CHAPTER TWENTY-FIVE

Theory and Use of Hydrophobic Interaction Chromatography in Protein Purification Applications

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Contents

Abstract

Hydrophobic interaction chromatography (HIC) is a valuable tool used in protein purification applications. HIC is used in the purification of proteins over a broad range of scales—in both analytical and preparatory scale applications. HIC is used to remove various impurities that may be present in the solution, including undesirable product-related impurities. In particular, HIC is often employed to remove product aggregate species, which possess different hydrophobic properties than the target monomer species and can often be effectively removed using HIC. In this chapter, we provide a description of the basic theory of HIC and how it is used to purify proteins in aqueous-based solutions. Following the theoretical background, the latest in HIC adsorbent technology is described, including a list of commonly used and commercially available adsorbents. The basic procedures for using HIC adsorbents are described next, in order to provide the reader with useful starting points to apply HIC in protein purification applications.

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1. Theory

Hydrophobic proteins will self-associate, or interact, when dissolved in an aqueous solution. This self-association forms the basis for a variety of biological interactions, such as protein folding, protein–substrate interactions, and transport of proteins across cellular membranes (Janson and Rydén, 1997). Hydrophobic interaction chromatography (HIC) is used in both analytical and preparatory scale protein purification applications. HIC exploits hydrophobic regions present in macromolecules that bind to hydrophobic ligands on chromatography adsorbents. The interaction occurs in an environment which favors hydrophobic interactions, such as an aqueous solution with a high salt concentration.

By itself, water (a polar solvent) is a poor solvent for nonpolar molecules. Under such an environment, proteins will self-associate, or aggregate, in order to achieve a state of lowest thermodynamic energy. Prior to self-association, water molecules form highly ordered structures around each individual macromolecule (Fig. 25.1A). The self-association of nonpolar molecules (such as proteins) in the polar solvent is driven by a net increase in entropy of the environment. During the aggregation process, the overall surface area of hydrophobic sites of the protein exposed to the polar solvent

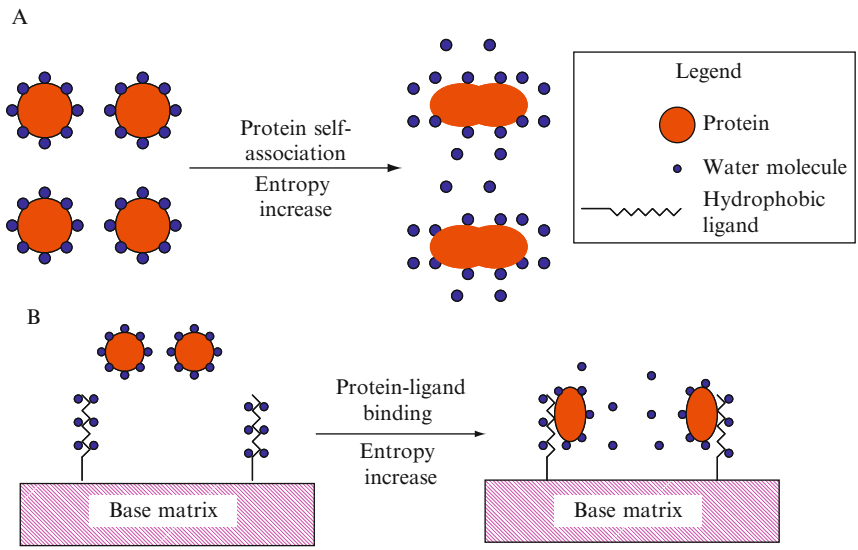

Figure 25.1 Schematic diagram showing hydrophobic interaction between proteins in an aqueous solution (A) and between proteins and a hydrophobic ligand on an HIC adsorbent (B).

is decreased, which results in a less structured (higher entropy) condition, which is the favored thermodynamic state.

This same concept is responsible for the interaction (association) between hydrophobic ligands attached to an adsorbent and the proteins of interest (Fig. 25.1B). Association, or hydrophobic interaction, between the protein and the hydrophobic ligand is driven primarily by an increase in the overall entropy (compared with the condition when no interaction is occurring between the protein and the adsorbent).

The polarity of the solvent can be controlled through the addition of salts or organic solvents, which can strengthen or weaken hydrophobic interactions between the HIC adsorbent and the protein. The influence of ions on hydrophobic interaction follows the well-known Hofmeister series (Hofmeister, 1988). Anions which promote hydrophobic interaction the greatest are listed (in decreasing strength of interaction) from left to right (Påhlman *et al.*, 1977):

$$PO_4^{3-} > SO_4^{2-} > CH_3COO^- > Cl^- > Br^- > NO_3^- > CLO_4^- > I^- > SCN^-$$

Ions which promote hydrophobic interactions are called lyotropes, while those which disrupt (weaken) hydrophobic interactions are called chaotropes. In the above series, phosphate ions promote the strongest hydrophobic interaction, while thiocyanate ions disrupt hydrophobic interactions.

For cations, the Hofmeister series consists of the following (listed in order of decreasing lyotropic strength):

$$NH_4^+ > Rb^+ > K^+ > Na^+ > Cs^+ > Li^+ > Mg^{2+} > Ca^{2+} > Ba^{2+}$$

Two of the most common Lyotropic salts used to promote hydrophobic interaction in aqueous solution are ammonium sulfate and sodium chloride. These salts are commonly employed when using HIC for protein purification.

In addition to salts, organic solvents can also be used to alter the strength of hydrophobic interactions (Fausnaugh and Regnier, 1986; Melander and Horvath, 1977). Organic solvents commonly used to weaken, or disrupt hydrophobic interactions include glycols, acetonitrile and alcohols. The organic solvents alter the polarity of the mobile phase, thereby weakening potential interactions that may occur. They may be added to the solution during the elution process, in order to disrupt hydrophobic interactions and elute the strongly bound protein of interest.

Protein hydrophobicity is a complex function of several properties, which include the amino acid sequence, as well as protein tertiary and quaternary structure in a given solution (Ben-Naim, 1980; Tanford, 1980). Hydrophobicity scales have been created for particular amino acids, which are based upon the solubility in water and organic solvents

(Jones, 1975; Nozaki and Tanford, 1971; Tanford, 1962; Zimmerman *et al.*, 1968). Empirical hydrophobic scales for proteins have also been created (Chotia, 1976; Krigbaum and Komoriya, 1979; Manavalan and Ponnuswamy, 1978; Rose *et al.*, 1985; Wertz and Scheraga, 1978) which are based upon the fraction of amino acids exposed on the protein surface, as well as the degree of amino acid hydrophobicity. The ability to predict the hydrophobicity of complex proteins has been only semiquantitative to date, and experiments are usually required to accurately understand protein hydrophobicity in a given aqueous solution.

2. Latest Technology in HIC Adsorbents

HIC adsorbents consist of a base matrix which is coupled to a hydrophobic ligand. The base matrix, which typically consists of porous beads with diameters ranging from 5 to 200 μm, provides high surface area for ligand attachment and protein binding. Common base matrices include agarose, methacrylate, polystyrene–divinylbenzene and silica (Table 25.1). For analytical applications, the bead size of the adsorbent is in the lower range (5–20 μm). Small beads are used in order to maximize resolution when performing analytical separations. For preparatory scale applications, larger bead sizes are usually required ($\geq$20 μm). Larger bead sizes are required for preparatory scale columns due to pressure drop limitations associated with the column hardware.

HIC adsorbents containing hydrophobic ligands with various degrees of hydrophobicity are available. The ligands consist of alkyl or aryl chains. As a

Table 25.1 Properties of commercially available HIC adsorbents

Base matrix	Available ligand types	Adsorbent manufacturers
Cross-linked agarose	Butyl	GE Healthcare
	Octyl	
	Phenyl	
Polystyrene–divinylbenzene	Phenyl	GE Healthcare Applied Biosystems
Methacrylate	Butyl	
	Ether	TosoHaas
	Phenyl	EM Industries
	Hexyl	
Silica	Propyl	J. T. Baker
	Diol	Synchrom
	Pentyl	Supelco
		YMC

general rule, the strength of hydrophobic binding of the ligand will increase with the length of the organic chain. Several of the most common ligands include butyl, octyl, and phenyl, which are linked to the base bead support through several different coupling approaches (Hjertén *et al.*, 1974; Ulbrich *et al.*, 1964). Aromatic ligands, such as phenyl, can also interact with the adsorbed compounds through so-called "π–π interactions," which can further strengthen the hydrophobic interaction (Porath and Larsson, 1978).

The hydrophobic interaction strength of the ligand can also be influenced by the ligand loading (ligand density) on the base matrix. The strength of interaction can increase with higher ligand densities. In order to have reproducible performance, manufacturers of HIC adsorbents must often produce adsorbents with narrow ranges of ligand density to ensure consistent performance from lot to lot.

3. Procedures for Use of HIC Adsorbents

3.1. Introduction

The application of HIC adsorbents for use in the purification of protein compounds is fairly straightforward. The purification process is composed of a series of subsequent steps, which are described in this section.

3.2. Choice of adsorbent

Since the hydrophobic properties of a given protein are often unknown, several HIC adsorbents may need to be screened prior to selection of a final adsorbent. The screening process is used to determine how strongly compounds bind to the adsorbent, and determine which adsorbents are viable candidates to purify the protein. Adsorbents with a broad range of hydrophobic binding properties should be included in the initial screening studies to determine the hydrophobicity of the compound, as well as which adsorbents effectively purify the protein.

Adsorbents may be available in the form of prepacked columns from the adsorbent manufacturer or may have to be packed by the user. In the case when columns need to be packed, the packing instructions from the manufacturer should be followed. Height equivalent to the theoretical plate (HETP) measurements can be used to verify that the column is packed correctly. Once packed, the adsorbent manufacturer often provides recommended operating ranges for use, which include allowable flow rate ranges, column conditioning, and column cleaning procedures. Acceptable operating conditions will vary for different adsorbents, and the vendor documentation should be consulted prior to use.

3.3. Feed/load preparation

Prior to column loading, the salt concentration of the protein mixture (which will be purified using the HIC adsorbent) must be increased to a level in which the target protein binds to the adsorbent using a high salt buffer. Proteins may precipitate in high salt solutions, so the compound solubility in a given salt solution should be evaluated prior to its selection. The influence of buffer pH on compound binding to HIC adsorbents has no general trend, but can influence the strength of interaction (Hjertén *et al.*, 1974). A buffer pH should be chosen in which the protein and the adsorbent are stable (e.g., avoid the use of extreme pH solutions). During the protein load adjustment step, the salt concentration may range from approximately 0.5 to 2.0 *M*, and will be increased high enough to ensure the protein binds effectively to the adsorbent. The salt concentration required to bind the protein to the adsorbent will depend greatly on the choice of salt, as described in Section 3.2. Selection of the appropriate salt concentration in which the protein binds to the adsorbent will require experimental work in most cases.

3.4. Adsorbent preparation

Prior to loading the protein feed, the column should first be equilibrated in a high salt buffer solution which possesses a similar composition (salt concentration) and pH as the feed solution to ensure the protein will bind tightly to the adsorbent. This step is referred to as the equilibration step.

Following the equilibration step, the adjusted feed (which contains the protein of interest) is loaded onto the HIC column at an appropriate velocity. After the protein-containing solution is loaded onto the column, the column is often washed with the equilibration buffer prior to product elution. Additional wash steps may be implemented prior to the elution step to remove undesirable impurity species. The wash steps may contain a salt concentration at an intermediate salt concentration—less than the load step, but greater than the elution step.

3.5. Product elution

After being bound to the adsorbent, the desired protein must be eluted and then collected in the column effluent. In many cases, the elution process is used to separate, or resolve, unwanted species from the desired protein. The unwanted species may bind less tightly to the adsorbent, and will be eluted prior to the product. In other cases, undesirable species bind more tightly to the adsorbent and will remain bound after the product is eluted. This is usually the case when HIC is used to separate protein aggregate species, which bind more tightly to the adsorbent than the desired protein monomer

species. During the elution step, a portion (or fraction) of the eluate may contain highly purified product, while fractions before and after contain higher levels of undesirable impurities. A schematic of an elution process (during a gradient elution) is shown in Fig. 25.2. Figure 25.2 illustrates that the column effluent collected during the elution step may need to be fractionated in order to achieve acceptable product purity when using HIC. The gradient elution process is described in more detail in Section 3.6.

The elution process can be done using either a stepwise (isocratic) or a gradient approach. The four most common methods (listed from most common to least common) used to elute the bound protein include the following:

1. *Decrease in the salt concentration (relative to the binding conditions)*. A decrease in the salt concentration will decrease the strength of hydrophobic interaction between the protein and the ligand, and the protein will be desorbed and eluted from the column.
2. *Addition of organic solvents*. Addition of an organic solvent (such as ethylene or propylene glycol) changes the solvent polarity, which disrupts the hydrophobic interaction.
3. *Increase in the salt concentration (using a chaotropic salt)*. Addition of a chaotropic salt will disrupt the hydrophobic interaction.
4. *Detergent addition*. Detergents are used as protein displacers, and have been used mainly for the purification of membrane proteins when using HIC (Janson and Rydén, 1997).

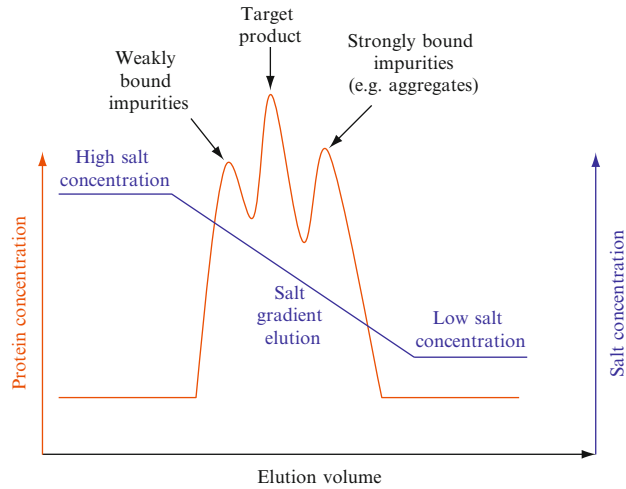

Figure 25.2 Schematic chromatogram showing a gradient elution of a protein mixture using hydrophobic interaction chromatography. In the diagram, the salt concentration is linearly decreased (from high salt to low salt), which results in elution of both impurities and the target protein.

This most common approach used to elute proteins from HIC adsorbents is by lowering the salt concentration during the elution step. This should be the first method that is attempted when using HIC for purification of a new protein compound. The other approaches described above have the disadvantage that an additional component (such as a chaotropic salt or an organic solvent) needs to be added, which may impact protein stability. However, such agents may be required in order to effectively elute a strongly bound protein species from the adsorbent. Each protein must be evaluated case by case to determine which elution method is appropriate. The HIC adsorbent used in the purification may also influence which elution method is effective.

3.6. Gradient elution

Gradient elutions are an extremely effective method useful for screening different HIC adsorbents in protein purification. During the gradient elution process, the salt concentration is decreased gradually (in a linear fashion) from a high salt concentration to a low salt concentration over a defined volume. During the initial screening of a bound compound on an adsorbent, the salt concentration may be decreased to as low as 0 m*M* to determine the salt concentration when the product elutes. As a starting point, a typical gradient elution process is performed over 10 column volumes, during which fractions are collected and evaluated for product purity. The gradient in salt concentration may be decreased (performed over a larger volume) in order to improve protein resolution (Yamamoto *et al.*, 1988).

In the event that the protein remains bound to the adsorbent following the gradient elution process, this may indicate that either a weaker lyotropic salt should be selected to bind the protein to the adsorbent or that a stronger elution condition is required to elute the protein. Stronger elution solutions may include the use of an organic solvent. For preparatory scale applications, nonflammable organic solvents (such as propylene or ethylene glycol) are often selected. Organic solvents in analytical scale applications may include such solvents as acetonitrile and alcohols. Alternatively, an adsorbent with weaker hydrophobic binding strength may need to be selected to decrease the strength of hydrophobic interaction.

3.7. Stepwise (isocratic) elution

After identifying the appropriate adsorbent and salt concentration to effectively elute the protein of interest, an isocratic elution can be used if desired. An advantage of using isocratic elution is its simplicity—it requires a simple switch in the inlet buffer (from a high to a low salt concentration). Use of an isocratic elution is a preferable approach to simplify the equipment

requirements, as gradient elution requires multiple pumps and additional process control to generate a linear change in the buffer salt concentration.

3.8. Adsorbent regeneration and sanitization

HIC adsorbents are reusable for multiple cycles and have a relatively long lifetime before having to be replaced. However, adsorbents must be cleaned and regenerated between uses in order to ensure reproducible performance over many cycles. The adsorbent manufacturers' provide regeneration procedures for the adsorbents, which should be consulted prior to use. In general, the cleaning procedures depend upon the stability of the base matrix and the hydrophobic ligand. For strongly bound proteins, 6 *M* guanidine hydrochloride is often recommended. If detergents have been used during the process, ethanol or methanol can be used to as part of the regeneration procedure (GE Healthcare, 2006). For sanitization, a caustic solution (1.0 *M* NaOH) can be used for most of the adsorbents (with the exception of silica). The manufacturer should also provide information on the appropriate storage conditions. Storage solution should be selected that prevents microbial growth, but does not impact ligand or base matrix stability.

REFERENCES

Ben-Naim, A. (1980). Hydrophobic Interactions. Plenum Press, New York.

Chotia, C. (1976). Surface of monomelic proteins. *J. Mol. Biol.* **105,** 112.

Fausnaugh, J. L., and Regnier, F. E. (1986). Solute and mobile phase contributions to retention in hydrophobic interaction chromatography of proteins. *J. Chromatogr.* **359,** 131–146.

GE Healthcare (2006). Data File No. 18-1127-63 AC.

Hjertén, S., Rosengren, J., and Påhlman, S. (1974). Hydrophobic interaction chromatography. *J. Chromatogr.* **101,** 281–288.

Hofmeister, F. (1988). On regularities in the albumin precipitation reactions with salts and their relationship to physiological behavior. *Arch. Exp. Pathol. Pharmakol.* **24,** 247–260.

Janson, J.-C., and Rydén, L. (eds.) (1997). Protein Purification: Principles, High-Resolution Methods, and Applications, 2nd edn., p. 284. Wiley-VCH, New York.

Jones, D. D. (1975). Amino acid properties and side-chain orientation in proteins. *J. Theor. Biol.* **50,** 167–183.

Krigbaum, W. R., and Komoriya, A. (1979). Local interactions as a structure determinant for protein molecules. *Biochim. Biophys. Acta* **576,** 204–248.

Manavalan, P., and Ponnuswamy, P. K. (1978). Hydrophobic character of amino acid residues in globular proteins. *Nature* **275,** 673–674.

Melander, W., and Horvath, C. (1977). Salt effects on hydrophobic interactions in precipitation and chromatography of proteins: An interpretation of the lyotropic series. *Arch. Biochem. Biophys.* **183,** 200–215.

Nozaki, Y., and Tanford, C. (1971). The solubility of amino acids and two glycine peptides in aqueous ethanol and dioxane solutions. Establishment of a hydrophobicity scale. *J. Biol. Chem.* **246,** 2211–2217.

Påhlman, S., Rosengren, J., and Hjerten, S. (1977). Hydrophobic interaction chromatography on uncharged Sepharose derivatives. *J. Chromatogr.* **131,** 99–108.
Porath, J., and Larsson, B. (1978). Charge-transfer and water-mediated chromatography. I. Electron-acceptor ligands on cross-linked dextran. *J. Chromatogr.* **155,** 47–68.
Rose, G. D., Geselowitz, A. R., Lesser, G. J., Lee, R. H., and Zehfus, M. H. (1985). Hydrophobicity of amino acid residues in globular proteins. *Science* **229,** 834–838.
Tanford, C. (1962). Contribution of hydrophobic interactions to the stability of the globular conformation of proteins. *J. Am. Chem. Soc.* **84,** 4240–4247.
Tanford, C. (1980). *In* The Hydrophobic Effect 2nd edn. Wiley, New York.
Ulbrich, V., Makes, J., and Jurecek, M. (1964). Identification of giycidyl ethers. Bis(phenyl-) and bis(a-naphthylurethans) of glycerol a-alkyl (aryl)ethers. *Collect. Czech. Chem. Commun.* **29,** 1466–1475.
Wertz, D. H., and Scheraga, H. A. (1978). Influence of water on protein structure. *Macromolecules* **11,** 9–15.
Yamamoto, S., Nakanishi, K., and Matsuno, R. (1988). Ion-Exchange Chromatography of Proteins Mercel Dekkar, New York.
Zimmerman, J.M, Eliezer, N., and Simha, R. (1968). The characterization of amino acid sequences in proteins by statistical methods. *J. Theor. Biol.* **21**(2), 170–201.