

Protein purification methods



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Living organisms are enormously complex. Proteins are fundamental for executing a wide variety of life controlling functions.

Proteins are of high interest both in the academic community as well as within the pharmaceutical industry, as malfunctioned proteins are often associated with causes of disease. Diabetes is a well-known example (insulin). By understanding the function/malfunction of proteins we can find possibilities to develop medicines to cure diseases.

The most common methods for preparative purification of proteins all involve chromatography. The method separate according to differences between the properties of the protein to be purified (the target protein) and the properties of other substances in the sample. Table 1 lists examples of protein properties used in different chromatography methods.

Table 1. Protein properties used for chromatographic purification

Protein property	Method
Specific ligand recognition (biospecific or nonbiospecific)	Affinity chromatography (AC)
Metal ion binding	Immobilized metal ion affinity chromatography (IMAC)
Charge	Ion exchange chromatography (IEX)
Size	Size exclusion chromatography (SEC, gel filtration)
Hydrophobicity	Hydrophobic interaction chromatography (HIC) Reversed phase chromatography (RPC)
Isoelectric point	Chromatofocusing (CF)

For example, affinity chromatography is considered to be the most common first purification step when the target protein is affinity tagged. If further purification is needed, additional methods are used to remove remaining impurities. Purification of untagged proteins often requires the use of several purification steps applying different methods in a suitable order. This document gives a brief overview of the key purification methods presented in Table 1. Further details regarding method descriptions and practical advice, including many examples, can be found in different handbooks from GE Healthcare's Life Sciences. Handbooks of interest are listed in the Related literature section.

Affinity chromatography (AC)

AC separates proteins on the basis of a reversible interaction between the target protein (or group of proteins) and a specific ligand attached to a chromatography matrix (Fig 1).

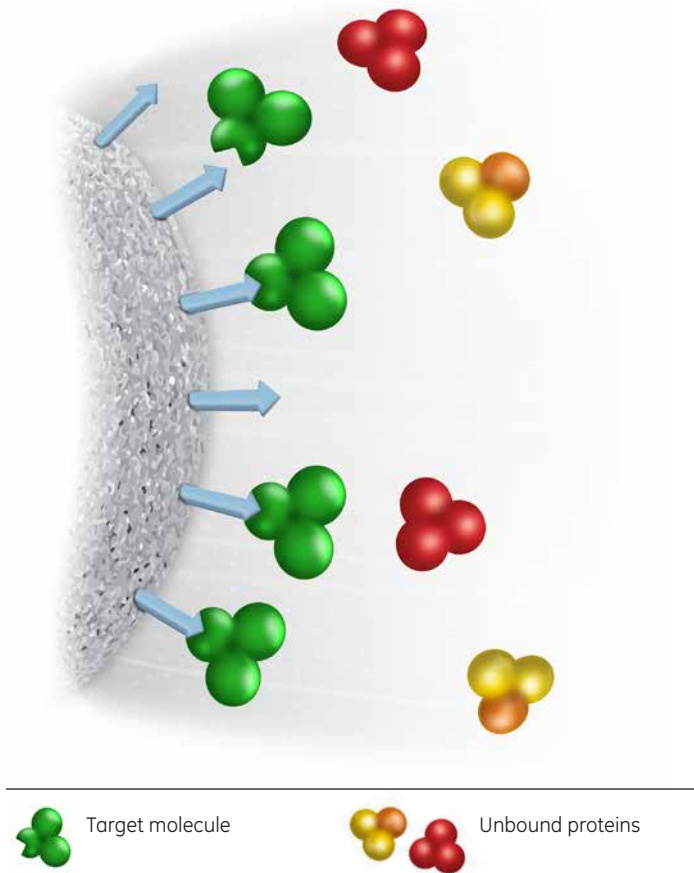


Fig 1. Schematic depicting AC.

The interaction can be biospecific, for example, antibodies binding Protein A or a receptor binding a hormone. It can also be nonbiospecific, for example, a protein binding dye substance or histidine-containing proteins binding metal ions (as in immobilized metal ion affinity chromatography, which will be described in a separate section due to its importance). AC offers high selectivity, and intermediate to high capacity. Elution can often be performed under mild conditions.

Principles

In AC, the target protein is specifically and reversibly bound by a complementary binding substance (ligand). The sample is applied under conditions that favor specific binding to the ligand. Unbound material is washed out of the column, and bound target protein is recovered by changing conditions to those favoring elution. Elution is performed specifically, using a competitive ligand, or nonspecifically, by changing, for example, pH, ionic strength, or polarity. The target protein is eluted in a purified and concentrated form. The key stages in an AC separation are shown in Figure 2.

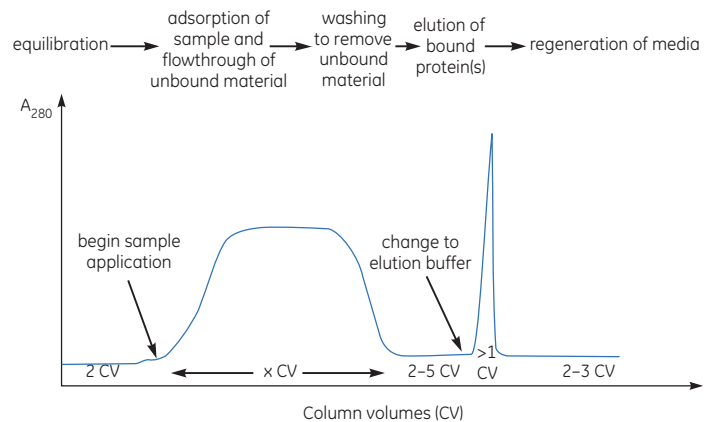


Fig 2. Typical affinity purification.

Ligands in AC are divided into two categories: mono-specific and group-specific. Ligands used for mono-specific AC are structurally and biologically closely related to the target molecule. This makes the selection of the ligand specific for each case. This also makes it commercially difficult to produce AC media (resins) for mono-specific separations. However, preactivated media ready for a variety of coupling chemistries are commercially available for the benefit of the user who intends to run mono-specific AC. Such media are for example CNBr-activated Sepharose™ or NHS-activated Sepharose, for immobilizing proteins via primary amino groups, or Epoxy-activated Sepharose where hydroxyl groups on the molecule are utilized for immobilization.

A group-specific ligand has an affinity for a group of related substances rather than for a single type of molecule. The same general ligand can be used to purify several substances (e.g., members of a class of enzymes) without the need to prepare a new medium for each different substance in the group. Ligands used for group-specific AC have a much wider applicability and affinity media for this purpose are commercially available. Table 2 shows some examples of group-specific ligands and their specificities.

Table 2. Examples of group-specific ligands and their specificities

Ligand	Specificity
Protein A or Protein G	Fc region of IgG
Concanavalin A	Gluconpyranosyl and mannopyranosyl groups
Cibacron™ Blue	Broad range of enzymes, serum albumin
Lysine	Plasminogen, ribosomal RNA
Benzamidine	Serine proteases
Calmodulin	Proteins regulated by calmodulin
Heparin	Coagulation factors, lipoproteins, lipases, nucleic acid-binding enzymes
Metal ions (e.g., Ni ²⁺)	Protein and peptides containing histidine

Due to its high selectivity, AC can sometimes be used for single-step purification or in instances when some impurities can be accepted. It is, however, more common for AC to be used as the first purification step, followed by a second step to remove remaining impurities or aggregates. In cases when very high purity is required, one or more additional purification step might be required.

Affinity-tagged proteins for lab-scale purification

Today, most laboratory-scale purifications are performed with affinity-tagged proteins. A large number of different affinity tags as well as media and prepacked columns are available to allow selection of optimal conditions for each target protein and purification task. A common task is purification of histidine-tagged proteins using IMAC or glutathione S-transferase (GST)-tagged proteins using a medium with immobilized glutathione. AC is also used to remove specific contaminants, for example, Benzamidine Sepharose 4 Fast Flow for removal of serine proteases. A preactivated medium can be used for covalent coupling of various ligands. For example, antibodies can be raised against the target protein and coupled on NHS-activated Sepharose for immunoaffinity purification of the desired protein.

Immobilized metal ion affinity chromatography (IMAC)

IMAC is based on the interaction of proteins with certain amino acid residues on their surface and divalent metal ions (e.g., Ni^{2+} , Cu^{2+} , Zn^{2+} , Co^{2+}) immobilized via a chelating ligand. The interaction is primarily between histidine and metal ions, but also, for example, tryptophan and cysteine. Histidine-tagged proteins have extra high affinity in IMAC because of the multiple (6 to 10) histidine residues. These proteins are usually the strongest binder among all the proteins in a crude sample extract (e.g., a bacterial lysate), while other cellular proteins will not bind or will bind weakly.

Principles

IMAC purification begins with equilibration of the column with a binding buffer containing a low concentration of imidazole. The concentration of imidazole depends on the selected medium (can be found in the instruction for the specific medium). The imidazole binds to the immobilized metal ion and becomes the counter ligand. The sample should be adjusted to the same imidazole concentration as the binding buffer before being loaded on the column. Proteins with histidines bind the column while displacing the imidazole counter ligands. The column is washed using the binding buffer. Elution of bound proteins is performed using a gradient of imidazole up to 100 to 500 mM or by step elution. Gradient elution (Fig 3) often gives two peaks, an early peak corresponding to naturally binding proteins in the lysate and a later peak corresponding to the histidine-tagged protein, which has higher affinity for the medium. Step elution (Fig 4) gives a single peak, with the histidine-tagged protein often of slightly lower purity. It is, however, a powerful capture step that can be combined with a second purification step to obtain higher purity, for example, size exclusion chromatography.

Histidine tags are small and generally less disruptive to the properties of the protein than other tags. Because of this, tag removal after purification of a histidine-tagged protein is not always of high priority. Histidine-tagged protein expressed in *E. coli* can accumulate in two main forms, as biologically functional soluble proteins or as large insoluble aggregates of more or less unfolded, inactive target protein.

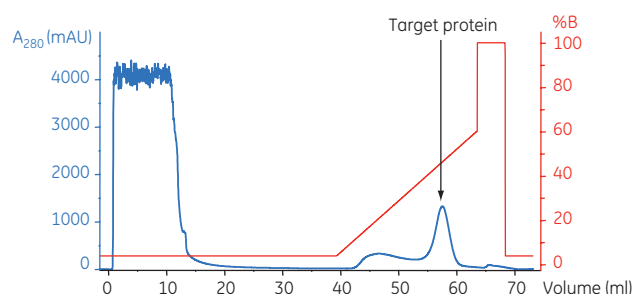


Fig 3. Typical IMAC purification with gradient elution.

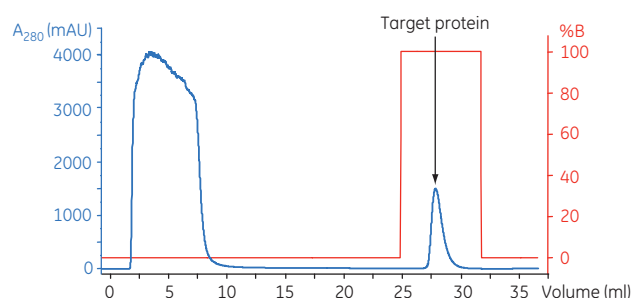


Fig 4. Typical IMAC purification with step elution.

Size exclusion chromatography (SEC)

SEC allows separation of substances with differences in molecular size, under mild conditions. The technique can be used for protein purification (Fig 5) or for group separation where the sample is separated in two major groups (Fig 6). Group separation is mainly used for desalting and buffer exchange of samples.

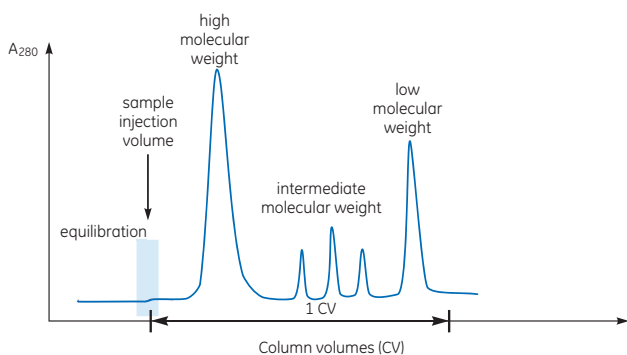


Fig 5. Principles of SEC purification.

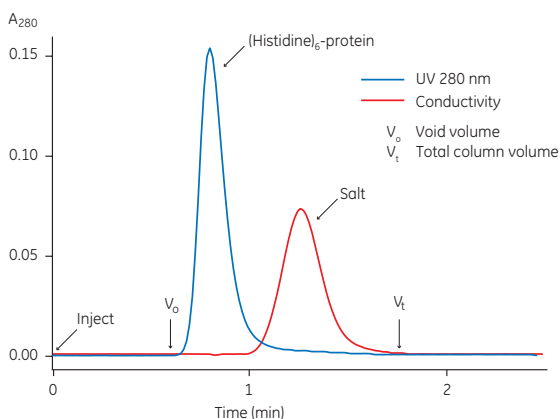


Fig 6. Typical example of group separation used for desalting of protein.

SEC is a nonbinding method (Fig 7), which means that no concentration of the sample components takes place. In fact, the sample zone is broadened during the passage through the column, resulting in dilution of the sample. The loaded sample volume must be kept small. In preparative SEC, maximum resolution can be obtained with sample volumes of 0.5% to 2% of the total column volume. However, up to 5% might give acceptable separation. Even larger sample volumes can be appropriate if the resolution between target protein and the impurities is high. To increase capacity, the sample can be concentrated before SEC. Avoid concentrations above 70 mg/ml, because viscosity effects might cause severe band broadening (so-called viscous fingering), which reduces the resolution.

Sample components are eluted isocratically (single buffer, no gradient). Separation can be performed within a broad pH, ionic strength, and temperature range. Furthermore, the medium accepts a variety of additives: co-factor, protein






-  Large molecule that cannot enter the pores of chromatography beads
-  Salt or other low-molecular-weight substances that can use the entire pore volume of the beads
-  Target protein that can use a fraction of the pore volume of the beads

Fig 7. Schematic depicting SEC.

stabilizers, detergents, urea, and guanidine hydrochloride. The buffer composition does not usually affect resolution, although including a low concentration of salt, for example, 25 to 150 mM NaCl, is recommended to eliminate weak electrostatic interactions between proteins and the SEC matrix. Buffer conditions are selected to suit the sample type and to maintain target protein activity. A benefit of SEC is that the sample does not have to be in exactly the same buffer as that used for equilibration and running. The buffer in the sample will be exchanged into the running buffer during the separation. Equilibration buffer can thus be selected according to conditions required for further purification, analysis, storage, or use. The selection of medium is the key parameter for optimization of resolution in SEC.

SEC applications

SEC is a powerful method for purification of proteins that have passed one or several initial purification steps. After those steps, the material has been concentrated and bulk impurities have been removed. SEC can be used to remove the remaining impurities. It will also remove oligomers or aggregates of the target protein. The purified target protein obtained after SEC will thus also be homogeneous in size. SEC is rarely used as a first purification step, but can be useful for small samples with moderate complexity.

Ion exchange chromatography (IEX)

IEX separates proteins with differences in surface charge to give high-resolution separation with high sample loading capacity (Fig 8). The separation is based on the reversible interaction between a charged protein and an oppositely charged medium. Target proteins are concentrated during binding and collected in a purified, concentrated form. IEX media can be used at high flow rates because binding kinetics for IEX are fast, and rigid chromatography particles can be used.

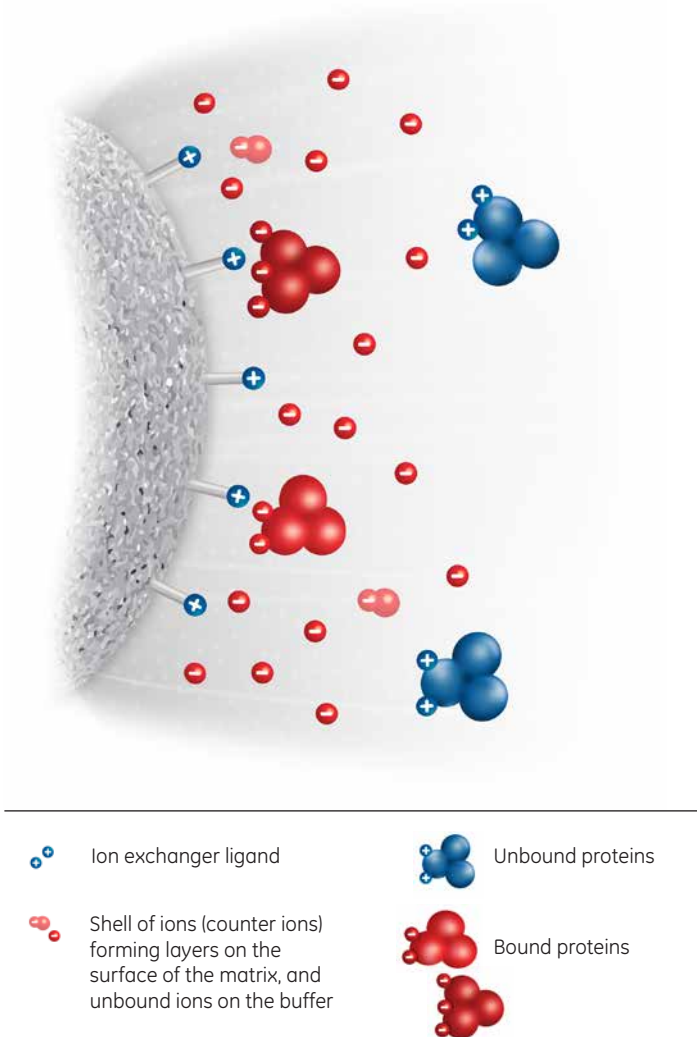


Fig 8. Schematic depicting anion exchange chromatography (AIEX) with a positively charged ligand. The same principle applies to cation exchange chromatography (CIEX), but the ligand is negatively charged.

The net surface charge of proteins varies according to the surrounding pH (Fig 9). Typically, when above its isoelectric point (pI) a protein will bind to a positively charged anion exchanger; when below its pI a protein will bind to a negatively charged cation exchanger.

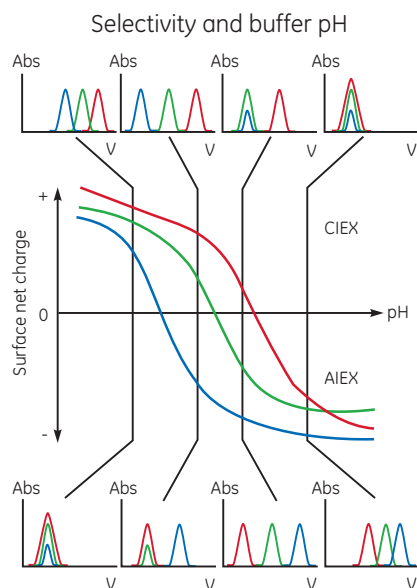


Fig 9. Schematic view of the effects of pH on protein elution patterns. The middle diagram shows the surface net charge of three proteins (blue, green, and red). The four chromatograms on top describe the behavior of these proteins in cation exchange chromatography (CIEX) with salt gradient elution run at varying pH values as indicated by the vertical lines. The bottom chromatograms show the behavior in anion exchange chromatography (AIEX).

Principles

Proteins bind as they are loaded onto a column at low ionic strength. The conditions are then altered so that bound substances are desorbed differentially. Elution is usually performed by increasing salt concentration or changing pH in a gradient (Fig 10), or stepwise (Fig 11). The most common salt is NaCl, but other salts can also be used to modulate separation, for example, salts containing K^+ , Ca^{2+} , Mg^{2+} , CH_3COO^- , SO_4^{2-} , I^- , or Br^- ions. The buffer used can also impact separation. Ions that bind to the protein might change its behavior in IEX.

Strong vs weak ion exchangers

Ion exchange matrices are categorized as strong or weak for both anion and cation exchangers. A strong IEX medium has the same charge density on its surface over a broad pH range, whereas the charge density of a weak ion exchanger changes with pH. The selectivity and the capacity of a weak ion exchanger are different at different pH values. A recommendation is to first try strong ion exchangers. If other selectivity is desired, try a weak ion exchanger.

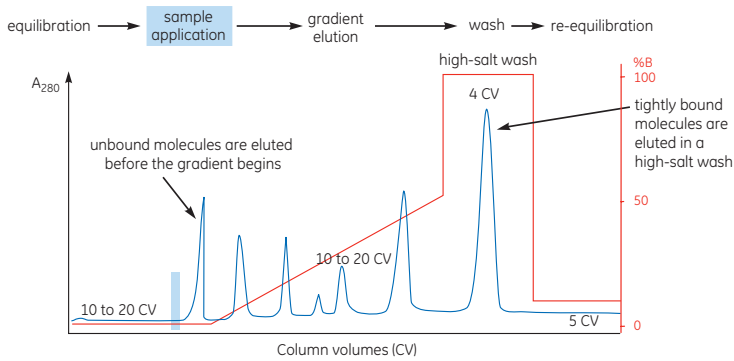


Fig 10. Typical IEX purification with gradient elution.

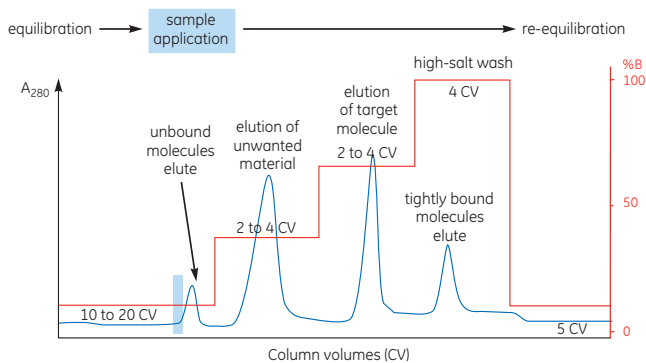


Fig 11. Typical IEX purification with step elution.

IEX applications

IEX can be used in any part of a multistep purification procedure: as a first step, in which high binding capacity and high flow rates allow capturing of both target protein and bulk impurities from a large-volume sample, as an intermediate purification step, or as a final step for high resolution purification to remove remaining impurities. Typically IEX is used to bind the target molecule, but it can also be used to bind impurities, letting the target protein pass through the column. IEX can be repeated at different pH values to separate several proteins that have distinctly different charge properties. Alternatively, a purification step using CIEX can be followed by a second purification step using AIEX at the same pH.

Multimodal ion exchangers

Multimodal ion exchangers, for example, Capto™ adhere and Capto MMC media, have been developed to offer novel selectivities. The charged ligands are complemented with extra functional groups that introduce additional cooperative interactions (combinations of hydrogen bond, hydrophobic, and van der Waals' interactions). Capto adhere can be used for the removal of aggregated monoclonal antibodies (MAbs) to obtain pure monomers.

Hydroxyapatite chromatography (HAC) can also be considered a multimodal ion exchange method. Crystals of hydroxyapatite ($\text{Ca}_3(\text{PO}_4)_3\text{OH}$) can be used as chromatography medium. Proteins are believed to bind cooperatively to both calcium and phosphate ions on the hydroxyapatite. The hydroxyapatite has a negative charge at neutral pH, and proteins that bind AIEX media tend to also bind to hydroxyapatite. HAC is a less common purification method, partly because of its unpredictable separation mechanism and low binding capacity.

Hydrophobic interaction chromatography (HIC)

HIC separates proteins with differences in hydrophobicity. The method is well-suited for the capture or intermediate step in a purification protocol. Separation is based on the reversible interaction between a protein and the hydrophobic surface of a chromatography medium (Fig 12). This interaction is enhanced by high-ionic-strength buffer, which makes HIC an excellent purification step after precipitation with ammonium sulfate or elution in high salt during IEX. There is no universally accepted theory on the mechanisms involved in HIC. For a brief discussion of the mechanisms, see "Hydrophobic interaction chromatography and reversed phase handbook: principles and methods" (11001269).

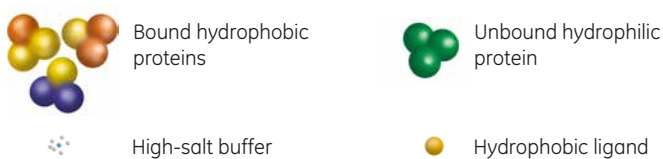
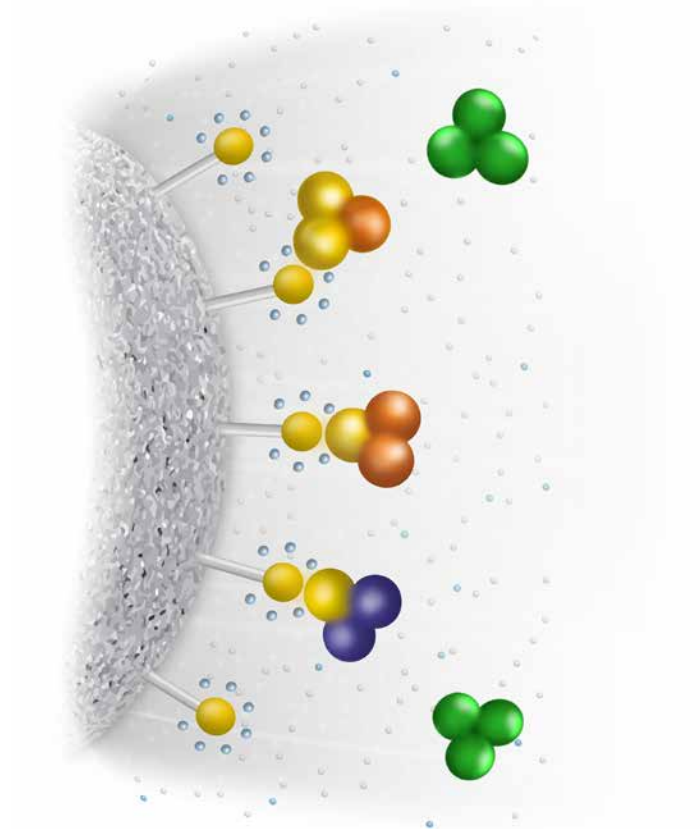


Fig 12. Schematic depicting HIC.

Principles

Many sample components bind a HIC column in high-ionic-strength solution, typically 1 to 2 M ammonium sulfate or 3 M NaCl. Conditions are then altered so that the bound substances are eluted differentially. Elution is usually performed by decreasing the salt concentration. Changes are made with a continuous decreasing salt gradient (Fig 13) or stepwise (Fig 14). Most commonly, samples are eluted with a decreasing gradient of ammonium sulfate. Target proteins are concentrated during binding and are collected in a purified and concentrated form. Other elution procedures include reducing eluent polarity (ethylene glycol gradient up to 50%), adding chaotropes (e.g., urea, and guanidine hydrochloride) or detergents, or changing pH or temperature.

Optimization involves screening several HIC media with different ligands and ligand concentrations of the HIC medium, and scouting conditions for best binding selectivity and capacity. High concentrations of salt, especially ammonium sulfate, might precipitate proteins. Therefore, check the solubility of the target protein under the binding conditions to be used.

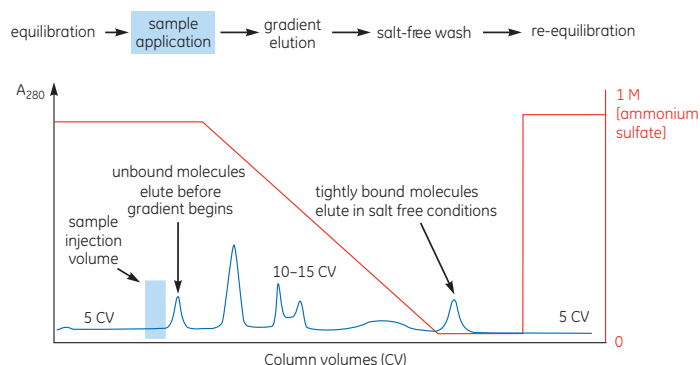


Fig 13. Typical HIC purification with gradient elution.

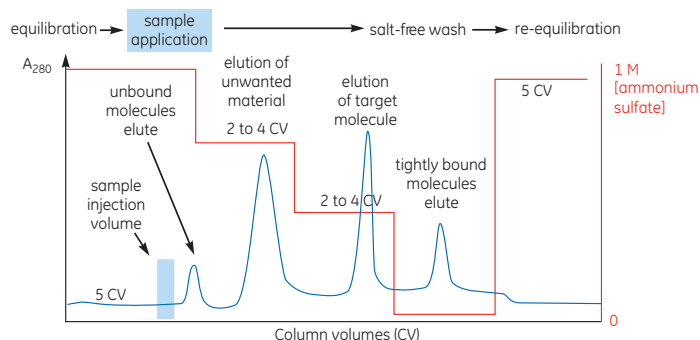


Fig 14. Typical HIC purification with step elution.

Reversed phase chromatography (RPC)

RPC separates proteins and peptides on the basis of hydrophobicity (Fig 15). RPC is a high resolution method, requiring the use of organic solvents.

Principles

The method is widely used for purity check analyses when activity and tertiary structure are not a focus. Because many proteins are denatured by organic solvents, the method is not generally recommended for preparative protein purification—the recovery of activity and native tertiary structure is often compromised. Proteins tend to denature and bind strongly to the RPC medium, and can be very difficult to elute. However, in the polishing phase, when the majority of protein impurities have been removed, RPC is excellent, particularly for small target proteins that are less commonly denatured by organic solvents. Sample components bind as they are loaded onto the column. Conditions are then altered so that the bound substances are eluted differentially. Due to the nature of the reversed phase matrices, binding is usually very strong.

Binding might be modulated by the use of organic solvents and other additives (ion pairing agents). Elution is usually performed by an increase in organic solvent concentration, where acetonitrile, methanol, ethanol, and propanol are most commonly used. The target protein is purified and concentrated in the process. The key stages in a separation are shown in Figure 16.

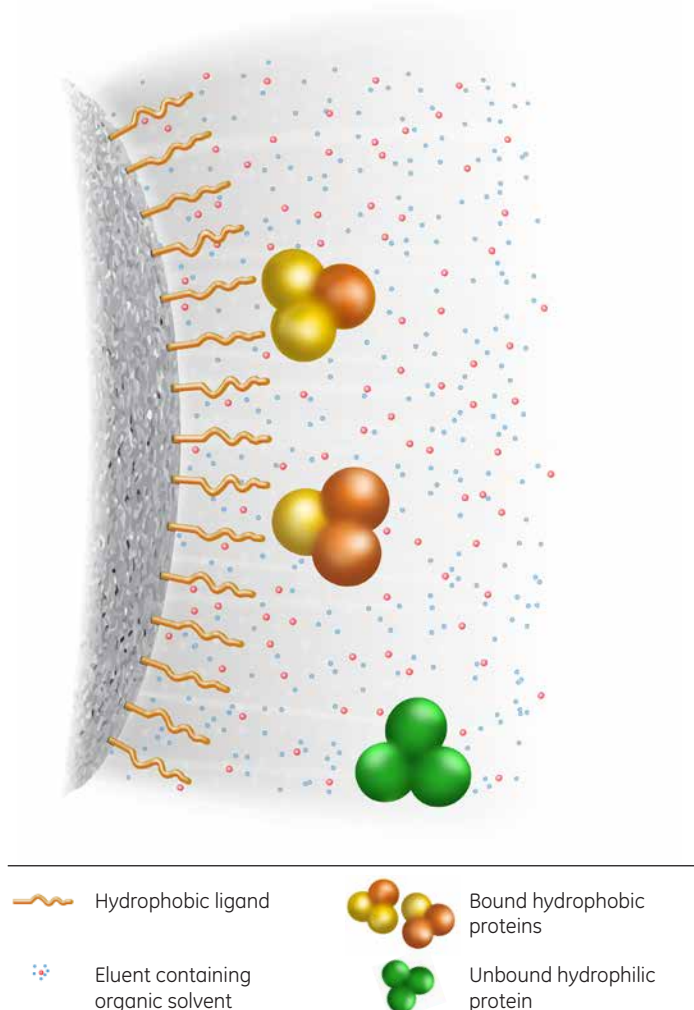


Fig 15. Schematic depicting RPC.

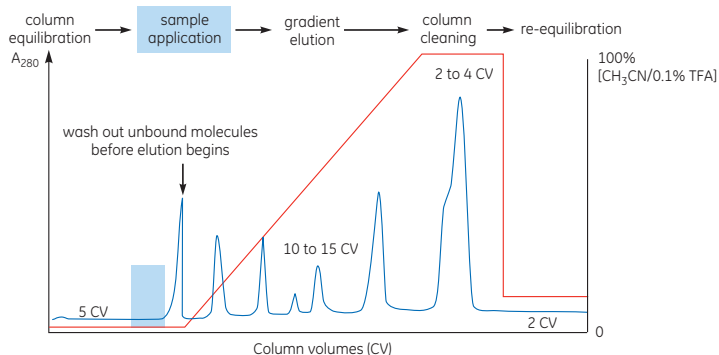


Fig 16. Typical RPC purification with gradient elution.

Chromatofocusing (CF)

CF separates proteins according to differences in their isoelectric point (pI). It is a powerful method and can resolve very small differences in pI (down to 0.02 pH units) and thus separate very similar proteins. However, the capacity of the method is low; CF should ideally be used for partially pure samples. A pH gradient is generated on the column as buffer and medium interact. The medium is a weak anion exchanger, and the buffer is a polyampholyte elution buffer containing a mixture of polymeric buffering species that buffers a broad pH range. Proteins with different pI values migrate at different rates down the column as the pH gradient develops, continually binding and dissociating while being focused into narrow bands and finally eluted.

CF applications

CF is useful for high-resolution, analytical separations and in preparative purification if IEX or other methods do not give a satisfactory purification.

Related literature

Handbook

Product code

Affinity chromatography handbook: principles and methods	18102229
Recombinant protein purification handbook: principles and methods	18114275
Size exclusion chromatography handbook: principles and methods	18102218
Ion exchange chromatography and chromatofocusing handbook: principles and methods	11000421
Hydrophobic interaction chromatography and reversed phase handbook: principles and methods	11001269



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