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Suitability of commercial hydrophobic interaction sorbents for temperature-controlled protein liquid chromatography under low salt conditions

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ABSTRACT

The effect of temperature in the range from 10 °C to 40 °C and comparatively low ammonium sulfate (AS) concentrations of up to 0.5 M on the adsorption of bovine serum albumin (BSA) on four different commercially available sepharose-based stationary phases was investigated. The determined isotherms were fitted by the Langmuir equation, and thermodynamic values were calculated by van't Hoff analysis. The adsorption of BSA onto the chromatographic resin Butyl Sepharose 4FF showed the strongest temperature influence; however, protein unfolding effects occurred when characterizing this system by dynamic column experiments, with an unfolded BSA fraction strongly attached to the sorbent. The percentage of the unfolding fraction was determined for different operating conditions and found to increase with the concentration of the cosmotropic salt, but even stronger with increasing temperature. Temperature-induced cyclic adsorption and desorption experiments were carried out to investigate the long-term performance of Butyl Sepharose 4FF by applying purely temperature-controlled regeneration. Over a period of five cycles, the working capacity remained stable, but BSA also started to accumulate on the column due to incomplete regeneration. Finally, the possibility to fractionate different proteins with a single temperature shift was shown by the complete separation of lysozyme and BSA. The results presented indicate that temperature-induced binding and elution may offer a possibility to shift the operation conditions of HIC resins toward reduced salt concentrations, thus saving chemicals and facilitating salt removal in further downstream processing stages.

1. Introduction

Hydrophobic interaction chromatography (HIC) is known to be an effective liquid chromatography technique for the separation of proteins [1,2], plasmid DNA, and DNA vaccines [3,4], as well as the removal of viruses from human plasma and the isolation of bacterial enzymes [5]. A number of applications are known for protein purification [1] and have been successfully put into practice in the lab and preparative scale [6–9]. The principle of HIC was described in 1949 by Shepard and Tiselius [10], but Hjertén [11] was the first to call it hydrophobic interaction chromatography. Much effort has been invested in the study of the different parameters influencing protein adsorption on HIC resins [12]. In contrast to reversed phase chromatography (RPC), which also uses hydrophobic interactions as the basic principle of separation, HIC does not face severe drawbacks concerning the purification of sensitive biomolecules [13] because mild aqueous buffers can be used as the mobile phase instead of harsh solvents. Many different HIC resin backbones currently exist, whereas commercially available functionalized sepharose (a cross-linked, beaded form of the polysaccharide agarose) is often used for the purification of proteins. HIC-specific ligands differing in chemistry, chain length, and constitution on the support are coupled onto this matrix, resulting in a variety of different protein adsorption characteristics. In general, linear-chain alkanes and aromatic groups, such as phenyl, are used. The retention mechanism of proteins onto HIC resins has been widely studied and several theories have been developed [14]. The increase in entropy originating from the release of water molecules, which are originally located in a more structured water layer around the ligands and proteins is currently thought to provide the main driving force for adsorption [5,15]. This theory can be thermodynamically described by the Gibbs–Helmholtz equation (Eq. (1)), which combines the change in entropy (ΔS) with the change in free energy (ΔG), the temperature (T), and the change in enthalpy (ΔH):

$$\Delta G = \Delta H - T * \Delta S \qquad (1)$$

By increasing the concentration of a cosmotropic salt in the mobile phase of a HIC column, the increase in entropy ($\Delta S > 0$), according to the mechanism, is more pronounced. Because the change in enthalpy is small compared to the second term, the adsorption

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process becomes thermodynamically favored because of a negative change in free energy ($\Delta G < 0$).

In addition to the well-studied effects of changing the salt concentration in HIC, temperature is known to influence the adsorption of proteins; high temperatures enhance binding and low temperatures promote protein elution [16]. According to Eq. (1), ΔG is influenced by temperature, and an increase in temperature has a similar effect as an increase in ΔS by increasing salt concentration. In a HIC process, a temperature increase is accompanied by an increase in retention factor k' according to Eq. (2):

$$\ln k' = \ln \varphi - \frac{\Delta G}{R * T} \quad (2)$$

where φ is the volume ratio of the sorbents and interstitial liquid, R is the ideal gas constant, and ΔG is the change in free energy according to Eq. (1) [1,15,17]. Even though temperature is known to be a parameter influencing protein adsorption onto HIC resins, few detailed investigations have reported the effect of temperature changes as a gentle method for switching between binding and elution conditions. Most publications describing the temperature effect of protein adsorption on HIC resins have dealt with changing retention times or profiles for the adsorption of reference proteins [18,19], the determination of adsorption and equilibrium parameters [20], and thermodynamic analyses to gain a deeper understanding of the driving forces underlying protein adsorption [21–23]. Furthermore, strong efforts were put into the investigation of conformational changes and protein unfolding via temperature-triggered adsorption onto HIC supports [24] and the mathematical modeling of these phenomena [25]. Only Muca et al. [13] described the application of temperature step changes as a possibility for the chromatographic separation of multi-component protein mixtures.

Here, the abilities of different commercial sepharose-based HIC resins to adsorb and elute proteins controlled mainly by temperature changes are described. The content of cosmotropic salts within the mobile phase was kept low, with the intention to study the potential of temperature control to shift the operation conditions of HIC resins toward reduced salt concentrations, thus increasing sustainability and economics. Investigations on protein separations using HIC at low salt concentrations have been reported before (see e.g. [26]). However, only a few of these reports include temperature as a variable parameter and none investigated multi-cycle operation using temperature-mediated resin regeneration. In the following we present a proof-of-concept of such multi-cycle operation, starting with quantitative equilibrium data for the adsorption of BSA, using temperature as the main variable. Afterwards, the use of temperature changes as a sole regeneration step for loaded HIC columns and the adaptability of temperature change as the driving force underlying the single-step separation of proteins BSA and lysozyme are also demonstrated.

2. Materials and methods

2.1. Instrumentation

For batch experiments, a Thermomixer comfort (Eppendorf, Germany) was used. FPLC experiments were conducted using an ÄKTA Purifier UPC 10 (GE Healthcare, Sweden). A thermostat Lauda E100 (Lauda, Königshofen) was used for tempering the separation column with an accuracy of ±0.5 °C for the temperature control. Protein concentrations were determined using a Nanodrop® ND-100 (Thermo Fisher Scientific, USA) photometer. The protein peak areas of the resulting chromatograms were evaluated using Unicorn® 6 (GE healthcare, Sweden) software. Water used in buffers was deionized by a Millipore Milli-Q Ultrapure system (Millipore, USA).

2.2. Chromatographic stationary phases

The stationary phases for batch experiments were Phenyl Sepharose 6FF high sub (PheHS), Phenyl Sepharose 6FF low sub (PheLS), Butyl Sepharose 4FF (But), and Octyl Sepharose 4FF (Oct). For further characterization of the temperature effect, a HiScreen Butyl FF column with a column volume (CV) of 4.7 ml was used. Resins and columns were purchased from GE healthcare (Sweden).

2.3. Proteins

Bovine serum albumin (BSA; 66 kDa) and lysozyme (Lys; 14.6 kDa) were used for the experiments. The purity of the proteins was ≥98%. Both proteins were purchased from Sigma–Aldrich (Poland).

2.4. Buffer and sample preparation

The buffer used for all experiments was a phosphate buffer with a molarity of 50 mM, made of disodium hydrogen phosphate with different amounts of AS (0.1 M, 0.3 M, 0.5 M) at a pH of 7.0. All buffers were filtered through a glass fiber filter with a pore size of 0.6 μm and degassed before use. Defined amounts of BSA and Lys were dissolved in buffer and filtered before use (regenerated cellulose filter with a pore size of 0.45 μm).

2.5. Protein concentration determination

Protein quantity was determined for adsorption isotherms and the equilibrium binding capacity (EBC) of the chromatographic column was measured spectrometrically at a wavelength of 280 nm using a Nanodrop® ND-100 (Thermo Fisher Scientific, USA) photometer. For BSA and Lys from hen egg white, the mass extinction coefficients for a 10 mg ml^{-1} solution were determined to be 6.7 L g^{-1} cm^{-1} and 26.4 L g^{-1} cm^{-1}, respectively. Protein was quantified in column experiments by evaluating the protein peak areas measured at a wavelength of 280 nm.

2.6. Adsorption isotherms

All resins were washed first with buffer, 50 mM sodium phosphate (pH 7.0) containing different amounts of AS (0.1 M, 0.3 M, 0.5 M). In a 2 ml microcentrifuge tube, 500 μl of various concentrations of BSA was added to 500 μl of a suspension containing a sedimented bed volume of 200 μl of the respective resin. The initial concentrations of the aqueous BSA solutions for the batch isotherm experiments were 0.5, 1.5, 3, 5, 7, and 10 mg ml^{-1}. The solutions in the microcentrifuge tubes were incubated at defined temperatures (10 °C, 25 °C, or 40 °C) and subjected to constant mixing at 1000 rpm for 60 min. After an additional 30 min of incubation without mixing, the supernatant was removed. Protein concentrations were determined at 280 nm by UV spectroscopy and could be calculated from these equilibrium loadings by mass balance.

The Langmuir model was used to fit the adsorption isotherm parameters $q_{\max}$ and K_L to the experimental data sets of the protein concentration in the mobile phase under equilibrium conditions (c^*) and the loading capacity under equilibrium conditions per unit stationary phase (q^*) as shown in Eq. (3):

$$q^* = q_{\max} \frac{K_L c^*}{1 + K_L c^*} \quad (3)$$

where $q_{\max}$ is the saturation capacity and K_L denotes the Langmuir constant, defining the affinity of a molecule for a certain surface. Data analysis was performed by the software SigmaPlot® 11 (Systat Software Inc., USA) using the least squares method.