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Hydrophobic interaction chromatography of proteins I. The effects of protein and adsorbent properties on retention and recovery

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Abstract

The contributions of protein and adsorbent properties to retention and recovery were examined for hydrophobic interaction chromatography (HIC) using eight commercially available phenyl media and five model proteins (ribonuclease A, lysozyme, α-lactalbumin, ovalbumin and BSA). The physical properties of the adsorbents were determined by inverse size exclusion chromatography (ISEC). The adsorbents examined differ from each other in terms of base matrix, ligand density, porosity, mean pore radius, pore size distribution (PSD) and phase ratio, allowing systematic studies to understand how these properties affect protein retention and recovery in HIC media. The proteins differ in such properties as adiabatic compressibility and molecular mass. The retention factors of the proteins in the media were determined by isocratic elution. The results show a very clear trend in that proteins with high adiabatic compressibility (higher flexibility) were more strongly retained. For proteins with similar adiabatic compressibilities, those with higher molecular mass showed stronger retention in Sepharose media, but this trend was not observed in adsorbents with polymethacrylate and polystyrene divinylbenzene base matrices. This observation could be related to protein recovery, which was sensitive to protein flexibility, molecular size, and conformation as well as the ligand densities and base matrices of the adsorbents. Low protein recovery during isocratic elution could affect the interpretation of protein selectivity results in HIC media. The retention data were fitted to a previously published retention model based on the preferential interaction theory, in terms of which retention is driven by release of water molecules and ions upon protein-adsorbent interaction. The calculated number of water molecules released was found to be statistically independent of protein retention strength and adsorbent and protein properties.

Keywords: Inverse size exclusion chromatography; Phenyl adsorbents; Pore size distribution; Phase ratio; Adiabatic compressibility; Protein recovery

1. Introduction

Although ion exchange chromatography (IEC) remains the most widely used chromatographic method for large-scale separations, hydrophobic interaction chromatography (HIC) is also widely used. HIC is usually assumed to separate proteins by the differences in their surface hydrophobic character. Therefore, it provides an orthogonal chromatography technique to IEC, affinity and size exclusion chromatography for biomolecule purification. HIC and reverse phase chromatography (RPC) are assumed to achieve protein separation based on similar principles. However, RPC employs highly hydrophobic surfaces and concomitantly harsh eluents, with the resulting protein denaturation making the method suitable mainly for analytical separations. HIC uses less hydrophobic surfaces, and because of the milder protein-ligand interactions, proteins purified by HIC usually retain their native conformations and biological activities. HIC has been used to purify such products as antibodies [1,2], recombinant proteins [3], and plasmid DNA [4,5].

Most quantitative data and analysis in HIC studies have focused on retention. The retention factor, k', under linear retention conditions can be expressed as

$$k' = K_{eq}\phi \quad (1)$$

where K_{eq} is the adsorption equilibrium constant of a solute on the stationary phase and ϕ is the phase ratio of the adsorbent.

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Thus, understanding the quantitative basis for linear retention requires probing both the thermodynamic factors captured by K_{eq} and the structural ones reflected by ϕ. The thermodynamic factors relevant to protein retention in HIC are dominated by the high sensitivity to salt concentration and the chemical nature of the salt. Protein adsorption in HIC is induced by high salt concentrations such as those used in precipitation, and indeed in its nascent stages HIC was sometimes referred to as salting-out chromatography [6]. Later experimental results showed that salts with higher "salting-out" ability increase protein retention in HIC [7]. Thus, analysis of retention has been guided by that of protein solubility, with solvophobic theory [8–12] and preferential interaction theory [13–18] the approaches most commonly used.

In addition to the solvophobic theory and preferential interaction theory, other retention models have also been developed [19,20], although they have not been widely used. The model developed by Staby and Mollerup [20] shows that salts that increase the activity coefficient of the protein in the mobile phase improve retention. This emphasizes an important general point, namely that protein hydrophobicity is not the only factor affecting retention in HIC.

Eq. (1) indicates a direct dependence of retention on the physical structure of the stationary phase in addition to the thermodynamics of protein solutions and adsorption. This explicit dependence on a quantitative stationary-phase property conceals the possibility that adsorbent physical properties may also affect retention in other ways. For example, it has been shown that cation-exchange adsorbents with pore size distributions (PSD) containing a significant amount of pore space with dimensions similar to those of the protein solute display increased protein retention [21].

Assuming that the phase ratio is the principal stationary-phase physical property of interest, it is the lack of comprehensive information on the physical properties of HIC media that makes it difficult to compare and analyze experimental results generated with different media. Mercury porosimetry and nitrogen adsorption are two methods commonly used to measure the PSD and surface area of porous solids. However, they are performed under conditions that are not relevant to chromatography applications. To obtain more accurate column void volumes, a NMR method was developed [22], but it tends to underestimate the intraparticle porosity for large probe molecules due to pore blockage caused by protein adsorption. Inverse size exclusion chromatography (ISEC) is an alternative method for determining the PSD and accessible surface area of chromatographic media [23–26] that has the advantages of simplicity and being performed under normal chromatographic conditions; this approach is used here.

The other contribution to Eq. (1) is the adsorption equilibrium constant, the estimation of which is the objective of retention models. The retention models outlined above are largely conceptual. They typically account for the salt concentration and salt type via parameters such as the molal surface tension increment or the preferential interaction parameter. However, they do not account for properties such as ligand type, ligand density and most notably, the structural properties of the protein. This issue was partially addressed by a mathematical model developed to predict retention based on protein average surface hydrophobicity [27]. A key additional complicating factor to be considered in HIC is that a protein's native conformation can change when it is adsorbed onto a hydrophobic surface [18,28–30]. Jones and Fernandez [31] and Wu et al. [18] showed that the adsorption of α-lactalbumin onto HIC surfaces could cause the protein to unfold. Therefore, understanding the correlation between protein and adsorbent physical properties and retention can shed light on the mechanisms of protein retention in HIC.

It is well known that proteins can undergo conformational changes under high pressure [32]. Dadarlat and Post [33] showed that proteins with high compressibilities have higher heat capacities per residue, ΔC_p, upon unfolding. Murphy et al. [34] showed that the change in entropy per residue, ΔS, of proteins decreases with increasing ΔC_p when proteins unfold. These two results suggest that proteins with high compressibilities also have high conformational entropies, hence flexibilities, in their native states. This is consistent with the correlation that proteins with higher compressibilities are more thermally stable [35]. On the basis of these results, adiabatic compressibilities, k_s, of proteins were used to reflect their flexibilities in this study. Studies also indicate that proteins with high $k_s/\Delta C_p$ tend to have more hydrophobic cores [35,36]. Adiabatic compressibility has been used to probe conformational changes in proteins adsorbed on silica particles [37], polystyrene particles [38] and reverse phase media [39]. It was found that higher adiabatic compressibilities lead to more extensive conformational changes when proteins are adsorbed onto solid surfaces [37,38]. Mahn et al. [40] showed that ribonuclease S has higher retention on a butyl adsorbent than ribonuclease A, ribonuclease T1 wild type and ribonuclease TI variant although they all have almost identical surface hydrophobicity. It was suggested that the higher flexibility of ribonuclease S could be responsible for the stronger retention. On the basis of these results, protein flexibility could have a profound effect on protein retention in HIC.

Extensive experimental studies have been conducted to investigate the effects of the salt [30,41,42], ligand type [7,42–47], ligand density [7,48], pH [7,18,42,49–51], and temperature [18,43,49,52,53] on protein retention in HIC. In general, high concentrations of kosmotropic salts promote retention. All these studies suggest that the performance of HIC can be optimized by using appropriate combinations of these factors, but not yet in a predictable fashion. Although many different sets of protein retention data in HIC are available in the literature, they were generated with different adsorbents and proteins under different conditions. The paucity of large sets of retention data in which individual factors are varied systematically makes it difficult to analyze how different factors contribute to protein retention in HIC. Two very large data sets providing retention curves for proteins on commercially available stationary phases have appeared recently [54–56], but little mechanistic analysis was provided.

In this paper, the physical structure and PSD of eight commercially available HIC media were determined. The effects of the adsorbent characteristics, as well as of the protein molecular size, surface properties and structural flexibility on retention on