Swayam Course - Analytical Techniques

Week: 4, Module10 - Affinity Chromatography

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1. Learning outcomes

- Principle of affinity chromatography
- Process and components of affinity chromatography
- Immobilization of affinity ligands
- Types of and factors affecting affinity chromatography
- Applications and precautions

2. Introduction

Affinity chromatography is also known as bio-selective adsorption. This technique uses specific binding affinity between two molecules. In this technique, a specific ligand is bound to an inert support matrix and immobilized in such a way that when a sample is passed through it, the molecules which have specific affinity to the ligand will bind to it. After washing off of non-specific molecules present in the sample, the bound molecule(s) can be eluted from the ligand attached to the matrix. Affinity chromatography utilizes a protein's biological structure for its purification; thus, it offers high capacity, selectivity and resolution. As a result, purification is often easily achieved with affinity chromatography. In 1968, affinity chromatography was introduced by Chris Anfinsen, Meir Wilchek and Pedro Cuatecasas. They described a method to purify an enzyme using immobilized substrates and inhibitors. This field is still developing. It is widely applied to explore fields such as post translational modifications and protein–protein interactions. In recent times, the development of mass spectrometry instruments and their coupling with reversed phase affinity chromatography has helped enormously in disease biomarker discovery.

3. Principle

Affinity chromatography is different from other types of chromatography as it does not depend on variations in the physical properties of the analytes. It uses very specific biological interactions for resolution of analytes. Therefore, it has the conceptual ability of giving absolute purification, even if the analyte of interest has many different impurities, in single process.
Affinity chromatography is based on the reversible interaction between the target molecule and the affinity ligand immobilized on the column matrix. Suitable ligand selection is determined by an inherent recognition site present on the target molecule, such that the binding between the target molecule and the ligand is very specific as well as reversible.

$$A + L \rightleftarrows AL \text{ complex}$$

Where:

A: Analyte of interest
L: Ligand attached to matrix

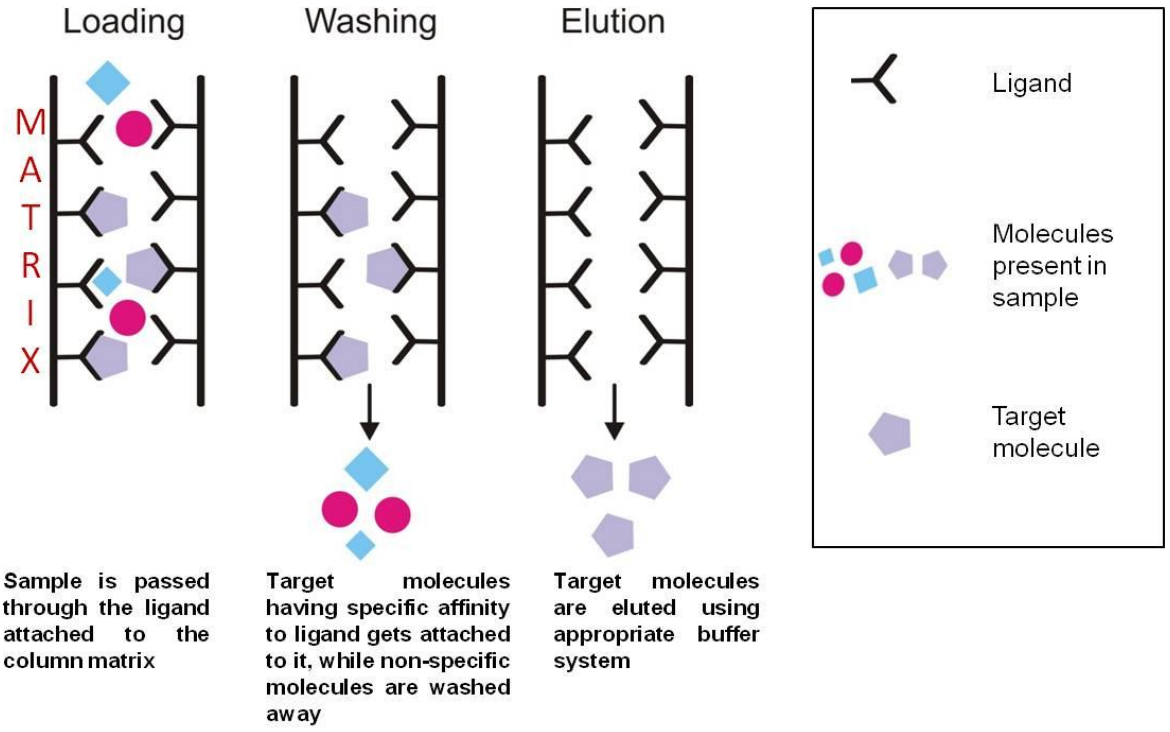

Figure: A simple schematic representation of affinity chromatography

When a mixture which has the analyte of interest is added to the immobilised ligand, only that analyte will bind to it. All other molecules will be washed off. For an analyte to be purified using affinity chromatography, its detailed structure and biological specificity should be known so that ideal separation conditions can be prepared.

4. Process of affinity chromatography

Affinity chromatography process has three basic steps – sample loading, washing off of non-specific molecules and elution of target molecule. Prior to starting a separation process, the affinity column (a column which contains an affinity resin, a specific ligand immobilized on an inert matrix) is equilibrated with the conditions that favour maximum binding between the target molecule and the affinity ligand. The sample is then loaded on to the column. This is followed by column washing to remove unbound substances, so that only the target molecule is attached to the affinity column. Finally, target molecule is eluted either by changing the buffer conditions (e.g. pH or ionic strength) that disrupt the interaction between the target molecule and the affinity ligand or by adding a competitive molecule which has a greater affinity for the ligand.

5. Components of affinity chromatography

There are several components of affinity chromatography, which are decisive for the purification of a specific biomolecule(s) from complex mixtures. The column matrix, spacer arms and affinity ligands are the most important components.

<u>Column matrix</u>
Affinity ligands are coupled to various supports, such as latex beads, nano and macro particle beads, magnetic particles, microarray surfaces and many other supports to capture specific biomolecules.

Traditionally used matrices for affinity chromatography are agarose, cellulose, polyacrylamide and silica. The commercially available matrices come in a wide range of particle and pore sizes. Some affinity resins with commonly used affinity ligands immobilized on them are also available e.g. protein A/G, heparin, Cibacron Blue, etc.
In addition to these porous matrices, non-porous and monolithic support matrices are also being developed. The factors which should be considered while selecting an appropriate affinity support material include pore size, stability, chemical inertness and mechanical stability.
An ideal matrix should-

- be mechanically and chemically stable during attachment and elution of the analyte
- be easily derivatized
- have a surface that is easily accessible to the ligand
- have good flow properties
- be stable over a wide range of buffers
- possess suitable and sufficient functional groups so that the ligand can be coupled
- have weak, if any, interactions with other molecules besides the analyte to minimise non-specific adsorption

Ligands

Different kinds of ligands are used to separate target molecules. These include antibodies, dye-ligands, DNA, RNA and peptides. The ligand is decided based on the biological specificity of the analyte. It may be possible to select a ligand that is extremely specific such that it will bind only to the analyte. Generally, a ligand having group selectivity is sufficient, such that it binds to a closely related group of molecules having similar structure/biological specificity. The ligand should also have a suitable functional group using which it can be attached to the matrix.
In addition to commercially available affinity ligand-matrix resins, customized affinity supports can also be designed to target unique biomolecules. To design custom affinity system, a ligand needs to covalently attached to a matrix. The type of linkage between the immobilized ligand and the matrix is critical as it significantly affects the performance of the affinity separation.

Ligand	**Affinity**
5'-AMP	NAD^+-dependent dehydrogenases, some kinases
2'5'-ADP	$NADP^+$-dependent dehydrogenases
Calmodulin	Calmodulin-binding enzymes
Avidin	Biotin-containing enzymes
Fatty acids	Fatty-acid-binding proteins
Heparin	Lipoproteins, lipases, coagulation factors, DNA polymerases, steroid receptor proteins, growth factors, serine protease inhibitors
Proteins A and G	Immunoglobulins
Concanavalin A	Glycoproteins containing a-D-mannopyranosyl and a-D-glucopyranosyl residues
Soybean lectin	Glycoproteins containing N-acetyl-a-(or b)-D-galactopyranosyl residues
Phenylboronate	Glycoproteins
Poly(A)	RNA containing poly(U) sequences, some RNA-specific proteins
Lysine	rRNA
Cibacron Blue F3G-A	Nucleotide-requiring enzymes, coagulation factors

Table: Examples of ligands used in affinity chromatography

Spacer arms

- A spacer arm, as shown in the figure below, is usually inserted between the support matrix and affinity ligand.

- It facilitates proper binding with target molecule and is helpful in providing a more effective binding environment.
- It is critical because in many cases binding sites of the target molecules are buried in the structure and thus, are restricted for ligand access.
- It is also useful when receptor area of the target molecule is too large for the ligand.
- Addition of a spacer arm helps the affinity ligand to move away from the surface of the matrix, which allows it to bind the target molecule more efficiently.
- The length of a spacer arm is critical as improper size may cause binding failure or non-specific binding.
- Its optimum length is 6-10 carbon atoms or equivalent.

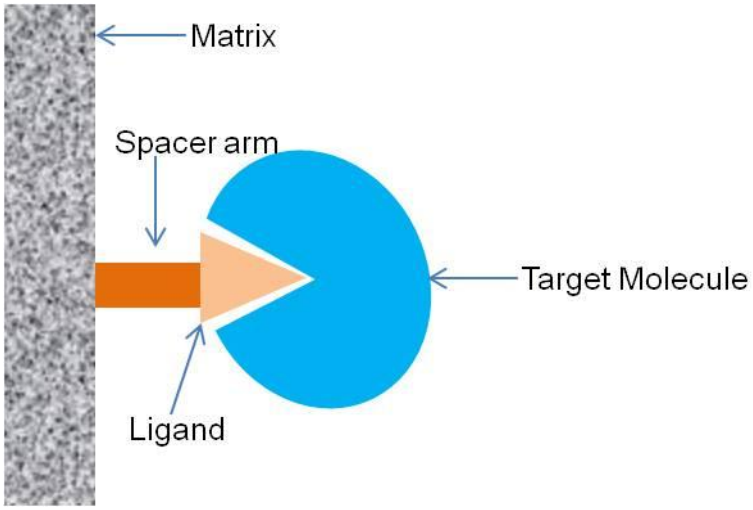

Figure: A Spacer arm

6. Immobilization of affinity ligands

- The type of linkage between the immobilized ligand and the matrix is critical as it significantly affects the performance of the affinity separation. Improper linkage may adversely affect the separation process.
- If linkage blocks the ligand structure, it may reduce the quality of affinity separation.
- Weak linkage may allow the release of immobilized ligand from the matrix and may contaminate the purified molecule.
- If a chemical reaction introduces a charged functional group into the matrix, it may result in nonspecific binding and thus, will generate ion-exchange effects.
- Care should be taken to ensure that the immobilized ligand is able to bind the target molecule easily.
- Ideally, a ligand is immobilized on a matrix in such a way that its binding pockets are fully exposed to bind the target molecules.
- Activity of a ligand can be affected by orientation of the ligand, multi-site attachment steric hindrance etc.
- Ligands can be immobilized by several methods, including covalently, co-ordination with metal ions, adsorption onto a surface or by entrapping inside a pore. However, covalent immobilization and adsorption are more commonly used strategies.

Covalent immobilization

- An affinity ligand specific for a target molecule can be attached covalently to a solid support matrix.
- It is one of the most widely applied methods to attach an affinity ligand on to a matrix.
- Various coupling chemistries for covalent-immobilization of ligands onto a matrix include amine, aldehyde, carboxyl, carbonyl, hydroxyl and sulfhydryl coupling which can efficiently link ligands onto support matrices.
- The matrix is first activated by an easily reactive compound (mentioned above) that is capable of binding with the ligand functional groups.
- This activated complex generates a covalent connection between the affinity ligand and the support matrix and thus, results in the immobilization of the ligand.

Adsorption

- Another method used for immobilization of a specific ligand on an affinity matrix is adsorption, which might be specific or nonspecific.
- Specific adsorption
  - In specific adsorption method, the attachment of the ligand is site-specific.
  - Common examples are:
    - Avidin or streptavidin are used for the adsorption of ligands containing biotin.
    - Protein A/G is usually used for immobilizing antibodies.
  - The primary ligands, such as streptavidin, avidin, protein A/G are first immobilized on the solid matrix using covalent immobilization (e.g. amine-reactive method).
  - Biotin or an antibody, as per the need, is then attached to them through adsorption.
- Nonspecific adsorption
  - In nonspecific adsorption, no specific sites are involved and the ligand molecules are adsorbed on the matrix surface by hydrogen bonding, coulombic or hydrophobic forces.

7. Types of affinity purification

Based on the different types of affinity targets, various affinity purification strategies are applied. Commonly available affinity chromatography procedures are described here -

Antibody purification

Antibodies are usually purified from serum or culture supernatants by applying affinity chromatography methods in 2 ways.

- Affinity purification with immobilized Protein A/G - Protein A and protein G are known to bind most of the IgG subclasses. IgG is produced by most of the mammals in response to various immunogens and is known to be the most abundant immunoglobulin. So, these proteins are widely used to capture antibodies using affinity strategies. Immobilized Protein A and G resins are available commercially. A specified antibody purified with this method might be contaminated with other IgGs or similar molecules if present in the crude sample. So, this method allows only partial purification of antibodies.
- Affinity purification with immobilized antigen - This strategy is used to purify a specific antibody from a crude sample. Antibody specific antigen is first covalently immobilized on a solid support matrix and then crude sample is passed through it. As the antigen -antibody reaction is extremely specific, the antibody specific for that antigen can be purified. The captured antibody is then eluted using appropriate buffers. Various activated resins and ready to use affinity kits are available commercially.

Antigen purification using antibodies

Antigens of interest can be purified at a small-scale using antibody affinity chromatography, commonly referred as Immunoprecipitation (IP). A typical IP method involves separation of an antibody-antigen complex using Protein A or G resin. In this method, a specific monoclonal purified antibody is covalently immobilized to the matrix by the cyanogen bromide method. It is then incubated with the sample in a neutral buffer. The antibody binds to its specific antigen present in the sample. Next, antibody-antigen complex is captured by Protein A or G beads. Finally, antibody-antigen complex is eluted using appropriate buffer, preferably by Lammeli buffer and separated by gel electrophoresis. Elution of the bound protein can be difficult because of the requirement of breaking its bond with the antibody. This can cause denaturation of the protein. High salt concentration with or without detergent; urea, guanidine hydrochloride, other chaotropic agents like thiocyanate etc; pH changes; organic solvents e.g. acetonitrile etc. can be used.

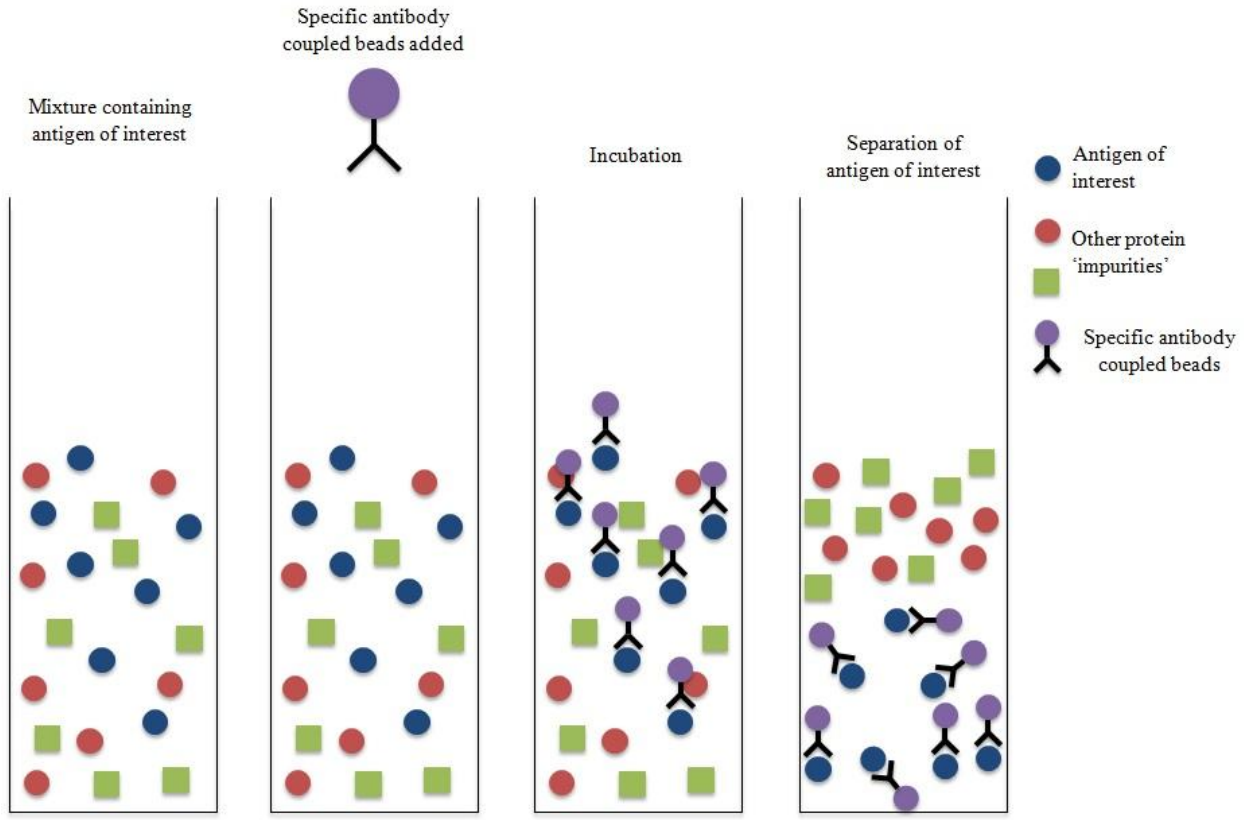

Figure: Antigen purification using antibodies

Co-immunoprecipitation

Co-Immunoprecipitation, commonly abbreviated as Co-IP is very similar to IP method. However, this method is used for studying protein-protein interactions. It involves not only capturing an antibody-antigen complex, but also the proteins attached to antigen in the crude sample. In contrast to IP, a soft buffer is used in Co-IP which is not so harsh that it destroys protein-protein interaction. A buffer with moderate pH range and less detergent (usually less than 100mM) is preferred.

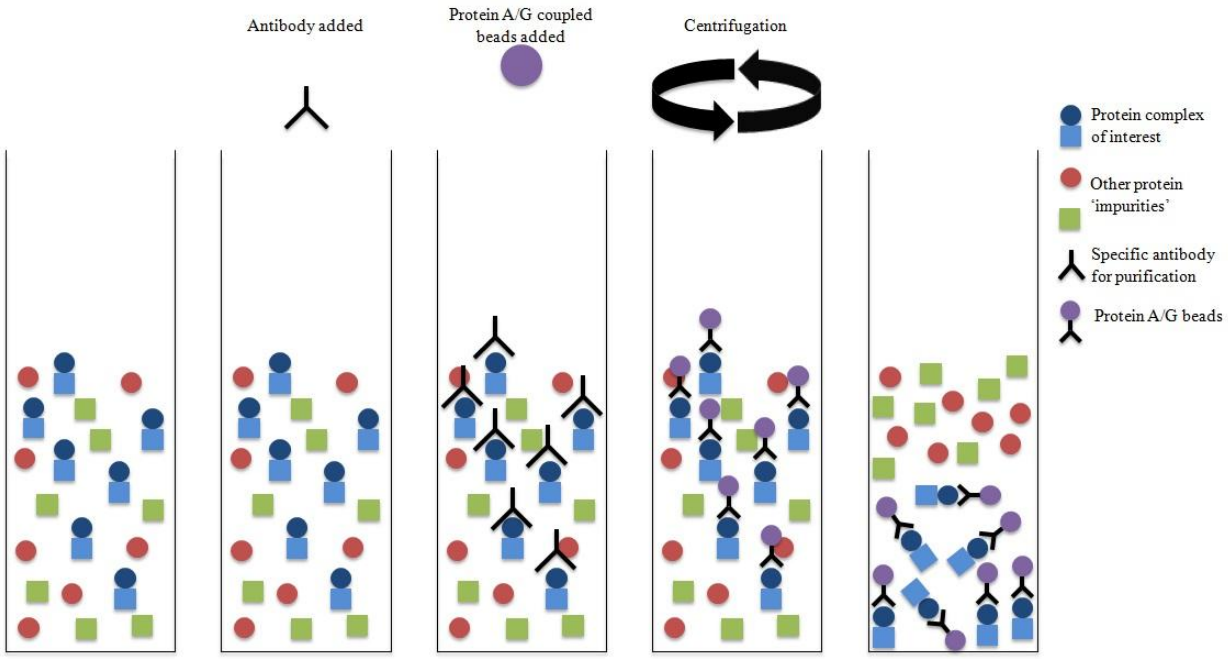

Figure: Coimmunoprecipitation

Pull-down assays

Pull-down assays are affinity methods commonly applied for analysis of protein interactions, specifically protein-protein complexes. A purified protein is essentially required for a pull-down assay. This purified protein is known as the bait. It is used to pull-down its interacting proteins in a sample. Interacting proteins(s) are commonly termed as the prey. The initial protein can either be purified from natural sources or it can be generated through cloning and expression. The bait protein is first immobilized on solid matrix and it is then mixed with the crude sample containing interacting proteins. The bait-prey protein complexes are separated using various available methods.

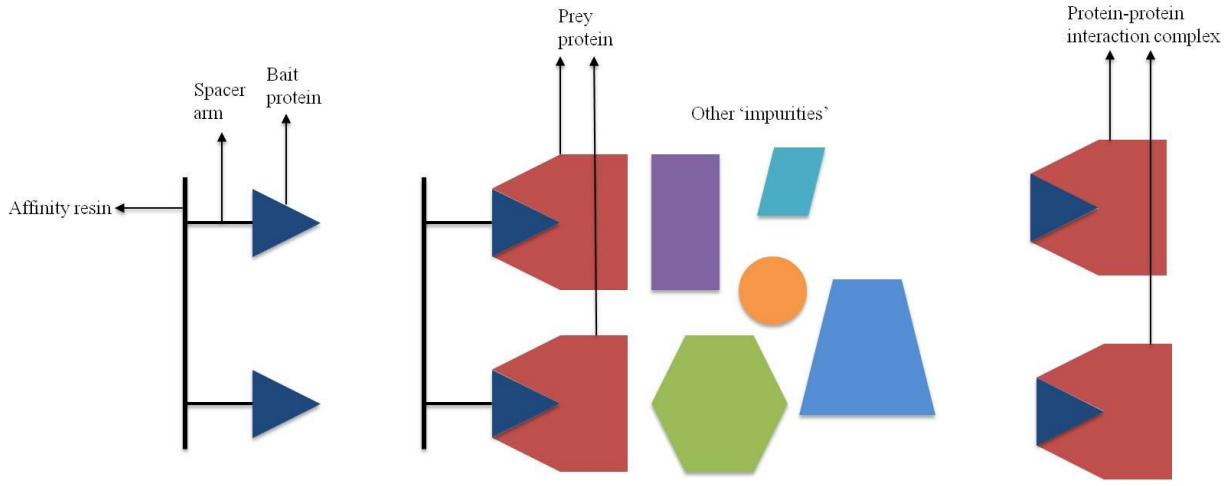

Figure: Pull down assay

Avidin-Biotin affinity systems

Avidin is a protein that has a strong affinity for biotin. Avidin-biotin interaction is the strongest known non-covalent interaction, that is unaffected by extreme pH ranges, high salt concentration, temperature, organic solvents and denaturing reagents. Also, bond formation between biotin and avidin is very rapid. Biotin is a co-factor which is known to have a crucial biological role in eukaryotes. Streptavidin is another protein that binds to biotin. These biotin-binding proteins have four biotin binding sites which make their interaction ideal for affinity purification. Other than affinity purification, avidin-biotin affinity system is widely applied in Western blotting, ELISA, immunohistochemistry, Fluorescence-activated cell sorting, electromobility shift assays and Cell-surface labelling.

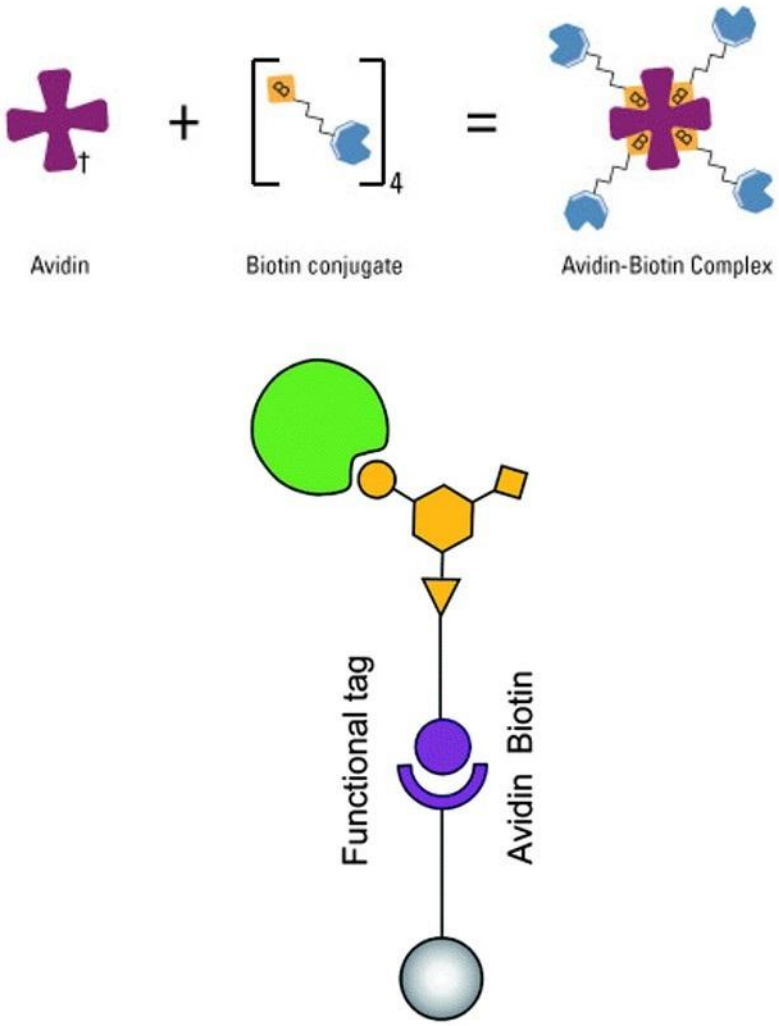

Figure: Avidin-biotin system

Recombinant Tag Protein Purification

In the process of recombinant protein expression, a functional domain (which is called a fusion tag) or some additional amino acids can be added to help in its purification. The fusion tags are usually appended to the DNA which encodes the specific protein sequence. For example, His tag is one of the most common fusion tags. It can be a string of six (6xHis tag) to nine (polyHis tag) histidine residues. These tags easily bind to metal ions, e.g. cobalt, nickel, etc. Other important fusion tags are GST, HA, Myc,

FLAG, MBP, SUMO etc. Unlike His and GST tags, epitope tags require specific antibodies for purification and are rarely used for purification at large-scale.

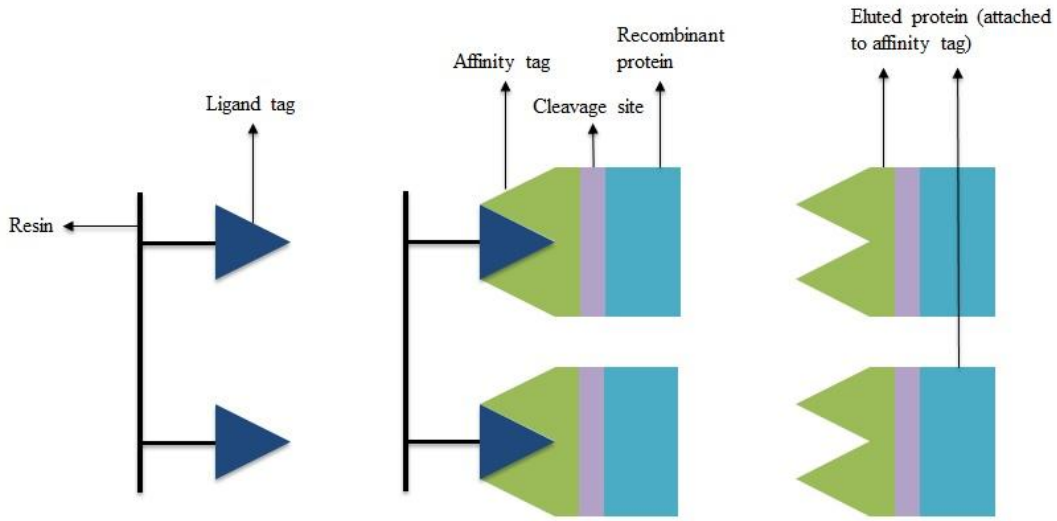

Figure: Recombinant Tag Protein Purification

Lectin affinity chromatography

Lectins are carbohydrate or sugar binding proteins usually containing two or more carbohydrate binding sites. Lectin affinity chromatography uses this sugar binding property to purify glycoproteins. It is a powerful method for studying post translation modification in proteins, specifically glycosylation. In lectin affinity technique, sugars (N-linked or O-linked) attached to a protein bind to an immobilized lectin. The bound glycoprotein is then eluted using a buffer containing sugar towards which the lectin has higher affinity. Several lectins are available commercially. Concanavalin A and wheat germ agglutinin (WGA) are widely applied lectins for purification of glycoproteins.

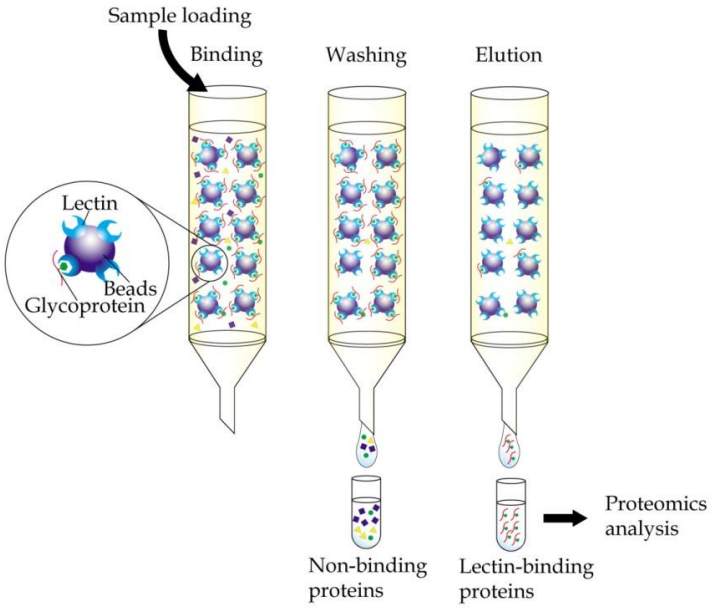

Figure: Lectin affinity chromatography

Metal chelate chromatography (immobilised metal affinity chromatography)

In this, an immobilised metal ion e.g. Cu^{2+}, Zn^{2+}, Hg^{2+} or a transition metal ion e.g. Co^{2+}, Ni^{2+} is used to bind proteins selectively. Usually, iminodiacetate- or tris(carboxymethyl)- ethylenediamine-substituted agarose is used as the solid support matrix. The metal ions react with the imidazole of histidine, thiol of cysteine and indole of tryptophan. A coordinate bond is formed between the metal ion and these amino acids present as residues in the protein of interest which allows its purification or separation. The protein can be then eluted by acidification i.e. decreasing the pH. EDTA can also be used which replaces the amino acid and makes a coordinate bond with the metal ion forming a complex. Nickel and cobalt are commonly used for His-tagged proteins.

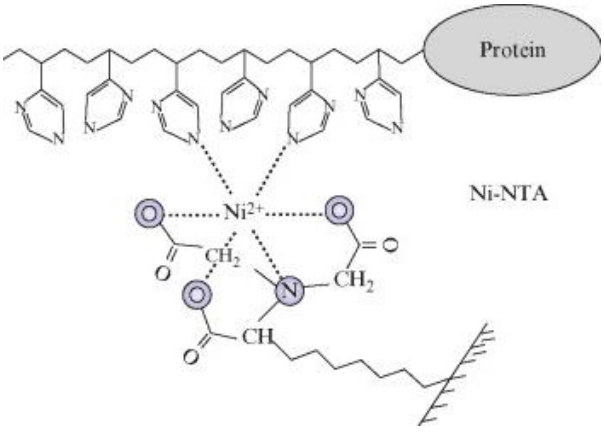

Figure: Metal chelate chromatography

Dye–ligand chromatography

Triazine dyes having both conjugated rings and ionic groups can bind to some proteins non-specifically. Hence, they are called pseudo-ligands. Selecting a dye for separating a specific protein is a trial-and-error process. It cannot be predicted if the protein of interest will bind to a given dye as the interaction is non-specific. The binding of a dye molecule to a protein molecule, usually at pH 7-8.5, increases its binding to other molecules like Sepharose and this property is used for their purification. The protein can be separated from the dye by a salt gradient or by affinity (displacement) elution. Using dyes has advantages as they are not costly, are stable and can be coupled to usually used matrices easily. The most commonly used dye is Cibacron Blue F3G-A.

Covalent chromatography

This is used to purify thiol (-SH-) containing proteins. Usually, a disulphide 2'-pyridyl group attached to an agarose matrix is used as a ligand. When this reacts with the protein having -SH-, pyridine-2-thione is released. This can be observed spectrophotometrically at 343 nm and the protein's adsorption can be known. Any unreacted thiopyridyl is eliminated by adding 4mM dithiothreitol or mercaptoethanol. The protein can be eluted by 20-50mM dithiothreitol, reduced glutathione or cysteine. The matrix can then be recycled by treating with 2,20-dipyridyldisulphide. This process can be employed for various proteins and can be used even for very impure protein preparations. The main limitations of this process are that it is very expensive and that recycling the matrix is quite difficult.

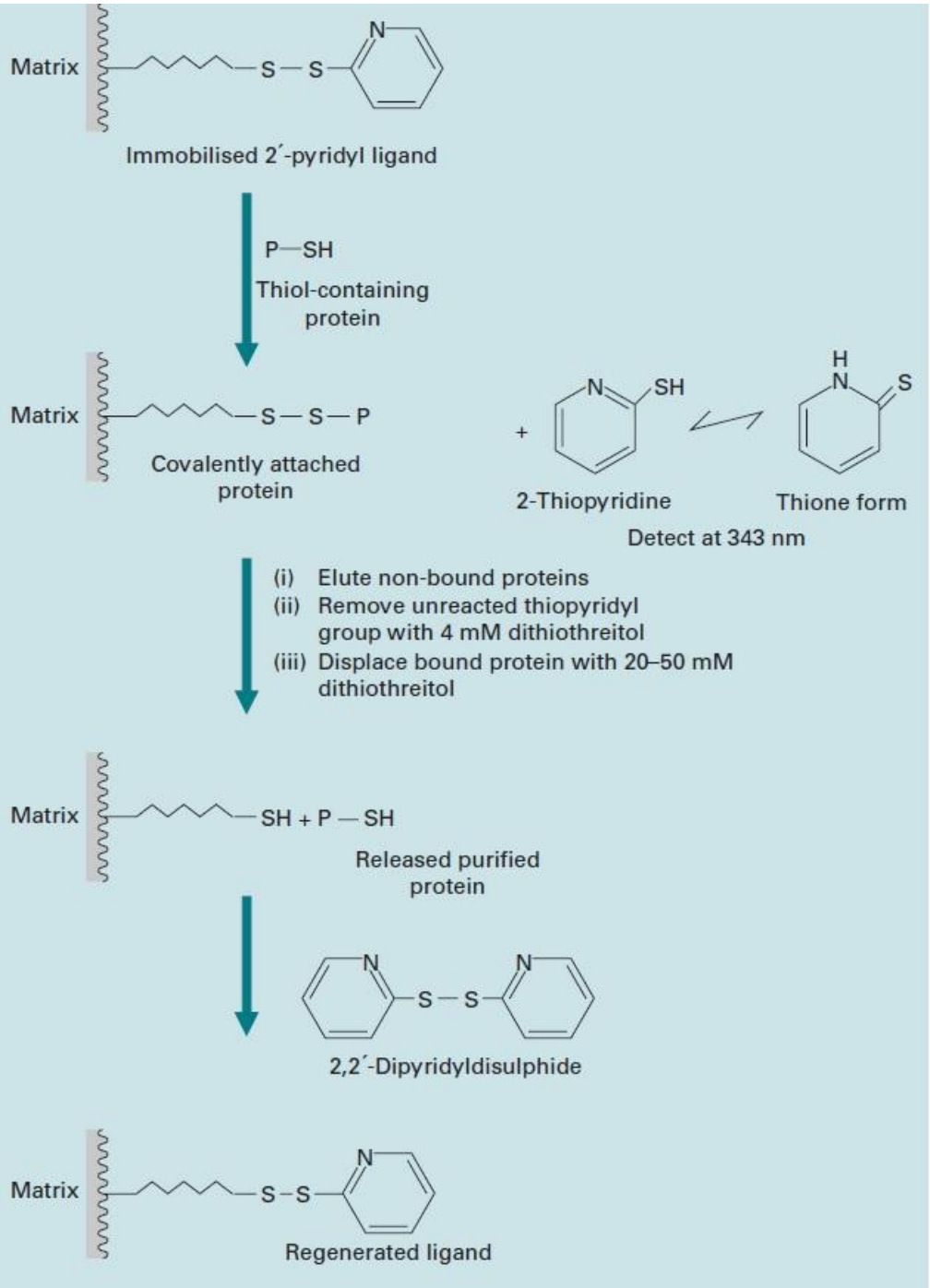

Figure: Covalent affinity chromatography (Reproduced from "Principles and Techniques of Biochemistry and Molecular Biology" p. 471, 7th edition, by Wilson K and Walker J., 2010, Cambridge University Press,)

8. Factors affecting affinity chromatography

Each type of affinity system requires its own set of conditions and faces its own challenges for a specific purpose. There are numerous factors which affect the separation quality in affinity chromatography, but selection of affinity ligands, buffer systems and sample preparation are the most important ones.

9. Applications

Affinity chromatography is widely used in several industries and research set ups. Common applications of are:

1) Depletion of high abundant proteins
2) Enrichment of proteins or protein groups
3) Study of protein-protein interactions
4) Purification of antibodies and proteins

10. Precautions

1) Selection of appropriate media and affinity ligand is very essential.
2) Buffer should be selected in such a way that it does not interfere with the affinity between the ligand and the biomolecule. It should also be capable of maintaining natural environment needed for their interaction.
3) Sample should be prepared in appropriate buffer and it should not be much concentrated as it may hinder the affinity due to overcrowding of other molecules.

11. Explanatory example

Suppose we have a mixture of 3 proteins, albumin, pepsin and lysozyme and we need to separate albumin. We can choose pre packed columns like CaptureSelect™ (Thermo Fischer) HiTrap™ Blue (GE) etc. The column is loaded according to the manufacturer's specifications. Albumin is the only component which binds to the column while pepsin and lysozyme flow through in the unbound fraction. Hence, albumin is separated from the mixture.
Another example is the use of Lectin (Concanavalin A column) affinity chromatography for the purification of N-glycosylated proteins. Cancanavalin A protein has four sugar binding sites. Thus, all the N-glycosylated proteins in a sample will specifically bind to the column while other proteins will flow through as unbound fraction. The bound proteins can be eluted with a buffer containing high affinity sugars e.g. Methyl-α-D-Mannopyranoside.

12. Comparison of three commonly used chromatographic techniques

S. No.	Affinity chromatography	Gel filtration chromatography	Ion exchange chromatography
1.	It is based on specific interactions between the molecule of interest and a specifically designed ligand.	It is based on separating molecules based on their molecular size and weight alone.	It is based on separating molecules pased on their charge which can be changed by adjusting the pH in relation to their pI.
2.	Specific ligands of the molecules of interest should be available.	The molecular weight/size of the molecules of interest should be different.	The pI of the molecules of interest should be different.
3.	It can also be used to study protein-	It can also be used to	It can also be used for

	protein interactions.	determine the molecular weight of the components of a mixture.	protein purification.
4.	It is used to deplete high abundant proteins like albumin from a sample. It can also be used to enrichment specific proteins or protein groups and in the study of protein-protein interactions or the purification of specific antibodies and proteins.	It can be used for purification, concentration and desalting.	It is used in water treatment and other applications like pharmaceutical research food industry, chemical industry etc.
5.	Upon elution, protein will be bound tightly to the ligand and may require severe conditions for separation which can cause denaturisation.	Upon elution, purified protein will be diluted and will have to be concentrated for further experimentation.	Upon elution, purified protein will always be in salt, which will have to be removed for further experimentation.

13. Summary

- Affinity chromatography is a technique that uses specific binding interactions between molecules.
- Affinity chromatography has three main components: solid matrix, spacer arm and affinity ligand.
- Immobilization of ligands on solid support can be achieved by various methods, such as covalent immobilization and adsorption.
- There are various types of affinity separation systems. These are antibody purification, IP & Co-IP, lectin affinity, avidin-biotin affinity and recombinant tag purification.