Lecture 6: Affinity Chromatography-I

Note : Affinity chromatography will be covered in two lectures. The first lecture will cover general discussion while second will be more specific.

1. Introduction
2. General Principle of Affinity Chromatography
3. Procedure
4. Specific examples of Affinity Purification
5. Application of Affinity Chromatography in Proteomics

1. Introduction

Affinity chromatography was introduced almost 50 years back as a powerful tool for purification of biologically active molecules like proteins. This technique has revolutionary impact on modern biological sciences such as molecular biology, biochemistry, medicine and biotechnology. This technique exploits molecular recognition principle of a biological compound to be separated by the specific ligand to purify it from a mixture of compounds.

The affinity chromatography is a type of liquid chromatography for the separation and specific analysis of sample components. This type of chromatography makes use of a reversible “biological interaction” (molecular recognition) for the separation and analysis of specific analytes within a sample e.g. enzyme with an inhibitor and antigen with an antibody. One of the components, the ligand is immobilized onto a solid matrix, which is then used to selectively purify the target protein. Including a competing ligand in mobile phase or changing pH that elutes the target protein out. For example, Ni-Affinity chromatography is applied for the purification of 6xHis tagged proteins in which Ni is the chelating metal which is attached on NTA matrix (more detail of Ni-Affinity chromatography in next lecture).

Theoretically affinity chromatography is capable of giving absolute purification in a single step. The technique was developed for purification of enzymes but now affinity chromatography is used for various other purposes like purification of nucleotides, nucleic acid, immunoglobulin, membrane receptors etc.

The biological interactions involve mostly non covalent interactions between the reactive groups of molecule targeted for purification and ligand with a dissociation constant K_d.

$$K_d = \frac{[A]\,[B]}{[AB]}$$

Where, A is assumed as molecule targeted and B as ligand and AB is the complex formed between them. K_d varies between 10^{-3} to 10^{-7}M for affinity binding.

The principle of affinity chromatography is that the stationary phase consists of a support medium (e.g. cellulose beads) on which the substrate (or sometimes a coenzyme) has been bound covalently, in such a way that the reactive groups that are essential for enzyme binding are exposed. As the crude mixture of proteins is passed through the chromatography column, proteins with binding site for the immobilized substrate will bind to the stationary phase, while all other proteins will be eluted in the void volume of the column.

Once the other proteins have all been eluted, the bound enzyme(s) can be eluted in various ways. We will discuss elution methods in during coming lectures.

Procedure

Preparation of column: The solid support (bead matrix) is a gel loaded into an elution column. Sepharose, agarose and cellulose are the most commonly used solid support, because the hydroxyl groups on the sugar residues can be easily manipulated to accept a

ligand. The ligand is then selected according to the desired isolate. In case a researcher planning to isolate antibodies specific for antigen A from an antiserum, antigen A can be used as ligand. There are several methods of immobilizing protein ligand on these solid support and some examples are given below (Fig. 1)

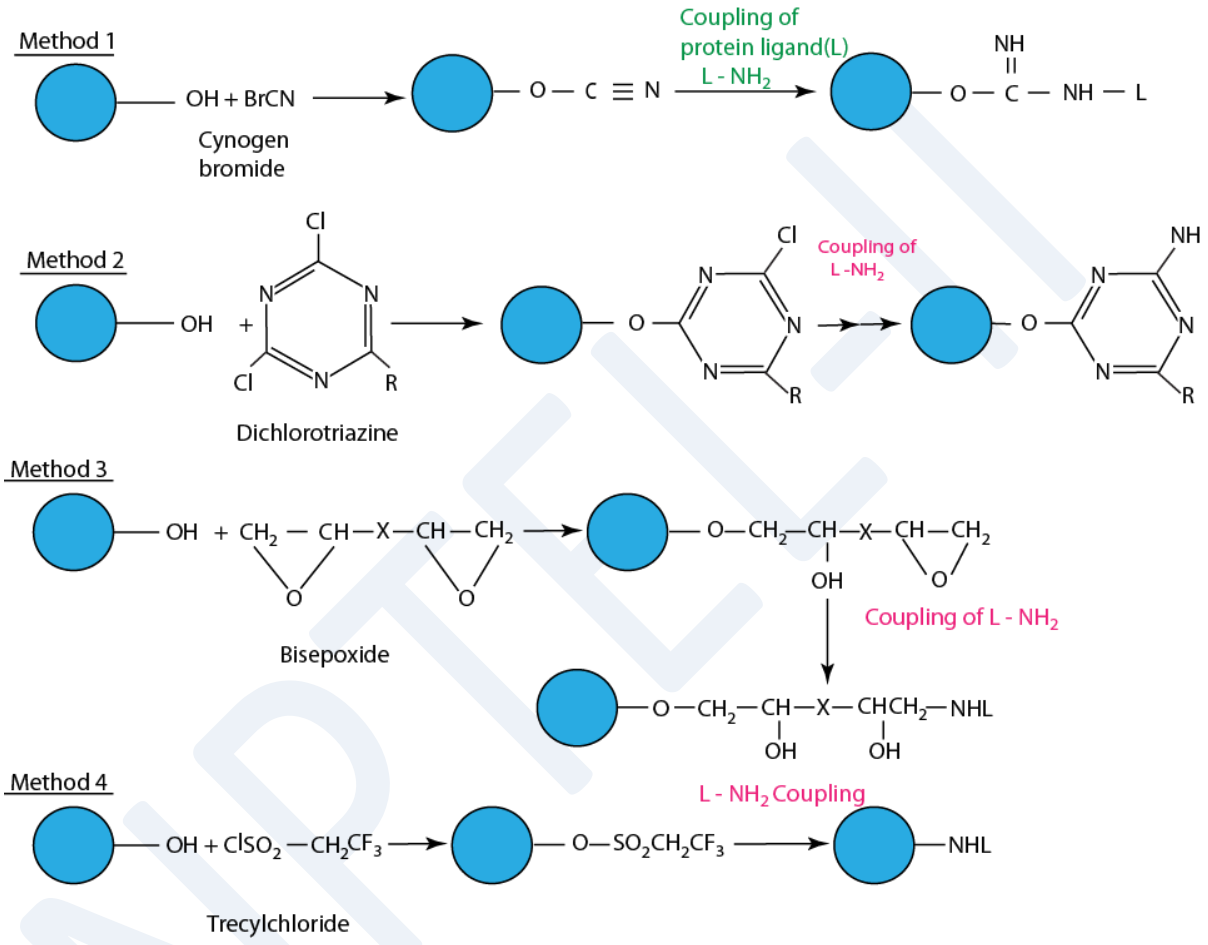

Figure 1: Immobilizing protein ligand on solid support

Sometimes flexible spacer-arm is attached between ligand and solid support to render better flexibility to ligand. For example NHS-activated Sepharose (agarose beads with 10-atom spacer arms (6-aminohexanoic acid) attached by epichlorohydrin and activated by N-hydroxysuccinimide) is used for protein ligand immobilization (Fig. 2)

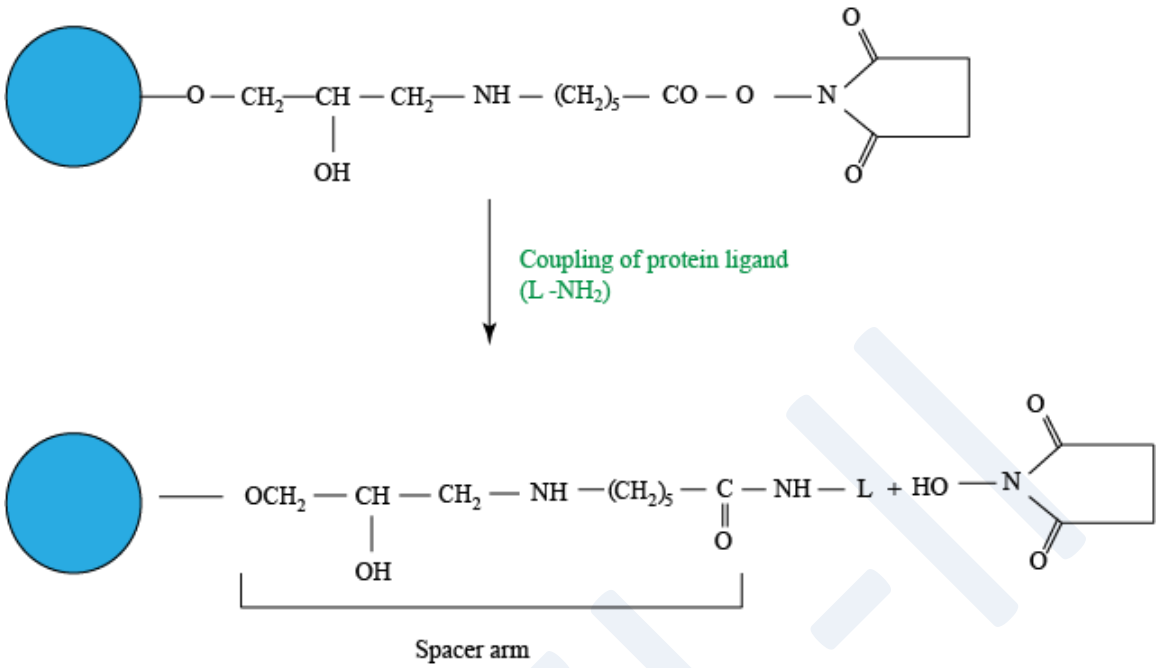

Figure 2: Flexible spacer-arm may be attached between ligand and solid support.

Likewise, if someone wants to separate specific enzyme, a strategy may involve immobilization of either its substrate, an inhibitor, or even a cofactor on solid support.

Two factors are required for the ligand:

I. Specific and reversible binding with the desired protein.
II. The ligand is capable of covalent bonding to the matrix without disrupting its binding activity. This is usually facilitated by the placement of spacer arms between the ligand and the matrix, so that in case the active site is buried deep within the ligand, it is not physically hidden from its binding substrate.

Steps of affinity purification

Loading of solution containing the substance to be isolated: The solution is usually a protein rich mixture such as antiserum, which is poured into the elution column and allowed to run through the gel, at a controlled rate. Proteins with specific affinity for immobilized ligand shall bind and other proteins will go in flow through. This follows

washing of column with buffer to remove all unbound protein (Fig. 3). Some affinity purification procedures are summarized in the table 1

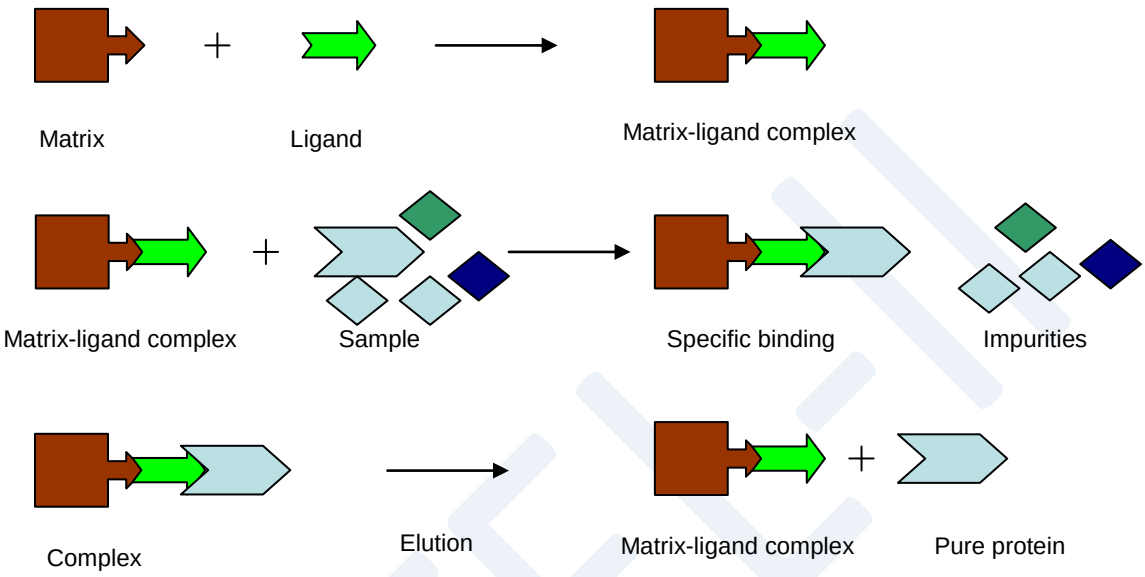

Figure 3. An outline of affinity purification scheme.

Table 1: List of a few affinity procedures.

Ligand	Affinity/purification process
Avidin	Avidin is a tetrameric protein deposited in the egg white of birds, reptile and amphibians. This protein has affinity for biotin, cofactor of several enzymes. Immobilized avidin column is used for purification of biotin containing enzyme. Non-biotin molecules do not bind to immobilized avidin and are washed away. Bound proteins with biotin may be competitively eluted using 2 mM biotin.
Calmodulin	Calmodulin, a regulatory Ca^{+2} binding protein, is present in all eukaryotic cells. Calmodulin binds proteins through their interactions with hydrophobic sites on its surface which is exposed after Ca^{+2} binding to the enzyme. Elustion is done by chelating agent such as EGTA or EDTA. Once Ca^{+2} bound to Calmodulin is chelated, a reversal of the conformational change which expose the protein binding sites takes place. This results in protein elution.
Concanavalin A	Concanavalin A is tetrameric metalloprotein. This protein is a carbohydrate-binding protein (lectin) originally extracted from the jack-bean. The immobilized Concanavalin A is used for affinity purification of glycoproteins containing a-D-mannopyanosyl and a-D-glucopyranosyl residues. Elusion of bound glycoprotein is achieved by increasing gradient of α-D-methylmannoside or α -D-methylglucoside.
Protein A and G	Immunoglobulins

We shall study some very common affinity purification methods in more details during next lecture.