Hydrophobic Interaction Chromatography: A Key Method for Protein Separation

Nermana Kovačević, Mohammad Tariq AL-Boush
International Burch University
Sarajevo, Bosnia, and Herzegovina
nermana.kovacevic@stu.ibu.edu.ba
tariq.boushi@stu.ibu.edu.ba

Literature review

Abstract: *This review's main goal is to increase theoretical knowledge and comprehension of the techniques used to separate important enzymes using chromatography. Protein separation, purification, and analysis frequently involve the use of hydrophobic interaction chromatography (HIC), in which the molecules are separated based on differences in hydrophobicity. It is conducted using a weakly hydrophobic, nonpolar stationary phase to which the proteins bind in an aqueous high-salt solution as the mobile phase. The protein integrity is thereby conserved during the process. HIC is usually used with a gradient from high to low concentrations of salt as an eluent and can be applied in a wide range of biotechnological processes. In this study, after a brief overview of hydrophobic interaction chromatography, we explain the HIC procedure, protein retention mechanism, factors affecting HIC, and applications of HIC.*

Keywords: Hydrophobic interaction chromatography, protein purification.

1. Introduction

Based on their hydrophobicity, biomolecules are separated and purified using the hydrophobic interaction chromatography (HIC) technique [17]. The term "salting-out chromatography" was initially used to describe this method in 1949 by Shepard and Tiselius [21]. Shaltiel and Er-el [20] renamed the process "hydrophobic chromatography" in 1973. Because proteins can stay on weakly hydrophobic matrices in the presence of salt, Hjerten (1973) coined the term "hydrophobic interaction chromatography" [11]. The efficacy of this approach has been the subject of numerous studies. Some research has found that this method can be used to separate and purify proteins in their native state [18], isolate protein complexes [5], and study protein folding and unfolding [3]. As we can see, this method is most commonly used for protein purification. Except for protein purification, hydrophobic interaction chromatography can be used to separate and purify cells, viruses, nucleic acids, and carbohydrates [6].

2. Hydrophobic Interaction Chromatography Procedure

In the hydrophobic interaction chromatography, the mobile phase passes through a stationary phase that contains a hydrophobic ligand. This allows the hydrophobic species found in the mobile phase to bind to the immobilized hydrophobic ligands found in the stationary phase [6]. HIC's stationary phase is made up of a resin matrix containing hydrophobic ligands. Linear chain alkyl groups such as ether, butyl, hexyl, and octyl are the most common and frequently used ligands in HIC [19]. Elution can be carried out by lowering the ionic strength of the mobile phase linearly or gradually and modifying the pH and temperature of the elution buffer [16]. The isolation of a particular HIC-compatible protein typically requires experimental research to identify the best chromatographic medium or buffer conditions. Among the commercially available HIC media, there are differences in the chemical composition of the functional groups, their hydrophobicity, density, and the size of the inert matrix beads to which the functional groups are linked. Unique chromatographic elution patterns are produced by these changes in the HIC medium.

Functional group densities range from 5 to 50 μmol/ml of medium, with matrix bead sizes ranging from 30 to 100 μm. Relatively small bead diameters and higher functional group densities provide better chromatographic resolution, as opposed to larger bead diameters

and lower functional group densities. Those operating conditions are suggested for highly concentrated mixture separations and faster flow rates. Numerous different factors that are routinely improved to enhance HIC protein purification elution features include elution buffer molarity, pH, and chromatographic flow rate [22]. Furthermore, the hydrophobic nature of the resin, the nature and structure of the protein sample, the prevalence and distribution of surface-exposed hydrophobic residues, and the type and quantity of salt in an aqueous binding buffer also have an important role in the separation of biomolecules by HIC [8].

The resolution of hydrophobic interaction chromatography is usually satisfactory. To improve resolution, it is critical to experiment with different operating conditions. Jennissen (2000) [13], suggested using the crucial hydrophobicity approach, which consists of three main steps: choosing an acceptable alkyl chain length, determining the critical surface concentration of alkyl residues, and determining the minimal salt concentration needed to achieve complete adsorption of proteins [15].

3. Hydrophobic Interaction

In an aqueous environment, hydrophobicity is defined as the association of nonpolar molecules that results from water's tendency to reject nonpolar molecules. Two hydrophobic or non-polar molecules will cluster when discovered in a polar environment to reduce contact with the polar solvent, such as water, methanol, etc. "Hydrophobic interaction" is the term given to this process. In biological systems, those interactions are extremely important. Hydrophobic interactions are important in many biological procedures, such as in antibody-antigen interactions, enzyme catalysis, protein aggregation and regulation. Furthermore, they are one of the primary driving forces responsible for the stability and folding of protein structures [23].

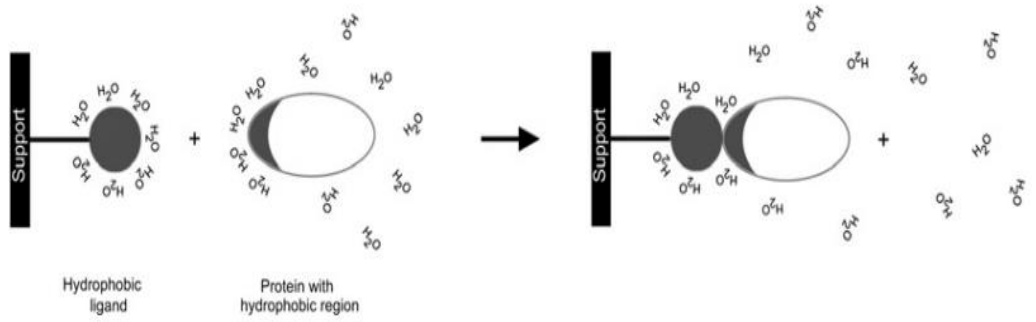

Figure 1. Illustration of hydrophobic interaction between immobilizing ligand and a protein [23]

4. Retention Mechanism in the HIC

In HIC, there is one very important mechanism known as a protein retention mechanism. This macromolecule retention occurs because of hydrophobic interaction. This interaction occurred between hydrophobic ligands which are found on a stationary phase and non-polar parts of the proteins, Figure 2. In HIC, several stationary phases are utilized, including agarose, polyacrylamide, cellulose, and dextran. Their primary characteristics include their high moisture absorption, high porosity, and potential for chemical modification.

Alkyl groups or aryl groups with 4–10 carbons act as ligands (weakly non-polar or hydrophobic) attached to the stationary phase. To prevent self-folding, there are no more than 10 carbon atoms in total [1]. In HIC, the hydrophobic ligands butyl (four carbons), octyl (eight carbons), and phenyl (the aromatic ring that encourages - interactions with the aromatic residues on the surface of a protein) are the most frequently utilized. Butyl is the least hydrophobic of the carbon chains used as HIC ligands because it is the shortest, octyl has an intermediate level of hydrophobicity, and phenyl displays the most hydrophobic interaction [1].

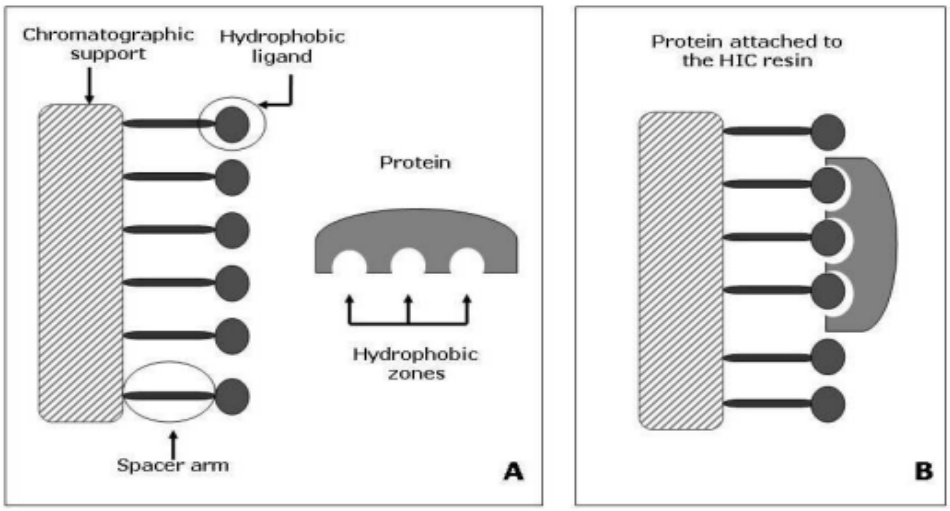

Figure 2. Retention mechanism in hydrophobic interaction chromatography [1]

The presence of neutral salts in the HIC enhances the protein retention mechanism. Whenever a neutral salt is added to a solution that contains a polar solvent, competition for the water molecules that hydrate the proteins occurs. This competition is more beneficial to salt. As a result, high salt concentration reduces the number of solvent molecules surrounding the protein, favoring the hydrophobic interaction between them. When that solution comes into contact with HIC resin, it enhances the interaction between

the proteins and the non-polar ligands on the resin surface. As a result, protein adsorption to the HIC stationary phase occurs. It is critical to select the appropriate salt type and concentration to reduce protein precipitation caused by solubility decrease in the presence of high salt concentration [1].

5. Factors that Impact HIC

A variety of factors influence HIC, including protein quality, matrix composition, ligand type and density, salt type and concentration, temperature, and pH. However, some factors, such as pH and temperature, are unpredictable and should be considered during technique optimization to improve selectivity, resolution, and binding capacity [8].

Type of ligands

The type of immobilizing ligands has a significant impact on the HIC adsorbent's selectivity for the protein. In hydrophobic interaction chromatography, alkyl and aryl chains that are covalently bonded to a base matrix are the most typical form of ligands [9]. While aryl ligands exhibit mixed mode behavior, which combines both aromatic and hydrophobic characteristics, alkyl ligands exhibit pure hydrophobic character [23]. The protein binding capacities of HIC adsorbents increase with longer alkyl chains at a constant degree of substitution [7]. For each specific circumstance, screening experiments should be used to determine whether to use aryl or alkyl ligands [23].

Base matrix

The matrix's characteristics are determined by its chemical structure and particle size. Different types of matrix materials can be used in HIC, like natural polymers such as cellulose, agarose, dextran, or chitosan, as well as synthetic polymers like polymethacrylate and inorganic compounds (silica). To enable reversible adsorption and prevent the HIC adsorbent from becoming much more hydrophobic, the matrix material must be hydrophilic. For reversible adsorption to be possible and for the matrix material not to considerably increase the HIC adsorbent's hydrophobicity, it must be hydrophilic. The most frequently used matrices in HIC are agarose or cellulose [23].

Type and Concentration of Salt

The Hofmeister series describes how ions affect hydrophobic interaction [12]. The salts at the beginning of the Hofmeister series, referred to as lyotropic salts, tend to induce

hydrophobic interactions by increasing "salting out" effects. Because of a "salting-in effect," the salts near the end (right) tend to reduce interactions. On the left side of Figure 3. anions and cations have a positive effect on HIC retention since they enhance ligand-protein interaction. On the other side anions and cations on the right side of Figure 3. despite increasing the surface tension of the water, do not promote HIC retention [23].

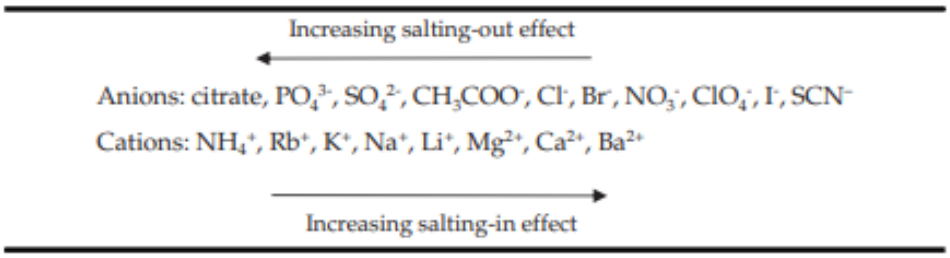

Figure 3. Hofmeister series. The impact of anions and cations on protein precipitation [20]

When a high concentration of lyotropic salts is added to a protein solution, the water molecules of the hydration shell may prefer to interact with the salt ions instead of the charged amino acid of the proteins. It thus exposes hydrophobic patches, which enable interaction between hydrophobic proteins or interaction between proteins ligands via Van der Waals interactions [10]. Among useful salts, sodium sulfate is the least viscous while sodium sulfate and sodium phosphate produce high molal surface tension. Ammonium sulfate is soluble in water and has high purity, but it can be degraded at an alkaline pH. Different salts can differently effect on retention of hydrophilic and hydrophobic proteins. In comparison to ammonium sulfate, sodium acetate tends to increase the retention mechanism of hydrophobic and hydrophilic proteins, whereas sodium citrate has the opposite effect [7].

pH

Higher pH tends to weaken hydrophobic interactions due to increased titration of charged groups and an increase in protein hydrophilicity. As a result, lower pH promotes hydrophobic interaction. Proteins that are unable to bind to HIC absorbents at neutral pH bind to them at acidic pH. Protein retention varies more noticeably at pH levels above 8.5 and below 5 than it does in the pH 5–8.5 range [11]. These results demonstrate that pH is a crucial separation factor for hydrophobic interaction chromatography optimization [9].

Temperature

Temperature increases usually have a positive effect on retention, while decreasing temperature favors elution. Also, protein conformational states and solubility may be affected by high temperatures. As a result, the temperature is frequently one of the characteristics that is kept relatively constant. However, in large-scale production, maintaining a constant temperature may be an expensive and difficult task. Retention in HIC was also discovered to be an entropy-driven process at low temperatures and an enthalpy-driven process at high temperatures [23].

Additives

Other methods can be applied in HIC for protein purification instead of lowering salt concentration [23]. In hydrophobic interaction chromatography, small concentrations of detergents, alcohols, and soluble compounds that possess salt promote a weakening of the interaction between proteins and ligands. This causes the bound solute to be deactivated and removed, allowing the elution of the desired product. The hydrophobic regions of alcohols and detergents compete with proteins for adsorption sites on the chromatography media. When highly hydrophobic proteins are linked to the gel medium, additives are also very important and efficient in cleaning HIC columns [7].

6. Literature Review

Hydrophobic interaction chromatography has proven to be a successful method for the purification of enzymes, especially β-glucosidases from plants, animals, insects and fungi, but also many other enzymes**.**
Below are several studies showing the successfulness of this method as a means of protein analysis.

Koffi et al. (2012) article was a study aiming to purify β-glucosidase from cockroaches, Periplaneta Americana, using hydrophobic interaction chromatography and other methods. Periplaneta americana were captured from rooms. They were collected directly from the nest and then stored. The enzyme was purified 9.93-fold to a specific activity of 40.33 (U/mg of protein) and an overall yield of 1.54 %. The optimal pH for studying Periplaneta americana β-glucosidase was 3.6, and the optimal temperature was 55 °C. The molecular weight for Periplaneta americana β-glucosidase was 43.8 kDa, meaning that the enzyme was monomeric (Table 1). The kinetic parameters km and Vma of β-glucosidase

were measured using three substrates: cellobiose, p-nitrophenyl-β-D-glucopyranoside, and pNP-N-acetyl-β-D-glucopyranoside. The km values for those substrates were found to be 3.29mM and 41.67 U/mg for pNP-β-D-Glucopyranoside, 0.37mM and 11.49 U/mg for pNP-N-acetyl-β-D-glucopyranoside and 8.52mM and 39.12 U/mg for cellobiose (Table 2). These findings reveal that the catalytic activity of β-glucosidase is significantly higher for pNP-N-acetyl—D-glucopyranoside than for cellobiose and pNP-D-glucopyranoside. Ultimately, as a result and conclusion, the article reported the successful isolation and purification of the enzyme using hydrophobic interaction chromatography [14].

Bešić et al. (2016) study aimed to isolate and purify β-glucosidase from brassica oleracea by salting out with ammonium sulfate and hydrophobic interaction chromatography. The main active fraction of the β-glucosidase was purified sevenfold with a yield of 4.1%. This study showed that β-glucosidase isolated from broccoli was a dimer (130 kD) made up of one major and one minor subunit (80 kD and 50 kD). Enzyme properties, such as the effect of different inhibitors, kinetic parameters, and optimum environmental parameters, were determined. The pH optimum was 6.0 and the temperature optimum was 35 °C (Table 1). The kinetic parameters km and Vmax of broccoli β-glucosidase were determined by using four substrates: 4-Nitrophenyl-b-D-glucopyranoside (p-NPG), ortho-Nitrophenyl-b-D-glucopyranoside (o-NPG), paraNitrophenyl-b-D-galactoside (p-NPGal), and ortho-Nitrophenyl-b-D-galact The km values for those four substrates (p-NPG, o-NPG, p-NPGal, and o-NPGal) were determined to be 0.755 mM, 0.174 mM, 0.988 mM, and 0.213 Mm, and the Vmax values were 604 U/mg, 38 U/mg, 556 U/mg, and 308 U/mg (Table 2). Considering that the Vmax values in all four instances were high, it is possible to conclude that β-glucosidase from broccoli has a strong affinity and interaction with those four substrates. Regarding inhibition experiments, p-NPG was used as a substrate and glucose and β–gluconolactone as inhibitors, with ki values of 0.64 mM and 0.038 Mm. A study showed that glucose and β–gluconolactone completely inhibit the broccoli β-glucosidase with ki values of 0.038 mM and 0.64 Mm, and inhibitions were competitive for both inhibitors. So this study eventually reported a successful purification of the beta-glucosidase using the hydrophobic interaction chromatography technique [4].

Table 1. Properties of β-glucosidase isolated using HIC

	Molecular mass (kDa)	Subunit molecular mass (kDa)	Quaternary structure	Optimum pH	Optimum temperature °C
Brassica oleracea	130	80 50	Dimer	6.0	35
Agaricus bisporus	110	46 62	Dimer	4.0	55
Periplaneta americana	43.8	-	Monomer	3.6	55

(-) Not determined

Ašić et al. (2015) study aimed to isolate and purify β-glucosidase from Agaricus bisporus (White Button Mushroom) using ammonium sulfate precipitation and hydrophobic interaction chromatography. Agaricus bisporus β-glucosidase was purified 10.12-fold during the precipitation and chromatography steps. They found that the enzyme was a dimer with two subunits of approximately 46 and 62 kDa. The enzyme functions best at a pH of 4.0 and a temperature of 55°C (Table 1). The enzyme was found to be exceptionally thermostable. To study enzyme activity, two substrates were used: p-NPGlu and o-NPGlu. The km values for those substrates were found to be 1.751 mM and 8.547 mM, and the Vmax values were 833 U/mg and 556 U/mg (Table 2). As compared to o-NPGlu, A. bisporus exhibits a much stronger affinity for p-NPGlu as a substrate. A. bisporus β-glucosidase was inhibited by both gluconolactone and glucose. Both function as competitive inhibitors, with gluconolactone being a considerably more effective inhibitor. This is supported by a comparison of the computed Ki values for glucose and gluconolactone, which were 9.402 mM for glucose and 0.0072 mM for gluconolactone. The β-glucosidase from Agaricus bisporus was successfully purified and biochemically characterized using ammonium sulfate precipitation and hydrophobic interaction chromatography [2].

Table 2. Kinetic parameters of β-glucosidases

	Substrate	Km (mM)	Vmax
Brassica oleracea	p-NPG	0.755	604 U/mg
	o-NPG	0.174	38 U/mg
	p-NPGal	0.988	556 U/mg
	o-NPGal	0.213	308 U/mg
	o-NPG	14.11	48.5 U/mg
Agaricus bisporus	p-NPGlu	1.751	833 U/mg
	o-NPGlu	8.547	556 U/mg
Periplaneta americana	pNP-beta-D-Glucopyranoside	3.29	41.67 Umg
	pNP-N-acetyl-beta-D-Glucopyranoside	0.37	11.49 U/mg
	Cellobiose	8.52	39.12 U/mg

7. Applications of HIC

HIC is frequently used in the production of highly purified biomedical products such as therapeutic proteins, monoclonal antibodies, and enzymes. To purify target proteins, hydrophobic interaction chromatography can be used as a single step or together with other chromatography methods. HIC is also a particularly helpful method in large-scale industrial applications. to purify antibodies.

Purification of plasmid by hydrophobic interaction chromatography was achieved using sodium citrate-based buffers. Successful HIC of ribonuclease A, ovalbumin, and lactoglobulin was performed at alkaline pH (9.5) using monosodium glutamate. Purification of monoclonal antibodies was successfully achieved by HIC. HIC was used to separate lysozyme from chicken egg white. Hydrophobic interaction chromatography was used to purify human PON1Q192 and PON1R192 isoenzymes. HIC together with ion exchange chromatography and ammonium sulfate are used for successful purification of recombinant HIV reverse transcriptase [5].

8. Conclusion

In conclusion, in biological systems, hydrophobic interactions are extremely important. They are the most critical factor in protein folding and structural stabilization, as well as other biological procedures such as in reactions between antibodies and antigens. HIC uses the protein's hydrophobicity to promote separation via hydrophobic interactions between non-polar ligands and hydrophobic areas on the proteins [21]. Hydrophobic interaction chromatography is currently a widely-used and effective separation method for enzyme purification on a laboratory and industrial scale.

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