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A comprehensive study to protein retention in hydrophobic interaction chromatography

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ABSTRACT

The effect of different kosmotropic/chaotropic salt systems on retention characteristics of intact proteins has been examined in hydrophobic interaction chromatography (HIC). The performance was assessed using different column chemistries, *i.e.*, polyalkylamide, alkylamine incorporating hydrophobic moieties, and a butyl chemistry. Selectivity in HIC is mainly governed by the salt concentration and by the molal surface tension increment of the salt. Typically, a linear relationship between the natural logarithm of the retention factor and the salt concentration is obtained. Using a 250 mm long column packed with 5 μm polyalkylamide functionalized silica particles and applying a 30 min linear salt gradient, a peak capacity of 78 was achieved, allowing the baseline separation of seven intact proteins. The hydrophobicity index appeared to be a good indicator to predict the elution order of intact proteins in HIC mode. Furthermore, the effect of adding additives in the mobile phase, such as calcium chloride (stabilizing the 3D conformation of α-lactalbumin) and isopropanol, on retention properties has been assessed. Results indicate that HIC retention is also governed by conformational in the proteins which affect the number of accessible hydrophobic moieties.

1. Introduction

The need for a comprehensive characterization of protein-derived macromolecules used in the biopharmaceutical and food industries is increasing rapidly [1,2]. More efficient analysis of new modified products may improve and accelerate innovation and may be translated into safer products and improved production processes. Liquid chromatography (LC) presents many exciting possibilities for the characterization of complex samples. Various forms of LC exist, allowing separation of sample constituents according to their polarity (normal-phase LC or reversed-phase LC), hydrodynamic volume (size-exclusion chromatography), charge in solution (ion-exchange chromatography), *etc*. Whereas denaturing LC conditions are typically applied, native protein separation technology maintains the 3D protein conformation [3]. Examples of native LC modes include aqueous size-exclusion chromatography [4], aqueous ion-exchange chromatography [5], and hydrophobic interaction chromatography (HIC) [6].

HIC capitalizes on the interaction between hydrophobic patches of proteins and weakly hydrophobic ligands attached to the stationary phase [7,8]. The separation is typically performed using aqueous (non-denaturing) buffer systems at pH = 7 and applying a linear salt gradient starting at relatively high salt concentration [9]. The origin of HIC technology can be traced back to 1948 when Shepard and Tiselius discussed the adsorption of proteins on silica gel in the presence of salt, called "*salting-out chromatography*" [10]. Other landmark contributions include the work of Shaltiel and Er-El, discussing protein retention by lipophilic interactions between accessible hydrophobic pockets of proteins with carbon side chains on the stationary phase [11], and the seminal work of Horvath et al. who developed a theoretical framework describing the effects of salt on hydrophobic and electrostatic interactions [12]. Since then most efforts have been directed to elucidating the retention mechanism of HIC and demonstrating the application possibilities of the technology. Extensive studies have been conducted to investigate mobile-phase [13–16] and stationary-phase contributions to protein retention [17,18]. It has been reported that the influence of nature of salt on retention is governed by the Hofmeister series [19].

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Table 1
Summary of stationary-phase properties.

Column	ProPac HIC-10	MAbPac HIC-20	MAbPac HIC-10	MAbPac HIC-butyl
Material	silica	silica	silica	polymethacrylate
Particle size (μm)	5	5	5	5
Pore size (Å)	300	1000	1000	non porous
Surface area (m^2/g)	100	20	20	1.36
Surface chemistry	polyalkylamide	polyalkylamide	alkylamide	butyl

Kosmotropic salts have higher polarity than chaotropic salts and interact with water strongly. This leads to the formation of strong hydration layer around the kosmotropic salt, hence leaving the hydrophobic patches of the stationary phase unexposed, promoting HIC interaction. Chaotropic salts disrupt hydrogen bonding and reduce the hydrophobic effect (destabilizing the native structure of the proteins) and therefore weaken the hydrophobic retention effects. However, recent studies have demonstrated that protein retention in HIC appears to be affected by an interplay of different contributions, such as pH [13,14], salt concentration and type [15,16], ligand type and ligand density [17,18], unfolding of proteins upon adsorption [20], kinetics of protein spreading [21], *etc*. Protein retention has also been linked to protein properties, including the hydrophobicity index. Different excellent reviews have appeared in the literature describing different approaches to determine hydrophobicity indexes [22–24] and retention-time models as function of different input parameters [25–27]. Fausnaugh and Regnier demonstrated the effect of amino-acid (AA) substitution on protein retention using lysozyme isolated from different bird species [28]. It was concluded that AA substitution on the protein surface affected the strength of the hydrophobic interaction rather than changing the contact area. Retention was furthermore influenced by the ionization state of histidine residues. More recently a number of key references have appeared in literature describing the application possibilities of HIC to analyze antibody variants [29,30]. Valliere-Douglass et al. described the application of HIC for assessing the heterogeneity, stability, and potency of monoclonal antibodies and Fab and Fc sub-domains [29]. An overview of application possibilities to profile therapeutic proteins with HIC was provided by Haverick et al.[30]. Recently, the group of Guillarme and Fekete described practical aspects of mobile-phase optimization for method-development purposes [31,32]

Although HIC shows great potential for native biomolecule separations, the effects of operating conditions such as mobile phase composition (type and concentration of salts and the possibility to add organic modifiers), and stationary-phase chemistry on retention are still not fully understood. In this study, the performance of four commercially-available HIC columns was assessed for protein HIC separations and the effects of eluent type and concentration on protein retention was investigated using different kosmotropic/chaotropic salt systems, including ammonium sulfate, sodium sulfate, potassium sulfate, sodium chloride, and sodium nitrate dissolved in phosphate buffer pH = 7.0. Using optimized column and mobile-phase systems the possibilities to separate intact proteins were explored. Finally, effects of mobile-phase additives (calcium chloride and isopropanol) on retention has been studied.

2. Materials and methods

2.1. Chemicals and materials

Sodium dihydrogen phosphate (≥99.0%), disodium hydrogen phosphate (≥99.0%), sodium hydroxide (HPLC grade, 50.0%), sodium chloride (≥99.0%), ammonium sulfate (≥99.0%), sodium sulfate (≥99.0%), potassium sulfate (≥99.0%), sodium nitrate (≥99.0%), and calcium chloride hexahydrate (98%), cytochrome *c* from bovine heart, myoglobin from equine heart, ribonuclease A from bovine pancreas, apo-transferrin from bovine pancreas, lysozyme from chicken egg white, trypsinogen from bovine pancreas, α-chymotrypsinogen A from bovine pancreas, α-chymotrypsin from bovine pancreas, calcium-depleted α-lactalbumin from bovine milk, and bovine serum albumin (BSA) from bovine pancreas were purchased from Sigma-Aldrich (Diegem, Belgium). Isopropanol (LC–MS grade) was purchased from Biosolve (Dieuze, France). Deionized HPLC-grade water was produced in-house using a Milli-Q water purification system (Millipore, Molsheim, France).

100 mm × 4.6 mm i.d. HIC columns (ProPac HIC-10, MAbPac HIC-20, MAbPac HIC-10, and MAbPac HIC butyl) and a 250 mm × 4.6 mm i.d. MAbPac HIC-20 column were provided by Thermo Fisher Scientific (Sunnyvale, USA). The stationary-phases properties are described in Table 1.

2.2. Instrumentation

HPLC experiments were conducted using an UltiMate 3000 HPLC system (Thermo Fisher Scientific, Germering, Germany) equipped with a membrane degassed, ternary low-pressure-gradient pump, a thermostatted split-loop autosampler (set at 6 °C), a forced-air column oven, and a diode-array detector equipped with a 3 μL UV flow cell (9 mm path length). 250 mm × 100 μm i.d. tubing was used to connect the autosampler to the column inlet, and to connect the column outlet to the UV flow cell. All isocratic and gradient separations were performed in duplicate applying a flow rate of 1 mL/min, 3 μL injection volume, a column oven temperature of 30 °C, and UV detection at λ = 230 nm with a data collection rate of 50 Hz and a response time of 0.2 s.

Differential scanning calorimetry (DSC) was performed on a TA Instruments (DE, USA) nano-DSC III instrument between 25 and 90 °C with a scanning rate of 1 °C min^{-1} at 3 atm. The capillary cell (V = 300 mL) was filled with the α-lactalbumin solution, final concentration 2 mg mL^{-1} in a 50 mM PBS buffer at pH 7 containing 1.2 M of $(NH_4)_2SO_4$. Experiments were run in the absence of any added reagents, with 5% isopropanol or with 5% isopropanol and 10 mM $CaCl_2$. The reference cell was filled with the corresponding protein-free solution. The samples were degassed for 7 min prior to measurement. For each sample, at least four cycles of heating and cooling were performed with 10 min of thermal equilibration between the ramps. The thermograms were processed and analyzed using NanoAnalyze software from TA Instruments.

2.3. Mobile-phase and sample preparation

Mobile phase A was prepared by dissolving the desired salt concentration (2 M sodium sulfate, 0.7 M potassium sulfate, 2 M ammonium sulfate, 5 M sodium chloride, or 2 M sodium nitrate) in a 0.05 M disodium hydrogen phosphate/sodium dihydrogen phosphate solution. The pH of the mobile-phases was adjusted to pH = 7.0 by the addition of 2 M sodium hydroxide solution. The ionic strength was adjusted using mobile phase B, which consists of 50 mM phosphate buffer pH = 7.0. Additionally, the effect of adding