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HPLC of Peptides and Proteins

Methods and Protocols

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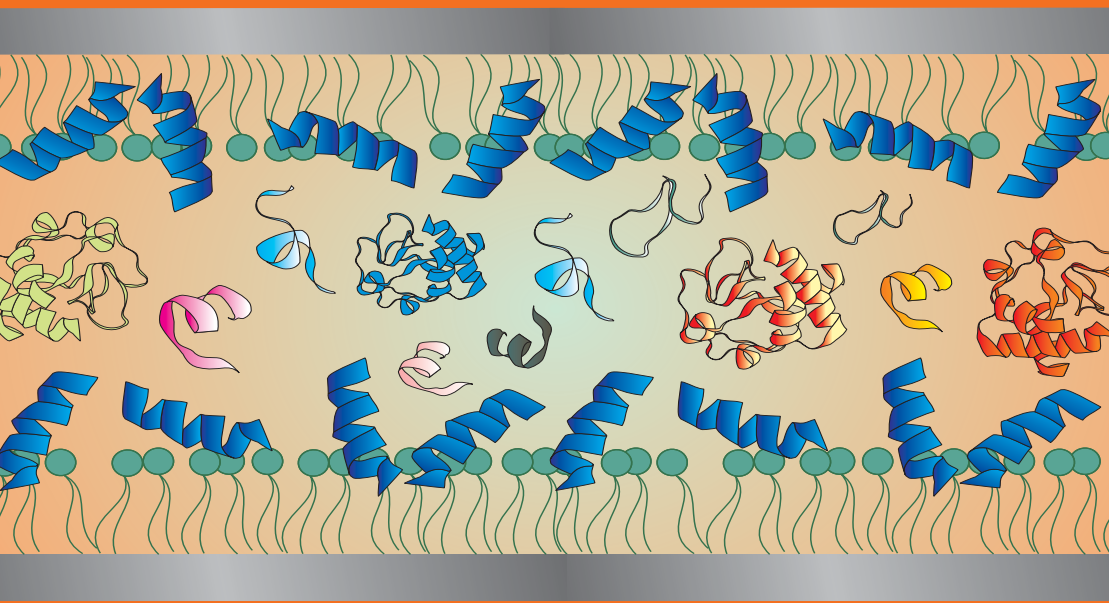

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High-Performance Hydrophobic Interaction Chromatography

Kálmán Benedek

1. Introduction

Hydrophobic interaction chromatography (HIC) is a column chromatographic separation technique frequently used for the purification of macromolecules such as proteins and polynucleotides. Purification schemes often are improved by incorporating HIC along with ion exchange, size exclusion, and affinity chromatography. HIC has found wide use for the purification of membrane proteins ***(1)***, serum proteins ***(2–4)*** nuclear proteins, polynucleotides ***(5)***, receptors ***(6,7)***, cells ***(8,9)*** and recombinant proteins ***(10–12)***.

HIC combines the nondenaturing characteristics of salt precipitation and the precision of chromatography to yield excellent resolution and activity recoveries. HIC is based on the adsorption of biomolecules to a weakly hydrophobic surface at high salt concentrations, followed by elution with a descending salt gradient. HIC was originally developed for protein separations using soft gel-based separation media. During the advance of modern high-performance liquid chromatography (HPLC), the technique was implemented into the new silica format and high-performance mode.

Conditions used in HIC are familiar to biochemists and similar to traditionally used salting out/in protein purification methods. Like in every type of interactive chromatography, the retention mechanism is based on adsorption–desorption equilibrium and preferred solubility conditions. The theoretical foundation of HIC is an extension of the solvophobic theory developed by Imre Molnár, Wayne Melander, and Csaba Horváth ***(13,14)***.

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2. Materials

2.1. Chemicals

1. Ammonium sulfate $(NH_4)_2SO_4$
2. Milli-Q water.
3. 0.1 *M* potassium phosphate buffer, pH 7.0.

2.2. Equipment and Supplies

1. HPLC solvent delivery system with binary gradient capability and ultraviolet (UV) detector.
2. Hydrophobic Interaction Column (*See* **Note 1**) 4.6 mm ID × 250 mm length, 5 µm particle size, 300Å pore size (*See* **Note 2**).
3. Controlled temperature column jacket is recommended.
4. Guard column made of the same packing material as the analytical column is recommended.
5. Solvent filtration apparatus equipped with a 0.45-µm filter.
6. Sample filters, 0.22 µm porosity.
7. Mobile Phase A: 2 *M* $(NH_4)_2SO_4$ in 50 m*M* buffer (*See* **Note 3**).
8. Mobile Phase B: 50 m*M* buffer (*See* **Note 3**).

3. Method

3.1 Sample Preparation

Dissolve 1 mg of sample in 1 mL of mobile phase B (not in mobile phase A!) and filter the sample through a 0.22-µm filter.

3.2. Solvent Preparation

Prepare all solvents and filter through a 0.45-µm filter before use. This removes any particles remained in the mobile phases after dissolution of the buffer salts. Particles can block solvent lines, inline filters, column and detectors. It is recommended to degas the mobile phases by vacuum or helium purging. $(NH_4)_2SO_4$ is usually dirty at this high concentration. The filtration of the high salt content mobile phase A is very important to extend the instrument and column lifetime.

3.3. Preparations for Chromatography

3.3.1. System Assembly

Connect the guard and the column to the solvent delivery system according to the HPLC system requirements and equilibrate under the following initial conditions:

Solvent:	100% Mobile phase A.
Flow rate:	1.0 mL/min (*see* **Note 3**).
Detection wavelength:	280 nm (*see* **Note 4**).
Temperature:	Ambient (*see* **Note 5**).

3.3.2. System Check

Start the pumps to wash the flow system and equilibrate the column. Once a stable baseline is obtained, inject 10 µL of water or buffer and run a blank gradient. It is recommended to repeat this a couple of times to establish the gradient profile (*see* **Note 6**).

3.4. Separation Optimization

Inject 10 µL of the sample and use a linear gradient from 0 to 100% mobile phase B over 30 min to elute the sample (*see* **Note 6**). Most of the components of a protein mixture will elute from the column under these general conditions. The resolution can be manipulated by the variable separation parameters. The most important parameters are the mobile phase pH, salt type, concentration, and the hydrophobic ligand of the stationary phase. Varying the mobile phase temperature could also lead to changes in the separation. The most critical column parameter is the nature of the immobilized ligand of the stationary phase.

3.5. Sample Analysis

Once the chromatographic conditions are optimized according to the objective of the project, the analysis of the individual samples can be performed. Reproducibility assessment of separation is recommended.

4. Notes

1. Modern HIC can be viewed as an extension of RPLC. The stationary phases suitable for hydrophobic interaction chromatography are much more hydrophilic than the alkyl phases used for RPLC. The stationary phases were developed based on earlier work with soft organic gels, and experience with reversed phase stationary phase syntheses. Varieties of silica-based mildly hydrophobic stationary have been developed and are routinely used for protein separations. The stationary phase could be also highly crosslinked polymer-based bead, which can be modified with appropriate surface chemistry. Ideal HIC stationary phases are nonionic and hydrophilic. The ligand density, charge characteristics and hydrophobicity are variable parameters of the stationary phase. HIC stationary phases usually contain alkyl chains attached to hydrophilic nonionic foundation. The length and density of the alkyl chain has a significant effect on retention and selectivity as was shown with soft gel chromatography ***(15)***. A similar result has been shown using silica-based stationary phases prepared by the same bonding chemistry, and attaching different-alkyl homologues to the same base material ***(16)***.

   Fig. 1 shows the effect of different ligands on the separation of standard proteins. **Table 1** lists some of the generally used HIC columns.
2. Column geometry has a similar role in HIC as in RPLC and ion exchange chromatography (IEX). Longer columns are recommended for isocratic separations,

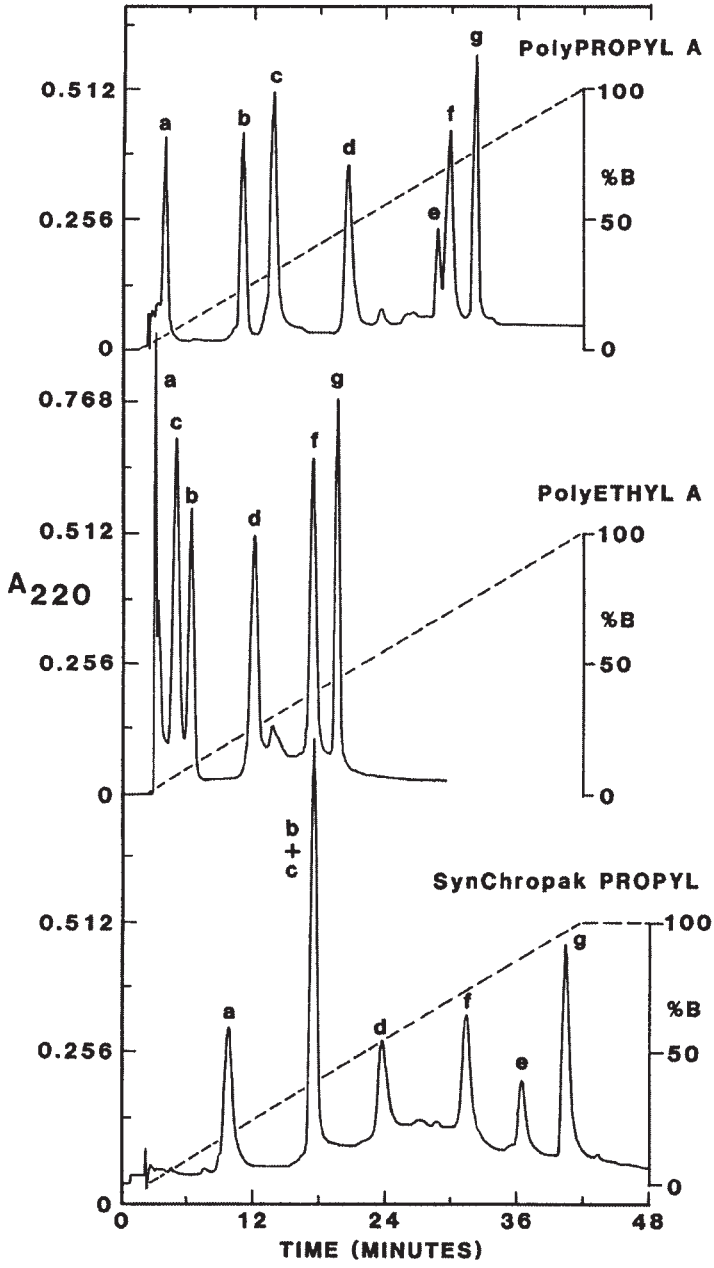

Fig. 1. HIC separations of standard proteins using different stationary phases. Stationary phases applied were PolyPROPYL and PolyETHYL Aspartamide and a PROPYL HIC column from other vendor. The elution condition consists of, mobile phase A: 1.8 *M* ammonium sulfate + 0.1 *M* potassium phosphate, pH 7.0 and mobile phase B: 0.1 *M* potassium phosphate, pH 7.0. A 40-min linear gradient was used from 0 to 100% B at 1 mL/min flow rate. The elution was followed at 220 nm.

Table 1
Stationary Phases[a]

Manufacturer	Product Name	Stationary Phase
Tosoh TSK HIC	Ether-5PW, Butyl-5PW, Phenyl-5PW, Butyl-NPR	Polymer Non-porous
PolyLC	PolyPROPYL Aspartamide, PolyETHYL Aspartamide, PolyMETHYL Aspartamide	Silica
Pharmacia	Butyl-, Phenyl-, Octyl-Sepharose	Polymer
BioChrom Labs	HYDROCELL C3-, C4-, Phenyl C3-NP10, C4-NP10, Phenyl-NP10	Polymer Non-porous

[a] The listed columns are only examples without attempting to be complete.

wider columns for preparative work and precolumns for column protection. More details regarding the role of the column parameters are described in Chapter 2. The pore size of the stationary phase basic material should be at least 300Å to allow the migration of proteins to the pore internal surface. For analytical separation 3–8 µm particles and for preparative purification the larger particles are suggested.

3. Hydrophobic interaction chromatography is fundamentally very close to the generally used salting-out and salting-in purifications method. The fundamental difference is the presence of an adsorptive surface and a flow system. Solvents play a critical role in hydrophobic interaction chromatography (HIC), because the separation of proteins by HIC is based on the hydrophobicity of the proteins presented to the solvents. The high salt content of the starting mobile phase increases the surface tension of the mobile phase, and the solvent-stationary phase interfacial tension. The free energy of adsorption of the protein to the stationary phase is negative. Decreasing the salt concentration thus decreases the interfacial tension and permits the proteins to elute. Various cosolvents have been tested as facilitating binding or elution of the proteins and solvent manipulation can affect retention, resolution, selectivity and peak shape.

   In mobile phase B, which is the elution buffer no sample retention should be observed. The sample adsorption generated by the high salt concentration where the solubility of the protein samples is at minimum. The most frequently used salt is $(NH_4)_2SO_4$, for the preparation of mobile phase A. Other chaotropic salts can also be applied. **Table 2** displays the chaotropic salt series. Strong chaotropic salts disrupt the structure of water and thus tend to decrease the strength of hydropho-

Fig. 1. *(continued)* Peaks: a = cytochrome-*c*, b = ribonuclease A, c = myoglobin, d = conalbumin, e = neochymotrypsin, f = α-chymotrypsin, g = α-chymotrypsinogen A. Reprinted from **ref. *16*** with permission from Elsevier Science, copyright 1986.

Table 2
Anionic and Cationic Ions Used in HIC

Anions	Cations
$(PO_4)^{3-}$	NH_4^+
$(SO_4)^{2-}$	Rb^+
CH_3COO^-	K^+
Cl^-	Na^+
Br^-	Cs^+
NO^-	Li^+
ClO_4^-	Mg^{2+}
I^-	Ca^{2+}
SCN^-	Ba^{2+}

bic interactions; the antichaotropic salts tend to favor them. Organic solvents are also commonly used to alter the polarity of water. Any of those salts can be used for the preparation of the solvophobic agent. The samples elute in the order of their solubility as a function of the salt concentration and their adsorption–desorption equilibrium ***(17)***.

Mobile phase A: The adsorption generating solvent is usually $(NH_4)_2SO_4$, dissolved in the mobile phase B buffer.

Mobile phase B: All sample components should be soluble in mobile phase B. The pH and ionic strength of B solvent should be selected accordingly.

Elution mode: During separation condition search, gradient elution mode is recommended. However, ones the elution conditions established than step gradient or isocratic elution can be applied. The elution mode primarily depends on the complexity of the samples.

Flow rate: The usually applied starting flow rate is 1 mL/min. However, higher flow rate can be used in isocratic separation mode.

4. UV or fluorescence detection is usually applied during HIC separations. Because of the high salt concentration, significant background noise can be observed without filtration and use of pure salts.
5. Temperature usually set at a comfortable laboratory temperature (25°C). However, the temperature can have significant effect on the retention, peak shape ***(18)***, and activity and mass recovery of proteins. **Figure 2** shows the retention time changes as a function of surface hydrophobicity. Nonlinear changes in retention as a function of temperature are an indication of conformational changes of the sample proteins. Some of the chromatographic changes can be originated from altering the conformational equilibrium of proteins. For sensitive samples, a study of temperature effect is recommended, in order to understand the phenomena involved ***(19)***. As an example, **Fig. 3** shows the chromatograms of α-lactalbumin as a func-

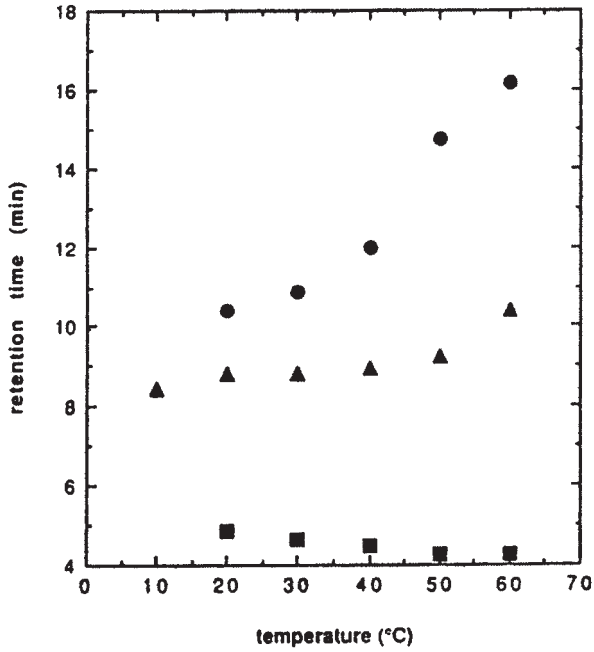

Fig. 2. Effect of surface hydrophobicity and temperature on the retention of α-lactalbumin on the PolyALKYL Aspartamide columns at pH 6.0 using ammonium acetate based mobile phase. Reprinted from **ref.** ***19*** with permission from Elsevier Science, copyright 1988.

tion of temperature. The displayed series of chromatograms clearly illustrate that the retention time and peak width significantly alters with increasing temperature. Normalized retention time-temperature curves are sigmoidal in those cases where denaturation occurs under the given chromatographic conditions. These denaturation curves are very similar to the classical transition curves of protein denaturation and have been analyzed by the same approach. Temperature studies also represent an example, that chromatography is a versatile experimental tool for not only the analysis and separation but physico-chemical studies of protein conformational changes as well ***(19)***.

6. The use of high salt could create erosions and plugging, which are usually not observed with other chromatographic systems. After separations the system should be thoroughly cleaned with the B solvent and water. The high salt mobile phase can precipitate at different part of the chromatographic system. Washing is critically important prior mobile phase and/or chromatographic mode changes. It is important to establish standard conditions to evaluate the separation system with protein standards. Standard conditions can provide information about system reproducibility, separation and system conditions, and column lifetime.

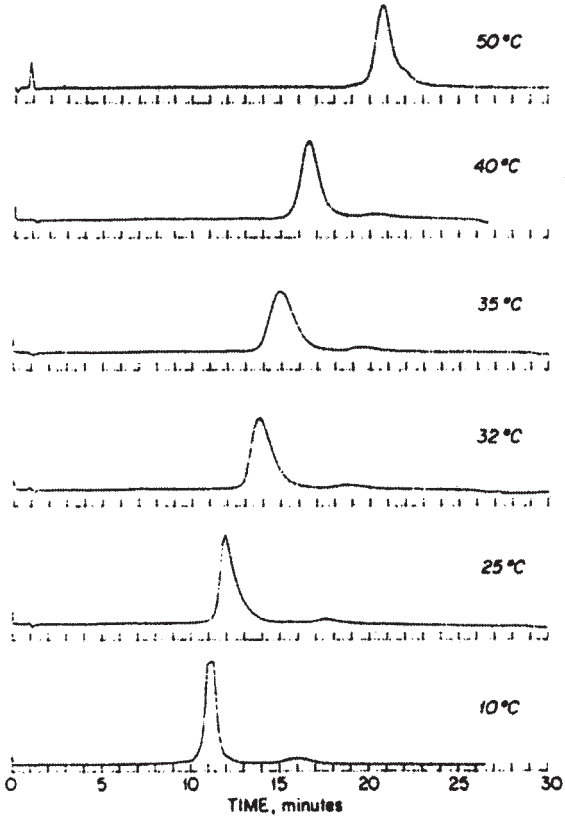

Fig. 3. Effect of temperature on the chromatographic peak width of α-lactalbumin on an HIC stationary phase. Reprinted from **ref.** ***18*** with permission from Elsevier Science, copyright 1986.

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