Affinity Chromatography

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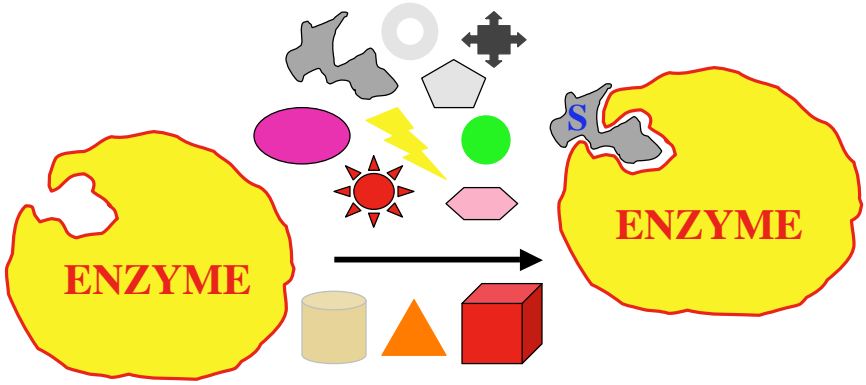
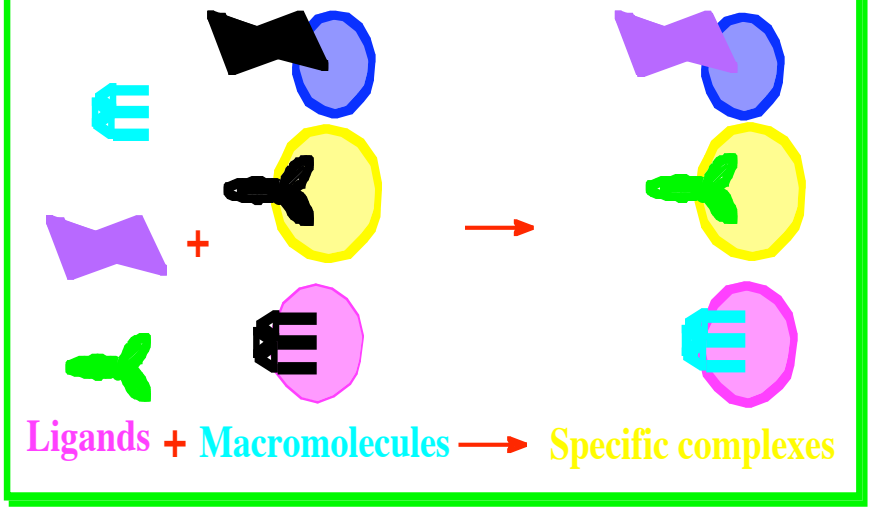
Binding of enzyme and substrate is specific



Of the numerous substrates available, enzyme binds specifically to only its substrate.

Biological interactions are highly specific



Affinity chromatography

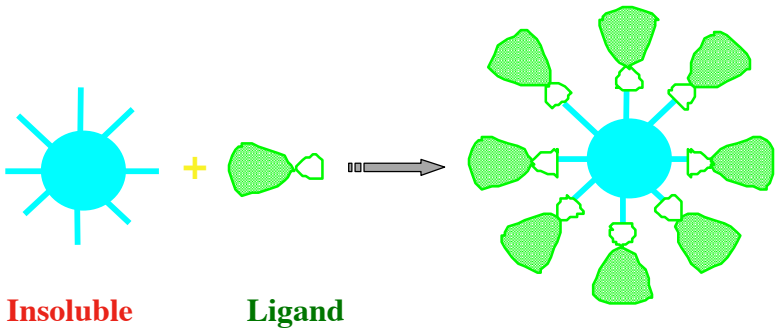
- It is a separation technique based on biological affinity.
- Also called biospecific chromatography.

Molecules that can be purified

Macromolecules	Ligands that can be used
Enzymes	Substrates, inhibitors, (cofactors)
Transport proteins	Specific transportable molecules
Antibodies	Antigens
Receptors	Hormones
Glycoproteins	Lectins (carbohydrate binding proteins)
Metal binding proteins	Metal ions
Hydrophobic proteins	Hydrophobic ligands
Nucleic acids	Complementary nucleic acids; Binding proteins
Nucleic acid Binding proteins	Nucleic acids with correct structure

Steps involved in affinity chromatography

A ligand exhibiting specific affinity to the macromolecule to be isolated is first linked covalently to an insoluble matrix.

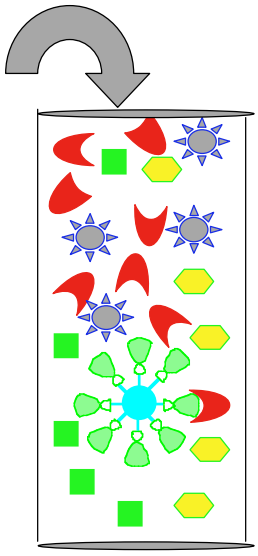


Insoluble Matrix with Spacer arm

Ligand

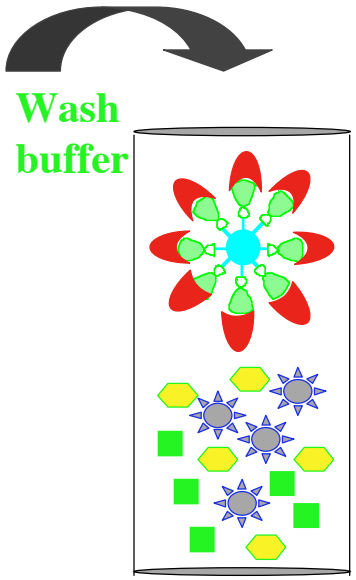
Immobilized ligand

Mixture of biomolecules are loaded on the column containing the ligand insolubilized matrix at the pH and ionic strength that is appropriate for specific binding of the ligand to the macromolecule

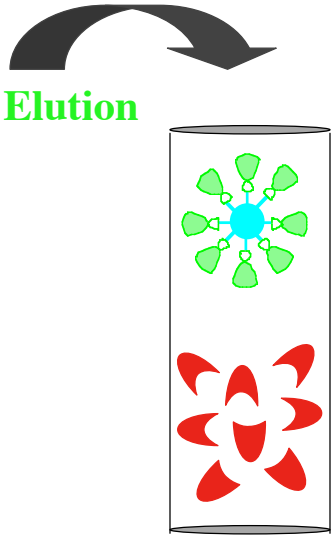


Specific binding occurs between the ligand and the macromolecule.

All other proteins are washed off of the column.



The bound macromolecule is specifically eluted from the column by buffers that dissociate the complex.
It could be change of pH, ionic strength, or inclusion of competing ligands.



Attention to pay:

1. Binding strength

- Too weak a binding, the macromolecule will not bind tightly to the ligand and might come out of the column during washing itself.
- Too strong a binding, the macromolecule binds so tightly to the ligand, separation of the complex may be very difficult.

Binding strength is important

$$E + S \underset{k_2}{\overset{k_1}{\rightleftharpoons}} ES \qquad K_d = \frac{k_2}{k_1} = \frac{[E]\,[S]}{[ES]}$$

K_d of 10^{-4} M to 10^{-8} M is good for performing affinity chromatography

K_d higher 10^{-4} M is weak binding. Enzyme might come out in washing.

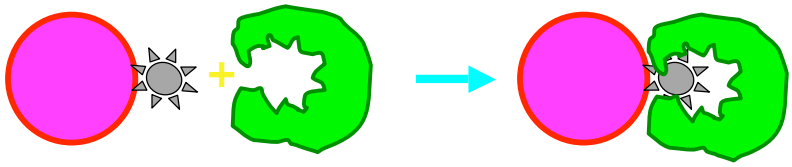
K_d lower 10^{-8} M is tight binding. Elution of enzyme will be difficult.

Attention to pay:

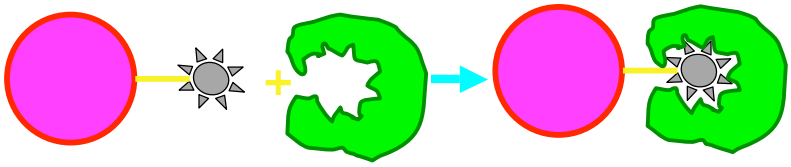
2. Spacer arm

- **With out a spacer arm, the bound ligand may not be positioned far enough to overcome any possible steric hindrance between the macromolecule and the matrix. This will prevent obviously the binding of the ligand to its specific macromolecule.**

Use of appropriate spacer arm

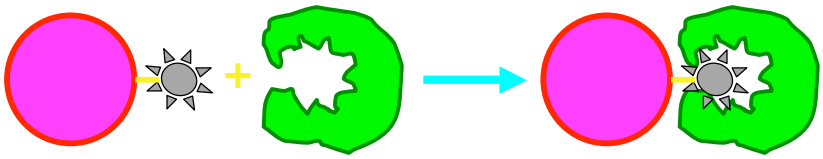


Ligand bound to matrix with out a spacer arm, prevents the binding of macromolecule due to steric hindrance



Spacer arm allows binding of the ligand to macromolecule.

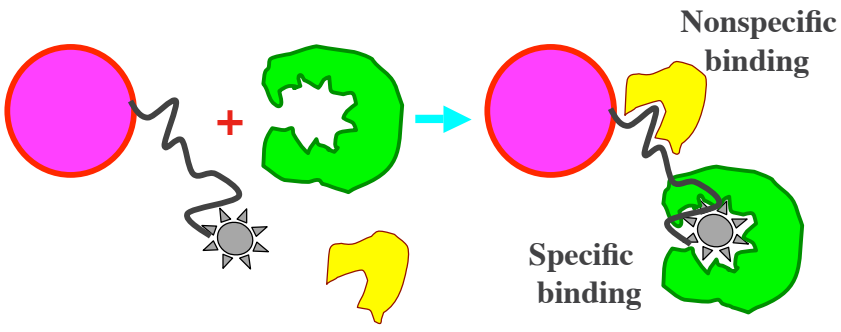
Problems with spacer arm



A small a spacer arm will still prevent the binding of ligand to its macromolecule.

Problems with spacer arm

A larger spacer arm will show nonspecific binding.

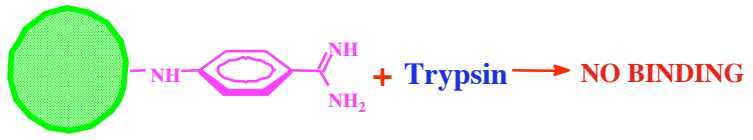


Ideal Spacer Arm

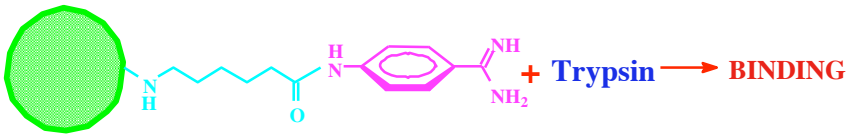
- Should not exhibit any nonspecific binding.
- Should allow for appropriate binding.

Usually a spacer arm of 6 - 12 methylene group is ideal

Example: Importance of Spacer Arm



p-Amino benzamidine linked directly to the bead
does not allow binding of trypsin



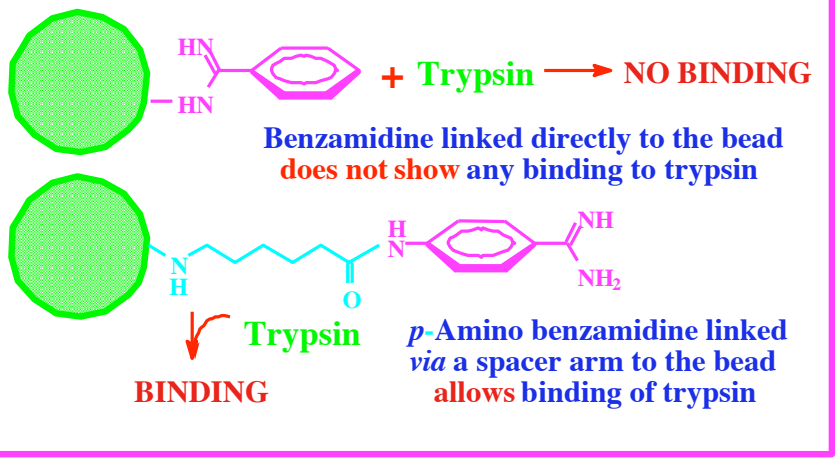
p-Amino benzamidine linked *via* a spacer arm
to the bead allows binding of trypsin

Attention to Pay:

3. Binding chemistry

- **The group(s) associated with the binding of ligand to the macromolecule should be kept free. If they were used for linkage to the matrix, they won't be able to bind the macromolecule. Such Immobilized ligands will be useless for affinity chromatography.**

Groups on the ligand necessary for binding to the macromolecule must be kept free



Attention to pay:
4. Binding Components

- **If binding requires any components other than substrate, such as a trace metal ions, then you have to make sure that such components are provided to the enzyme to promote its binding to the affinity column.**
- **This requirement is essential in addition to the normal pH and ionic strength required for optimal binding of the enzyme to its substrate.**

Case of homogentisate dioxygenase

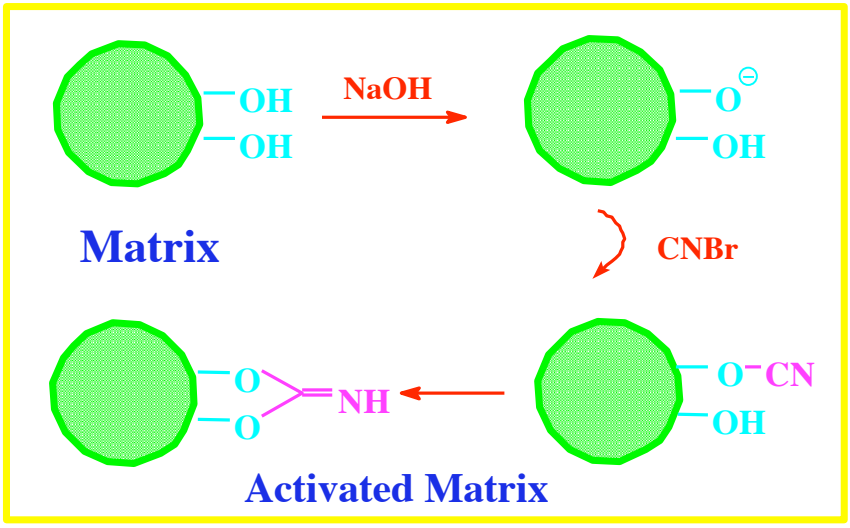
- Homogentisate dioxygenase from *Aspergillus niger* binds to *o*-hydroxy phenylacetate linked Sepharose column only after pre-incubation with ferrous iron. With out this treatment, the enzyme will not bind to the column.
- Since ferrous iron on the enzyme is lost during preliminary purification, it is important to replace the essential ferrous iron for the enzyme so that it can bind to its substrate (and analogs).

Methods of Immobilization

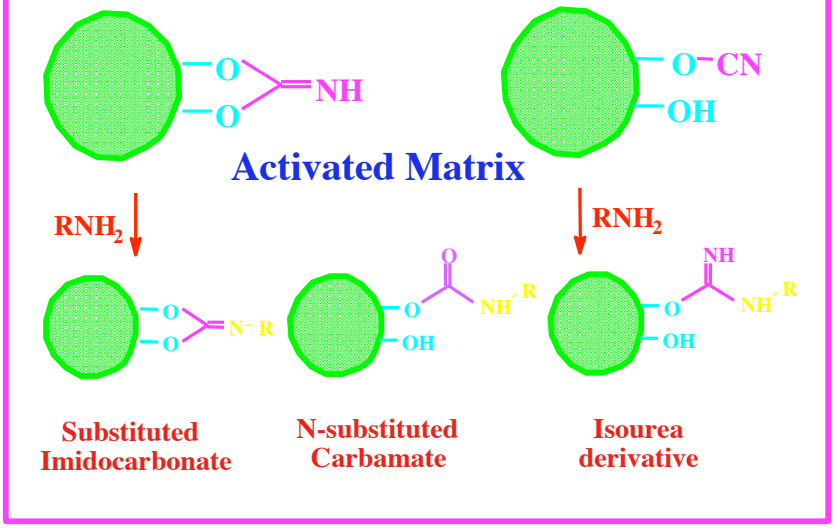
There are numerous methods to immobilize the ligands.

One of the method is outlined below

CNBr - Activation of the Matrix

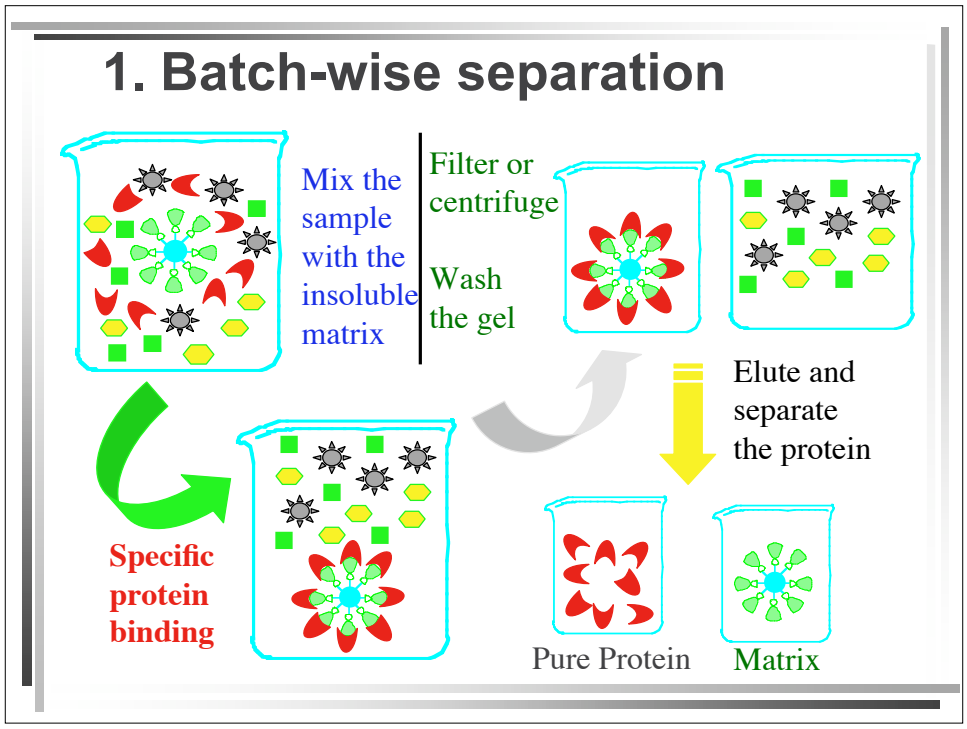


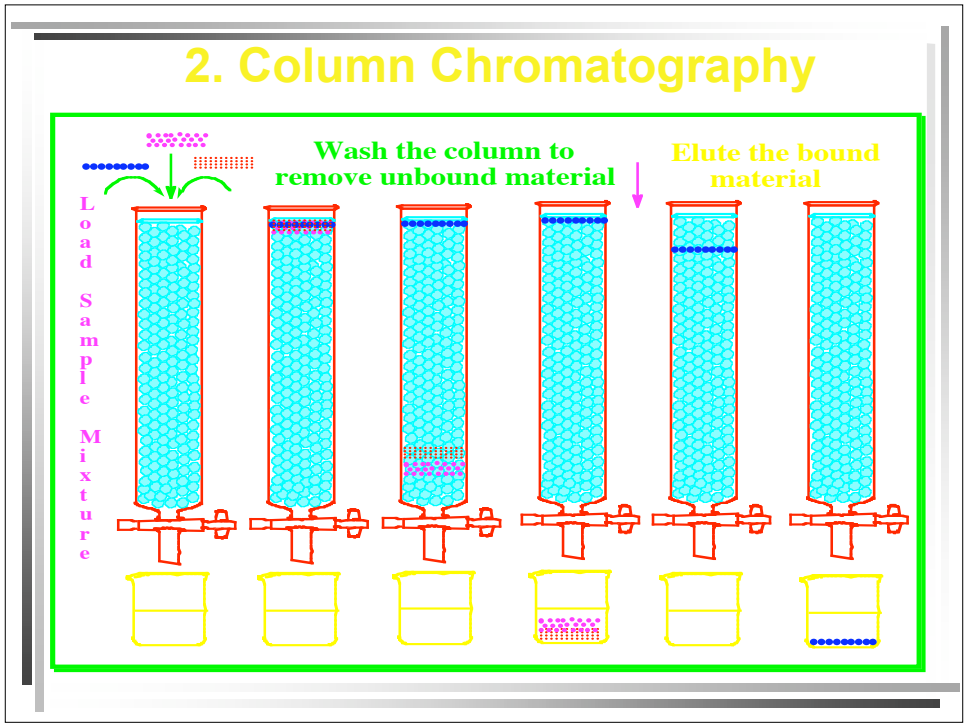
CNBr - Ligand binding to matrix



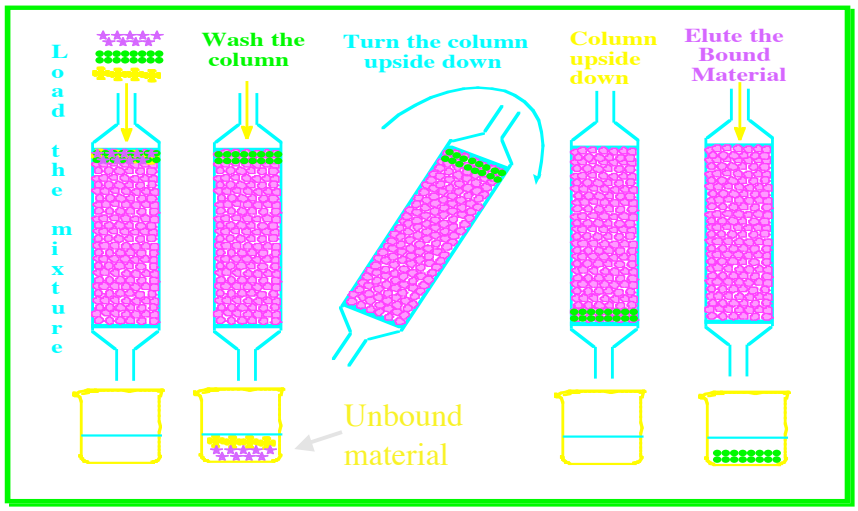
Three ways of performing affinity chromatography

1. Batch wise Separation
2. Column Chromatography
3. Magnetic Separation

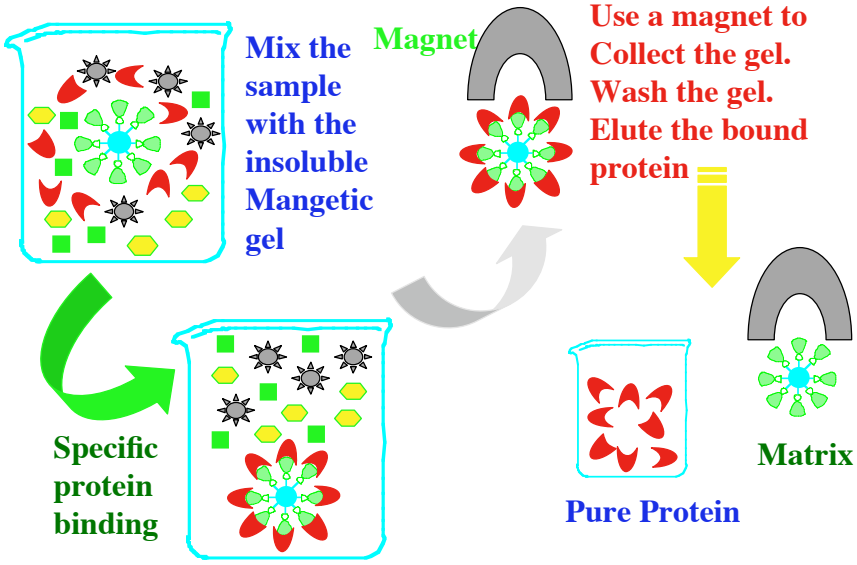

1. Batch-wise separation
Mix the sample with the insoluble matrix
Filter or centrifuge
Wash the gel
Elute and separate the protein
Specific protein binding
Pure Protein
Matrix


2. Column Chromatography
Wash the column to remove unbound material
Elute the bound material
Load Sample Mixture

Unique way of eluting bound material from columns



3. Magnetic Separation



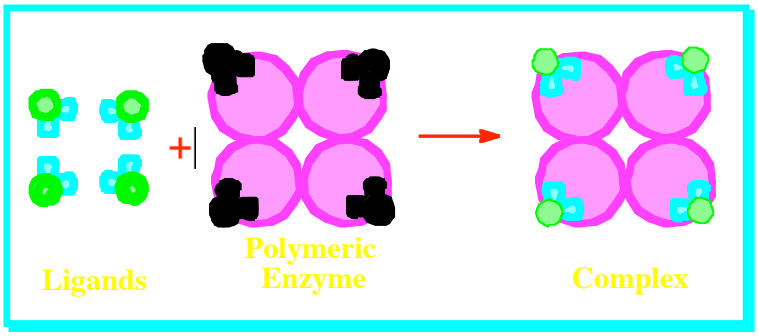
General Affinity Chromatography

Instead of targeting a single protein, a group of proteins exhibiting specific binding for a specific ligand is targeted here.

Some general affinity chromatography ligands

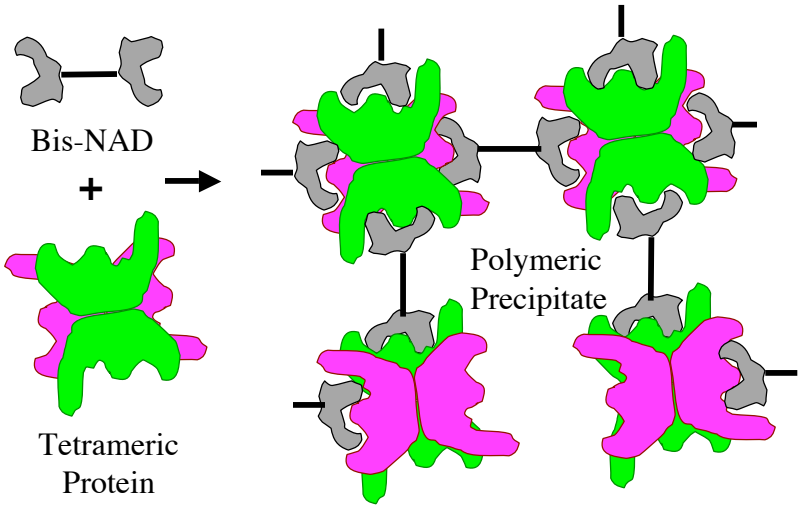
- **Concanavalin A - terminal glucose & mannose**
- **Lentil lectin - Similar to above.**
- **Wheat germ lectin - N-acetylglucosamine**
- **Poly U - mRNA with poly A tails.**
- **Lysine - Plasminogen; ribosomal RNA**
- **Protein A - Immunoglobulin G**
- **Cibacron Blue - nucleotide enzymes, serum albumin**
- **5'-AMP - enzymes binding to NAD and kinases.**
- **2'5-ADP - Enzymes binding to NADP.**

Affinity Precipitation

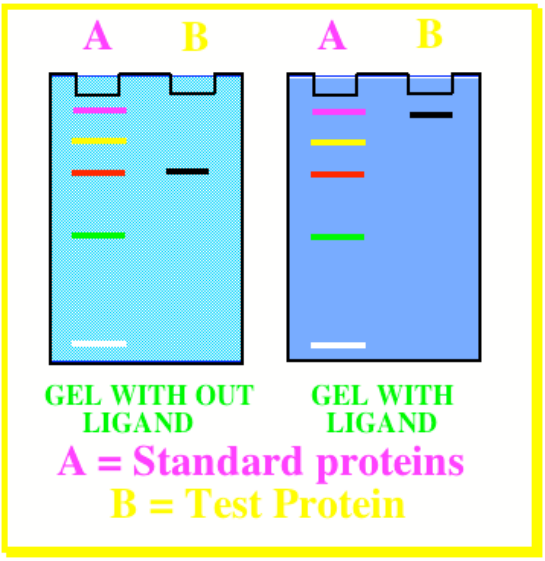


If a protein has multiple binding sites for a ligand, one can make a dimeric ligand that will allow binding of the enzyme through different molecules. This causes dimerization, trimerization and eventual polymerization.

Example:
Lactate Dehydrogenase



Affinity Electrophoresis



Polyacrylamide gel is polymerized with and without the ligand which shows affinity for the test protein. Test protein is subjected electro-phoresis on both gels. Test protein will show reduced mobility on the gel with the ligand.

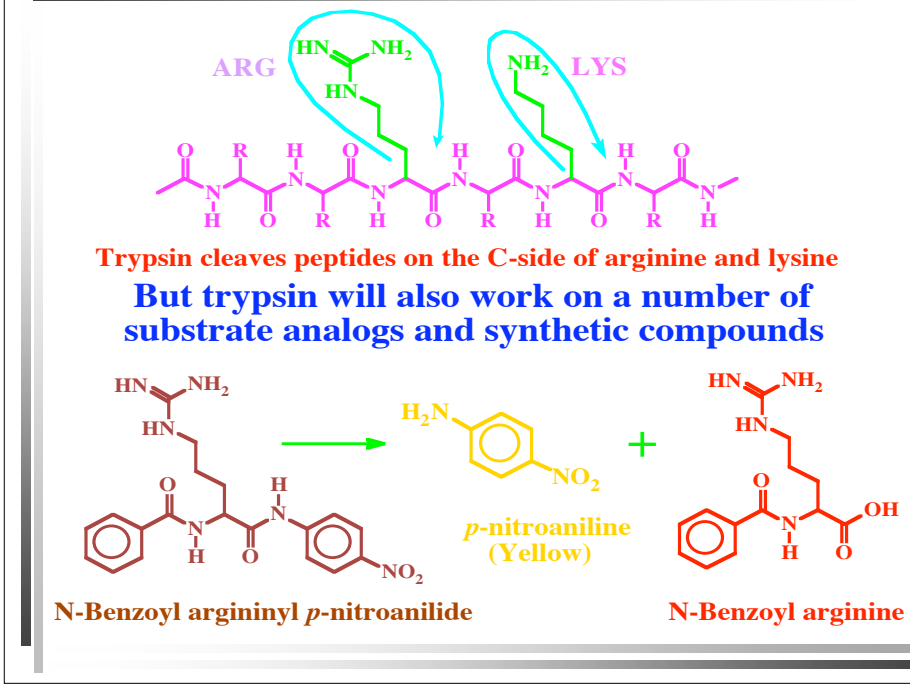
Affinity electrophoresis- uses

- to find out binding affinity between two macromolecules.
- to identify even weak interactions between macromolecules.

LIMITATION:
The ligand that is copolymerized in the gel has to be a macromolecule. It should not migrate in the gel during electrophoresis and should remain trapped in the gel. Above all, it should retain its biological activity to exhibit binding.

Trypsin affinity chromatography

- Trypsin is a proteolytic enzyme which cleaves peptides at arginine and lysine.
- It also hydrolyzes artificial substrates which meet some structural requirements.
- You will separate active form from the inactive form of trypsin using *p*-aminobenzamidine sepharose column.
- Active form binds to the above column, while inactive form comes out in the wash.

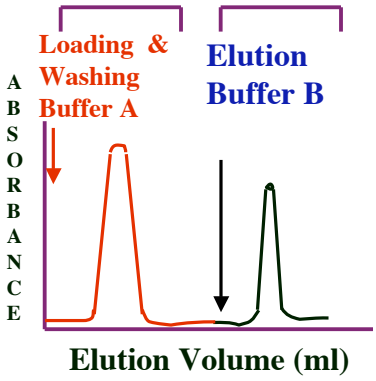


Protocol

- Pre-equilibrate the given column with Tris HCl buffer, pH 8.0 (Bufferf A). At this pH, trypsin will bind to the column.
- Wash the column with the same buffer to get ride of the unbound materials.
- Elute trypsin specifically with 0.01 M HCl containing 0.05 M KCl (Buffer B). (elution is done by altering the pH and ionic strength).

Separation of active form of trypsin from inactive form.

Inactive form **Active form**

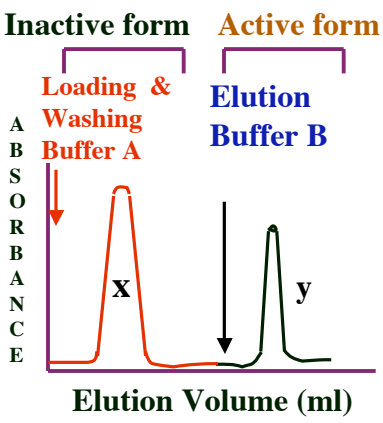


Active form of trypsin binds to Benzamidine Sepharose column tightly while inactive form comes out in the wash (0.1 M Tris-HCl, pH 8.00, Buffer A). Bound trypsin is eluted with 0.1. M HCl + 0.5 KCl (buffer B).

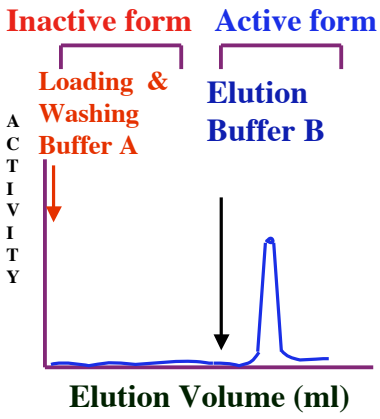
Hixson., HF. and Nishikawa, A. Arch. Biochem. Biophys. 154, 501-509 (1973).

The results of two assays - Protein estimation and trypsin activity determination

Graph 1.
Protein estimation (595 nm)



Graph 2.
Trypsin assay (410 nm)



Calculation

- Determine the protein concentrations in the bound and the unbound fraction (Graph 1).
- Determine the activity trypsin in the bound and unbound fraction (Graph 2).
- If trypsin is present only in the bound fraction, then percent active trypsin is given by the formula: protein in bound/total protein (multiplied by 100).(area y and area x from graph 1 are used for this calculation)

$$\text{Percent active trypsin} = \frac{y}{x + y} \times 100$$