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Protein losses in ion-exchange and hydrophobic interaction high-performance liquid chromatography

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Abstract

Protein losses in ion-exchange and hydrophobic interaction HPLC were examined. The supports were all non-porous, packed in columns of identical dimensions. Two ion-exchange chromatography (IEC), anion and cation, as well as a hydrophobic interaction chromatography (HIC) columns were tested. Proteins included cytochrome *c*, bovine serum albumin (BSA), immunoglobulin G and fibrinogen. Temperature effects on HIC supports were studied for cytochrome *c* and BSA. Both retention times and recoveries of the proteins were measured. The influence of column residence time on the recovery of proteins was also investigated. We found a linear relationship between the amount of protein recovered and the log of the molecular mass. Retention times also generally increased with temperature for both HIC and IEC. Other trends in retention behavior and recoveries are discussed.

Keywords: Hydrophobic interaction chromatography; Proteins

1. Introduction

Liquid chromatography (LC) has become an essential tool for biochemistry. Numerous proteins are separated by LC or high-performance LC (HPLC), often with high recoveries and good activity. Sometimes, separations are much less successful and proteins are lost [1–3]. Enzyme activities may also be lost, especially in more aggressive separations such as reversed phase [4]. Reversed-phase HPLC can denature proteins during elution, a temperature dependent process [5]. However, sorption to polystyrene can also cause losses in enzyme activity [6].

The process of sorbing proteins to solid supports can be most easily explained if the proteins behave as rigid spheres. However as the proteins become more flexible, they also adsorb differently, and can bind more easily to hydrophilic surfaces [7]. Several recent articles have examined the adsorption behavior of large proteins on solid supports [6,8–13].

Chromatographic retention is determined by a relatively small number of amino acids located in a contact region on the surface of a polypeptide [14]. One rigorous model for the separation of proteins by ion-exchange HPLC incorporates both detailed protein and surface structure [15]. This model examines contributions of more than one possible conformation of the protein. This and other molecular-level models for ion-exchange properties of proteins [16] contribute to our understanding of the HPLC separations. As our understanding of the ion-exchange process continues to improve models such as this will be able to more completely predict both retention and losses of proteins.

Hydrophobic interaction chromatography (HIC) was originally thought to be a much less denaturing

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separation process than reversed-phase chromatography [17]. It was later demonstrated that HIC can denature proteins [18–20]. Yet, HIC was still shown to be a much less denaturing chromatographic medium than reversed-phase chromatography. It was further suggested that ion-exchange chromatography (IEC) should be a more gentle separation process for proteins than HIC since the exposed amino acids in soluble proteins are polar, more like a weak ion-exchange than a hydrophobic support [18].

Binding processes between proteins and solids are important in nature [21]. In this case, transmembrane proteins bind to iron oxide and convert the oxide to its metallic form while transferring electrons to oxidize organic species during bio-remediation. Other examples can be found in biomaterials and blood coagulation and bio-fouling processes [7,22].

We have recently examined the influence of ion-exchange chromatography on losses of plasma proteins [2]. In these studies, larger proteins were lost on the sorbent within a few minutes, and smaller proteins were more easily recovered. For example, 50% of the fibrinogen (M_r=340 000) was lost within 2 min, whereas bovine serum albumin (BSA) (M_r 68 000) was fully recovered over that time period. Subsequent experiments with cytochrome *c* (M_r 12 400) on a similar but oppositely charged sorbent showed losses only when both the temperature and residence time were elevated [3].

BSA adsorption on a metal oxide surface has been shown to involve both reversible and irreversible binding [23]. A model was generated to describe both reversible and irreversible binding processes. Results agreed with the model. It was concluded that the irreversible binding was due to the protein being introduced to the surface in an orientation that favored strong binding. The rate of desorption was also found to be inversely proportional to the square root of the free area. This suggested lateral interaction between adsorbed proteins in which neighbors promoted their desorption.

It has been suggested that protein losses in ion-exchange HPLC are due to hydrophobic effects [24]. Correlations between temperature, molecular mass and losses have been examined for ion-exchange HPLC [2]. Differences in retention times but not recoveries were examined in HIC [25]. In the present study, we explore how protein recoveries are influenced in HIC and compare these results to those of IEC.

2. Materials and methods

The HPLC systems used in this study were from Bio-Rad (Hercules, CA, USA), Waters (Milford, MA, USA), and Hewlett-Packard (Palo Alto, CA, USA, Model 1090). All three systems were designed to operate in the gradient mode and were equipped with a multi-solvent delivery system. Detection and quantification of protein in all systems was by UV absorbance in a 9 μl flow cell at 280 nm (for all proteins). The standard flow-rate was set between 0.5 and 1 ml/min. Temperature was regulated using either a Bio-Rad accessory column heater (Bio-Rad and Waters HPLC systems) or an internal column heater (Hewlett-Packard HPLC system).

Chemicals and reagents were of the highest purity available from Sigma (St. Louis, MO, USA) unless indicated otherwise. Buffer A consisted of 5 m*M* Tris, pH 7.4 (reagent grade). Buffer B was identical to buffer A, except that it also contained 1.0 *M* NaCl. For HIC, the initial buffer consisted of buffer A with ammonium sulfate. The ammonium sulfate concentration varied for each protein. An initial concentration of 1.7 *M* ammonium sulfate was used for human serum albumin (HAS), 3 *M* for cytochrome *c*, and 0.5 *M* for fibrinogen. All buffer solutions were standardized to pH 7.4 with concentrated HCl, and filtered through a 0.45-μm membrane (Millipore, Bedford, MA, USA).

Sample preparation for each protein consisted of solubilizing the protein in its starting buffer. Protein concentrations were 1 mg/ml. Protein solutions were mixed daily for freshness. Some protein solutions were also stored at 2–8°C when not in use, but were warmed to room temperature for at least 2 h before each analysis. We observed identical results from freshly prepared samples, and those kept 2–3 days under these conditions. Proteins used were human fibrinogen (National Scientific Supply, San Rafael, CA, USA); bovine fibrinogen (fraction 1); BSA (fraction V); and bovine immunoglobulin G (IgG); cytochrome *c* was from horse heart.

Although our cytochrome *c* was the highest purity available from Sigma, we were concerned that the

losses we observed in previous experiments [3] may have been due to impurities. We found that both the purified and unpurified cytochrome *c* behaved identically, but we chose to use purified cytochrome *c* for these experiments. Our method for purifying cytochrome *c* was using a 100 cm×1 cm column and gel permeation (Bio-Gel P-30, 100–200 mesh, Bio-Rad Labs.). Dialysis was also used to remove unnecessary buffer or salt components. Purity was further monitored using sodium dodecyl sulfate–polacrylamide gel electrophoresis (SDS–PAGE). The SDS gels revealed a very small amount of protein material in the molecular mass region of 24 000. The purified cytochrome *c* did not contain detectable levels of this material. The contaminating material may have been a dimer of cytochrome *c*.

The column materials consisted of predominately quaternary amine, sulfopropyl or butyl (HIC TosoHaas column, Montgomeryville, PA, USA) functional groups. All columns were pre-packed by the manufacturer and had dimensions of 35×4.6 mm. The particle size was 2.5 μm.

We routinely cleaned the columns, using either a solution of 0.1% SDS, 0.1 *M* NaCl, and 5 m*M* Tris or 6 *M* urea. The cleaning solution was eluted for 10 min to 1 h. A separate solution of 20% methanol in water was used to clear the column of remaining SDS. Occasionally, 0.1 *M* NaOH was flushed through the column for approximately 2 h as a cleaning technique. As a conclusion to each clean-up process, a solution of 5 m*M* Tris–HCl, pH 7.4 was eluted until equilibrium between mobile and stationary phase was reached, usually overnight.

3. Methods

The HPLC gradients for IEC began with buffer A and ended with 1.0 *M* NaCl in buffer A. For the HIC experiments, each gradient began with high concentrations of ammonium sulfate and ended with buffer A.

Most IEC analyses were conducted at room temperature with a flow-rate of 0.5 ml/min. The injection volume for each experiment was 20 μl of 1.0 mg/ml protein. The gradient consisted of a linear increase in the concentration of NaCl from 0 *M* to 1 *M*. After each gradient, the column was washed for 5 min with buffer B to ensure elution of all recoverable protein. Bovine fibrinogen, human fibrinogen, bovine albumin, bovine IgG, and horse heart cytochrome *c* were examined in this study.

The HIC study used a flow-rate of 1.0 ml/min, often at room temperature.

Human serum albumin and horse heart cytochrome *c* were studied for temperature effects in HIC. The HIC column temperature was adjusted to 0°C (ice bath), 25°C, 40°C and 55°C. The gradient profile consisted of a 10-min linear gradient of 100% ammonium sulfate to 100% Tris–HCl. Experiments were carried out using a flow-rate of 1.0 ml/min.

Absorbance was recorded to monitor protein recoveries. Protein losses were calculated as the difference in absorbance between that from a low-dead-volume connector and from a column. Protein absorbance was monitored at 280 nm [2,3].

Peaks of eluting proteins were integrated, using HPLC software and constant flow as described previously [2]. Retention time (t_R) was calculated as the time between the injection of the protein into the system and the elution of the protein. Residence time was the time the protein spent on the column and was calculated by subtracting the dead volume t_R from the observed t_R.

4. Results and discussion

The elution profiles of both cytochrome *c* [18] and BSA [2,25] were examined as a function of temperature in HIC. Since the specificity of each column material is slightly different, we repeated these experiments for this HIC support. Results are shown in Fig. 1. Here, the continually increasing elution time with temperature was similar to previous observations [17,18,20]. The previous study showed increasing retention times with temperature for cytochrome *c*, myoglobin and BSA, but not lysozyme [17]. A few subtle differences could be seen in the chromatographic profiles as shown in Fig. 1. In Fig. 1, the single eluted peak at 55°C shifted to a lower retention time at 40°C. However, at 25°C a shoulder appeared at a greater retention time. This shoulder was larger and more pronounced at 0°C, where another shoulder appeared. Ingraham et al. [18] examined the temperature range 15 to 50°C in the

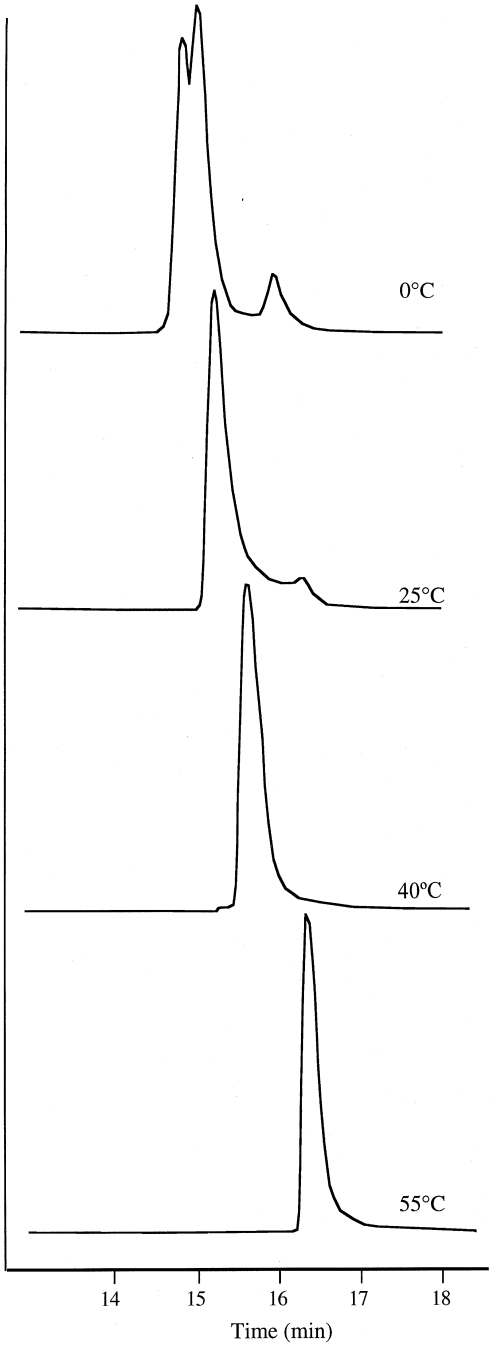

Fig. 1. The HIC separation of cytochrome *c* as a function of temperature. Horse heart cytochrome *c* was eluted through a Butyl NP column using a linear gradient from 3.0 *M* to 0 *M* ammonium sulfate in 5 m*M* Tris buffer. Temperatures were adjusted with a column heater. All three peaks in these chromatograms had identical absorbance spectra, indicating the separation was not due to the oxidation state of the protein.

(porous) Phenyl 5PW column. In this case, a single peak was observed at 15°C with additional smaller peaks forming with increasing temperature. A large transition in the peak profile occurred between 30 and 35°C when the Phenyl 5PW column was used. In the present study we only observed one elution peak throughout this temperature range. The different behavior of albumin in these two columns may be due to differences in selectivity of the butyl and phenyl groups. This change was the loss of the large peak observed at the lower temperatures, and its replacement with a peak eluting much later in the gradient at higher temperatures. In both the earlier work [18], and the present study, the peak area (and recovery) appears to diminish with temperature (see Fig. 1). This trend is consistent with our earlier study in which we attributed losses of cytochrome *c* with increasing temperature to surface-mediated protein unfolding [3]. Thus, two proteins adsorbed more strongly to different sorbents as the temperature increased.

In a previous study, when the Phenyl 5PW column was used to analyze BSA [18], there were two peaks, which began to separate from one another at a temperature between 25 and 30°C. The explanation for this peak splitting was an ammonium sulfate-induced structural transition of albumin [17]. When the butyl column was used in the present study, a second peak was not observed. The same HIC gradient was used for both separations (using decreasing concentrations of ammonium sulfate in a linear gradient), and both columns were polymer-based. The Phenyl 5PW support contained 5 μm particles with 1000 Å pores [17], whereas in the present study the butyl column contained a non-porous support with 2.5 μm particles. The different surface chemistries (phenyl vs. butyl) are one plausible cause of the variation in selectivity. Another is the density of hydrophobic residues on the support.

The albumin peak increased in retention time with temperature, using a linear gradient. This increase in retention time was observed with both sorbents. In the butyl column, the increase in retention time was linear with temperature (Fig. 2). Protein structural perturbations have been suggested as the cause of the elution time shifts [26]. We have observed similar results in HIC for cytochrome *c*, myoglobin, BSA, and lysozyme [17]. Differing slopes for each protein

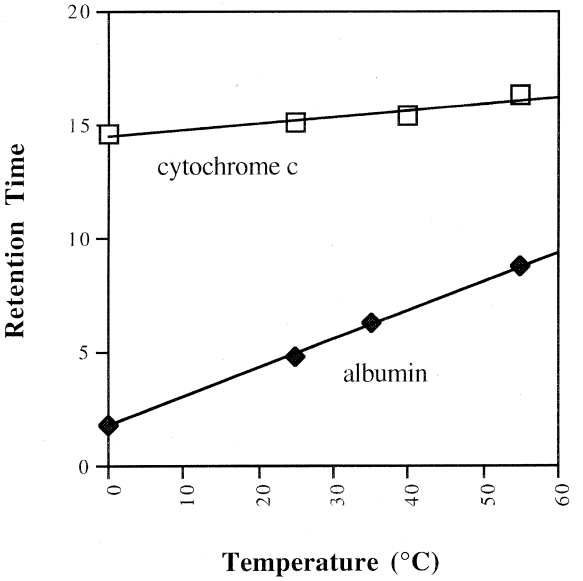

Fig. 2. Retention time of cytochrome *c* and BSA as a function of temperature. Separations were performed by HIC according to the conditions described in Fig. 1, except that BSA was separated using a linear gradient from 1.7 *M* to 0 *M* ammonium sulfate in 5 m*M* Tris buffer.

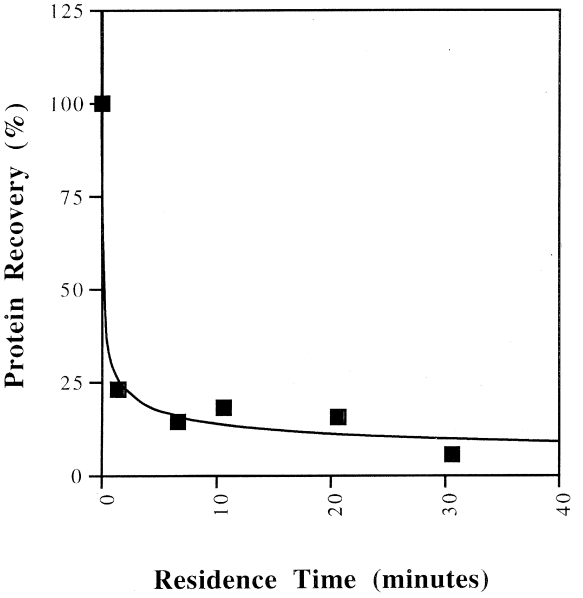

Fig. 3. Recovery of human fibrinogen as a function of time on the HIC support at room temperature. The initial data point indicates recovery of protein when a low-dead-volume connector was used. Recoveries of human fibrinogen from this column were 90% when the protein passed through the column unretained at 0°C (in the absence of ammonium sulfate). The remaining data points were obtained from passing human fibrinogen through the Butyl NP column using a linear gradient from 0.5 *M* to 0 *M* ammonium sulfate in Tris buffer at room temperature.

defines the influence of the kinetic energy of the protein and eluent on protein hydrophobicity. Proteins exhibiting greater retention with temperature bind with more of their surface exposed to the sorbent [27].

We have studied the shifts in retention time for cytochrome *c* [3,28], BSA, IgG and fibrinogen [2] in ion-exchange HPLC. In HIC, we had previously observed a retention time shift of approximately 0.4 min for BSA between 0 and 55°C. This retention time shift may be due to the exposure of hydrophobic regions in the proteins. Proteins will also flex more throughout their structure as the temperature is increased, allowing for more attachment points, as we have discussed previously for cytochrome *c* in ion-exchange HPLC [3].

The recovery of human fibrinogen at 25°C, as a function of the time the protein was exposed to the sorbent in HIC, is shown in Fig. 3. Here, recovery diminished almost immediately with time exposure, and continued to decrease with longer exposure times. This is similar behavior to that observed for bovine fibrinogen in ion-exchange HPLC [2]. In the temperature range 0 to 55°C, approximately 80% of cytochrome *c* was recovered using the HIC column. The large difference between losses of fibrinogen and cytochrome *c* in HIC were similar to those observed in IEC previously [2,3].

Recoveries of several enzymes and enzyme activities have been reported in the 80–100% range using HIC [26]. Methods used to obtain those recoveries were considerably different from the method used in the present study. Enzyme recoveries and activities are commonly measured after the enzymes have passed through the column under non-binding conditions. Many of the enzymes with high recoveries had low molecular mass (e.g., lysozyme M_r=14 500, chymotrypsin M_r=25 000). In contrast, the M_r values of two proteins studied here range from 12 400 to 340 000.

As we compared the recoveries of proteins from the various sorbents, there appeared to be a strong correlation with molecular mass. The higher the M_r,

the lower the recovery. We chose room temperature and a residence time of 9 min and plotted the recoveries with the log M_r. Similar plots were not examined at other temperatures. Other residence times gave similar results, but whether the slopes are significantly different may require the collection of more data points. At holding times much shorter than 9 min, albumin was not lost in IEC [2]. However, at a 9 min holding time, all proteins demonstrated a linear pattern with log M_r (Fig. 4). Here the loss of protein from the ion-exchange column appears to have a steeper slope than that of the HIC column, but the (% lost) intercept was greater in the HIC case than for IEC. A greater (% loss) intercept suggests smaller molecules are lost more easily. A greater slope suggests molecular mass has a stronger influence on recovery. Therefore, Fig. 4 suggests that IEC induced protein losses to a greater extent as molecular mass increased than did HIC. This would make the most sense if the losses were partly due to surface-mediated unfolding and the number of contact points. Others have suggested chromatographic losses are due to protein unfolding on the solid support [4,29,30]. If hydrophobic interactions caused unfolding in ion exchange, we should observe a similar behavior in IEC to that of the HIC curve. Or, since the IEC support was less hydrophobic than the HIC support by design, the slope should be the same or smaller if hydrophobic interactions were responsible for the unfolding process in IEC. The opposite appears to be true. That is, more unfolding appears to have occurred in lower-molecular-mass proteins on this HIC support than on the non-porous IEC support.

The slope of these lines at other temperatures should be investigated further. Others have found that the proteins that are strongly bound to polymeric supports can retain their thermal properties [11]. It has also been suggested that protein loading may influence unfolding on the surface with less unfolding as more protein is bound [6,11].

When additional proteins are examined by this technique, it would be surprising if they would fit precisely on these two (HIC and IEC) lines. How-

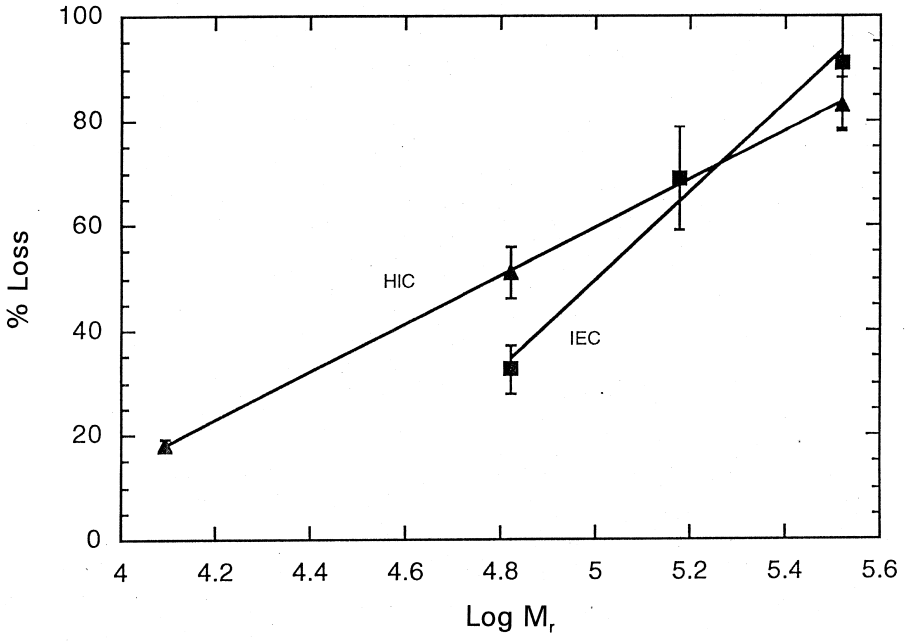

Fig. 4. Loss of several proteins as a function of molecular mass for both IEC and HIC. Proteins depicted are cytochrome *c*, BSA, IgG and fibrinogen. Conditions were as described in Fig. 1 for HIC, and as described elsewhere for IEC [2,3]. All analyses for this figure were carried out at room temperature. Losses were calculated at a retention time of 9 min of exposure to the column support. The figure shows data for both anion-exchange (fibrinogen, BSA and IgG) and cation-exchange (cytochrome *c*) supports. Standard deviations are indicated.

ever, we can conclude that higher-molecular-mass proteins are more susceptible to losses for both types of surface chemistry. Furthermore, these two types of column chemistries have different slopes and *y*-intercepts which are characteristic of the sorbent rather than the protein.

The tendency of the log of the molecular mass to be related to unfolding rather than some other function is surprising. If proteins were perfect rigid spheres, then the contact area would be related to approximately the M_r of the protein (volume of the sphere) and the kinetic energy would be related to the contact area, since all proteins were analyzed at the same temperature for the data points in this figure. Protein flexibility and instability should also increase with molecular mass, related to a number of parameters including the number of degrees of freedom in each of the bonds. The relationships shown in Fig. 4 suggest that as the molecular mass increased an order of magnitude, losses to the surface only increased linearly. Whether this is consistent with the flexibility or stability of a protein as the key variable responsible for the degree of loss is not clear. These relationships should be examined more fully so that the observed phenomena can be correlated with a plausible model.

Recent studies suggest that in IEC, proteins do not need to contact the support to be retained [31]. This may be especially true in isocratic separations in which the migration of proteins along the column only need be slowed to be successfully separated. When gradients are used, the protein should have more time and opportunity to migrate to the solid–liquid interface. Since either way, the protein would be retained, whether proteins actually touch the surface may not be of critical importance, except in the ability of the proteins to bind strongly enough to the sorbent to remain attached. We have typically observed several minutes of delay between the time it takes for a protein to elute fully recovered, to the time needed to lose some portion of the protein to the sorbent [2,3]. This delay may either be related to protein unfolding kinetics, protein diffusion through the ''affected layer'', or layer of protein and sorbent and the liquid–solid interface, or a combination of the two. Diffusion is an unlikely explanation for the delayed losses we have observed since smaller proteins should diffuse more rapidly than the higher-M_r components. The trend we observe is the opposite. That is, higher-M_r proteins were lost more quickly than those of lower M_r (Fig. 4). Therefore, losses are more likely delayed, not because of diffusion, but because larger proteins tend to have more flexibility. This gives them more opportunities over time to find more binding sites so that eventually they may be too strongly bound to desorb under normal elution conditions.

In a related study, the structural effects of heating IgG on adsorption were studied [32]. It was found that structural changes in IgG caused by heating and by adsorption were different, but that after heating the structure of adsorbed protein approached that of thermally denatured protein [32]. Zoungrana and co-workers [6,11] have shown that various sorbents cause proteins to unfold, but that this process is minimized when the protein concentration in maximized on the surface. In our investigations, we made no effort to maximize the surface concentration of the proteins.

An alternate explanation to surface-mediated unfolding is that proteins may be less stable in solution and more likely to aggregate as molecular mass and temperature are increased. Our controls were low dead volume connectors, not ''inert'' filters. An inert filter could show a similar effect to that observed in Fig. 4 (assuming negligible influences from surface chemistry). It is difficult to find a truly inert filter. In a previous study, we examined anion and cation exchangers of identical particle size (and column dimensions). The losses between ion exchangers were significantly different even though the elution buffers and conditions were identical [2]. We have also clearly demonstrated time-dependence on the observed losses. We have also shown that cytochrome *c* is lost to an anionic support at a much lower temperature than expected in solution. Therefore the instability of proteins in solution is an unlikely explanation. A more consistent explanation for the losses we have observed is that the sorbent acts as a catalyst for protein unfolding [3] or that the proteins become unstable as they adhere to the sorbent.

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