AFFINITY CHROMATOGRAPHY

By-

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Principle

- Separation and purification of analytes by affinity chromatography is unlike most other forms of chromatography in that it does not rely on differences in the physical properties of the analytes.
- Instead, it exploits the unique property of extremely specific biological interactions to achieve separation and purification.
- As a consequence, affinity chromatography is theoretically capable of giving absolute purification, even from complex mixtures, in a single process.
- The technique was originally developed for the purification of enzymes, but it has since been extended to nucleotides, nucleic acids, immunoglobulins, membrane receptors and even to whole cells and cell fragments.

...Principle

- The technique requires that the material to be isolated is capable of binding reversibly to a specific ligand that is attached to an insoluble matrix:

$$\underset{\text{macromolecule}}{\text{M}} + \underset{\substack{\text{ligand}\\(\text{attached}\\\text{to matrix})}}{\text{L}} \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} \underset{\text{complex}}{\text{ML}}$$

- Under the correct experimental conditions, when a complex mixture containing the specific compound to be purified is added to the immobilised ligand, generally contained in a conventional chromatography column, only that compound will bind to the ligand.
- All other compounds can therefore be washed away and the compound subsequently recovered by displacement from the ligand.
- The method requires a detailed preliminary knowledge of the structure and biological specificity of the compound to be purified so that the separation conditions that are most likely to be successful may be carefully planned.
- In the case of an enzyme, the ligand may be the substrate, a competitive reversible inhibitor or an allosteric modifier.

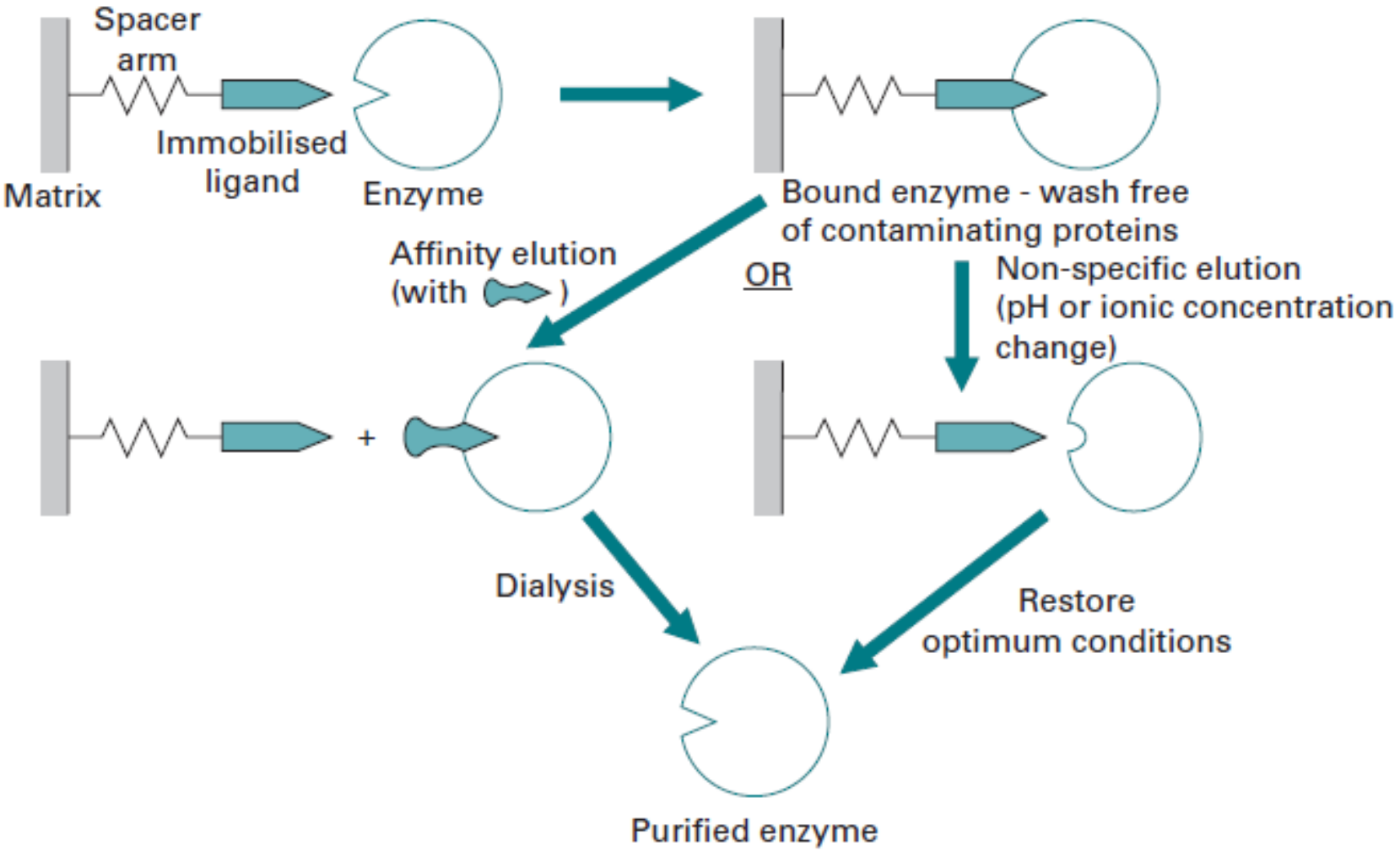

Fig. 11.10 Principle of purification of an enzyme by affinity chromatography.

Matrix

- An ideal matrix for affinity chromatography must have the following characteristics:
- possess suitable and sufficient chemical groups to which the ligand may be covalently coupled, and be stable under the conditions of the attachment;
- be stable during binding of the macromolecule and its subsequent elution;
- interact only weakly with other macromolecules to minimise non-specific adsorption;
- exhibit good flow properties.
- In practice, particles that are uniform, spherical and rigid are used.
- The most common ones are the cross-linked dextrans and agarose, polyacrylamide, polymethacrylate, polystyrene, cellulose and porous glass and silica.

Ligand

- In practice it is sometimes possible to select a ligand that displays absolute specificity in that it will bind exclusively to one particular compound.
- More commonly, it is possible to select a ligand that displays group selectivity in that it will bind to a closely related group of compounds that possess a similar in-built chemical specificity.
- An example of the latter type of ligand is 5'-AMP, which can bind reversibly to many NAD^+ -dependent dehydrogenases because it is structurally similar to part of the NAD^+ molecule.
- It is essential that the ligand possesses a suitable chemical group that will not be involved in the reversible binding of the ligand to the macromolecule, but which can be used to attach the ligand to the matrix.
- The most common of such groups are $-NH_2$, -COOH, -SH and -OH (phenolic and alcoholic).

Spacer arm

- To prevent the attachment of the ligand to the matrix interfering with its ability to bind the macromolecule, it is generally advantageous to interpose a spacer arm between the ligand and the matrix.
- The optimum length of this spacer arm is six to ten carbon atoms or their equivalent.
- In some cases, the chemical nature of this spacer is critical to the success of separation.
- Some spacers are purely hydrophobic, most commonly consisting of methylene (-CH2) groups; others are hydrophilic, possessing carbonyl (-CO) or imido (-NH) groups.
- Spacers are most important for small immobilised ligands but generally are not necessary for macromolecular ligands (e.g. in immunoaffinity chromatography),as their binding site for the mobile macromolecule is well displaced from the matrix.
- Several supports of the agarose, dextran and polyacrylamide type are commercially available with a variety of spacer arms and ligands pre-attached ready for immediate use.

Practical procedure

- The procedure for affinity chromatography is similar to that used in other forms of liquid chromatography.
- The buffer used must contain any cofactors, such as metal ions, necessary for ligand–macromolecule interaction.
- Once the sample has been applied and the macromolecule bound, the column is eluted with more buffer to remove nonspecifically bound contaminants.
- The purified compound is recovered from the ligand by either specific or non-specific elution.
- Non-specific elution may be achieved by a change in either pH or ionic strength.
- pH shift elution using dilute acetic acid or ammonium hydroxide results from a change in the state of ionisation of groups in the ligand and/or the macromolecule that are critical to ligand–macromolecule binding.
- A change in ionic strength, not necessarily with a concomitant change in pH, also causes elution due to a disruption of the ligand–macromolecule interaction; 1M NaCl is frequently used for this purpose.

Applications

- Many enzymes and other proteins, including receptor proteins and immunoglobulins, have been purified by affinity chromatography.
- The application of the technique is limited only by the availability of immobilised ligands.
- The principles have been extended to nucleic acids and have made a considerable contribution to developments in molecular biology.
- Messenger RNA, for example, is routinely isolated by selective hybridisation on poly(U)-Sepharose 4B by exploiting its poly(A) tail.
- Immobilised single-stranded DNA can be used to isolate complementary RNA and DNA.
- Whilst this separation can be achieved on columns, it is usually performed using single stranded DNA immobilised on nitrocellulose filters.
- Immobilised nucleotides are useful for the isolation of proteins involved in nucleic acid metabolism.

Immunoaffinity chromatography

- The use of antibodies as the immobilised ligand has been exploited in the isolation and purification of a range of proteins including membrane proteins of viral origin.
- Monoclonal antibodies may be linked to agarose matrices by the cyanogen bromide technique.
- Protein binding to the immobilised antibody is achieved in neutral buffer solution containing moderate salt concentrations.
- Elution of the bound protein quite often requires forceful conditions because of the need to disrupt the very tight ionic or hydrophobic binding with the antibody and this may lead to protein denaturation.
- Examples of elution procedures include the use of high salt concentrations with or without the use of detergent and the use of urea or guanidine hydrochloride, both of which cause protein denaturation.
- The use of some other chaotropic agents (ions or small molecules that increase the water solubility of nonpolar substances) such as thiocyanate, perchlorate and trifluoroacetate or lowering the pH to about 3 may avoid denaturation.
- Organic solvents such as acetonitrile can also be used to disrupt the hydrophobic interaction.

- **Metal chelate chromatography (immobilised metal affinity chromatography)**
- This is a special form of affinity chromatography in which an immobilised metal ion such as Cu^{2+}, Zn^{2+}, Hg^{2+} or Cd^{2+} or a transition metal ion such as Co^{2+}, Ni^{2+} or Mn^{2+} is used to bind proteins selectively by reaction with imidazole groups of histidine residues, thiol groups in cysteine residues and indole groups in tryptophan residues sterically available on the surface of the proteins.
- **Dye–ligand chromatography**
- A number of triazine dyes that contain both conjugated rings and ionic groups fortuitously have the ability to bind to some proteins.
- The term pseudo-ligand has therefore been used to describe the dyes.
- The attraction of the technique is that the dyes are cheap, readily coupled to conventional matrices and are very stable.
- The most widely used dye is Cibacron Blue F3G-A.

Covalent chromatography

- This form of chromatography has been developed specifically to separate thiol(–SH)-containing proteins by exploiting their interaction with an immobilised ligand containing a disulphide group.
- The most commonly used ligand is a disulphide 20-pyridyl group attached to an agarose matrix such as Sepharose 4B.
- On reaction with the thiol-containing protein, pyridine-2-thione is released.
- This process can be monitored spectrophotometrically at 343 nm, thereby allowing the adsorption of the protein to be followed.
- The protein is then released by displacement with a thiol-containing compound such as 20-50mM dithiothreitol, reduced glutathione or cysteine.
- The matrix is regenerated by reaction with 2,2’-dipyridyldisulphide.
- The method has been used successfully for many proteins but its use is limited by its cost and the rather difficult regeneration stage.
- It can, however, be applied to very impure protein preparations.