive charge of albumin excludes it at pH 4. At pH 4, the net number of positive charges on albumin is approximately 20. This is apparently just electropositive enough to repel albumin from the cationic surface. The net number of available anionic charged groups on albumin at pH 5.0 is close to 0 [11]. This number increases to approximately 5 negative charge groups at pH 7.0, 20 at pH 9.0 and 30 at pH 10.0 [11]. If these charged groups were evenly distributed around the external surface of the protein, a relatively flat surface (e.g., the support) would probably only be able to interact with a few of these, if we assume that the protein is rigid and spherical. In this case, all albumin molecules at any given pH should be identical, but the surface probably contains a much more random array of charged groups. Since both supports in the present study are weak ion-exchangers, only a small percentage of the surface sites are ionic. The rest are expected to be polar but neutral. If the surface charges were randomly arranged, then the arrangements between oppositely charged species (the interface between the protein and surface) would vary by some distribution function. This anisotropy of charge groups on both ion-exchange supports and proteins has been discussed previously [22]. The strength of interaction and thus the [NaCl] required for desorption should therefore follow a distribution function. If this is

true, then as the number of interaction sites between the protein and the surface increases, so should the distribution of force between the two surfaces. The result should be peak broadening.

Fig. 4 represents the broadening of the albumin peak as the pH was increased. This is consistent with the above discussion because the number of interaction sites should increase with pH and retention time. From a chromatographic perspective, peak broadening with increased retention is well understood in isocratic separations. However, the relationship between peak broadening and protein–surface interactions may be instrumental in understanding the adsorption process. In reversed-phase chromatography, bandwidth has been related to the interconversion of different protein conformations [23]. A similar explanation could apply to peak broadening in ion-exchange chromatography. Deconvoluting this information will help understand the molecular interactions responsible for adsorption phenomenon between proteins and synthetic surfaces and lead to a deeper understanding of the molecular interactions at interfaces.

Albumin unfolds in solution below pH 4 [11]. There may be more subtle changes in the tertiary structure of albumin with pH, but using this chromatographic approach, no changes in retention were observed in the region of pH 5 to 9. Our explanation for this phenomenon is that in this region, the same number of charged groups from the protein bind to the support. This may be partly because of the low charge density of the support as well as the number of available charged groups in the binding site of the protein. Therefore, it is consistent with our observations that while some changes may take place in albumin tertiary structure between pH 5 to 9, the ionic appearance of the protein to the chromatographic support is relatively unaltered.

Throughout this paper, we have discussed the nature of the interaction between the proteins and the solid support. Kopaciewicz et al. observed both weak and strong binding of proteins to an ion-exchange support that was silica-based [7]. The results in Tables 1 and 2 were on two polymer-based ion-exchange supports. Kopaciewicz et al. [7] observed strong binding of their proteins that they believed was due to hydrophobic interactions. This was because the protein would not desorb using high concentrations of NaCl, but would desorb in the presence of organic solvent and trifluoroacetic acid (TFA). We did not attempt to use these eluents in our experiments because they would damage the support material. Yet, proteins were removed in our system using a 0.1% solution of sodium dodecyl sulfate (SDS). Neither of these methods preclude the protein binding being due to ionic interactions because TFA and SDS are ionic and both are capable of denaturing proteins. It is equally likely that an alternate binding mechanism is responsible for the strong binding observed in this study. As an example, consider fibrinogen. On the quaternary amine surface, some fibrinogen was clearly retained by an ion-exchange process (weak binding). It eluted in the presence of 0.18 *M* NaCl (Table 1). The same protein unfolded on the quaternary amine surface quickly (strong binding) in 1.75 min (Table 1). If the only interaction between the protein and the surface was ionic, then one could argue that as the protein unfolds, more ionic sites interact and prevent the protein from desorbing. However, Table 2 shows a contrasting situation. On the sulfopropyl surface, the same protein did not bind at all in the ion-exchange (weak binding) mode, as evident by the lack of retention. Yet, as the percentage recovery indicates, fibrinogen quickly unfolded on this surface. One argument that may explain this apparent discrepancy is that fibrinogen unfolds more easily near the sulfopropyl binding site than near the quaternary amine binding site. Strong binding at this site could have been ionic even though there was apparently no weak binding. It may have just involved a large density of sites on the protein that precluded weak desorption.

In contrast, this apparent discrepancy between the two types of binding could also be explained if the strong and weak binding were by entirely different mechanisms. Ionic binding between fibrinogen and the sulfopropyl groups may not have occurred at all, as indicated by its elution in the void volume (Table 2). The binding that was observed may have been from a different mechanism. Some possible alternate binding types include hydrophobic, Van der Waals [24], London dispersion, hydrogen, or other types. Any one or more of these may have been stronger in the sulfopropyl than in the quaternary amine column. These alternate explanations will be the subject of future work.

4. Conclusion

Adsorption properties of both weak cation- and anion-exchange materials were investigated. These studies have so far involved only two polar surfaces, but the method can be easily adapted to study any number of surfaces. The technique used is ideally suited for studying protein adsorption because flow and temperature parameters can be carefully controlled to meet almost any condition while measuring binding properties to already well defined surfaces. Chromatography has the unique capability to relate various structural parameters and unfolding characteristics of proteins in solution. This technique is advantageous when HPIEC is used in that the proteins may be characterized in what is close if not identical to their native state. Based on recovery, it may be determined if the protein resists unfolding, unfolds linearly with time, or quickly binds and unfolds to the surface. This technique is also invaluable for studying the actual processes of adsorption by polar, or hydrophilic, interactions. The influence of charges involved in an interaction can further help us understand the adsorption process by identifying the number of charge groups associated with binding (e.g., Z number). This can possibly lead to identifying the binding site, for example. Future work will focus on a more comprehensive theory of the protein adsorption process.

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