

Tools of Biochemistry

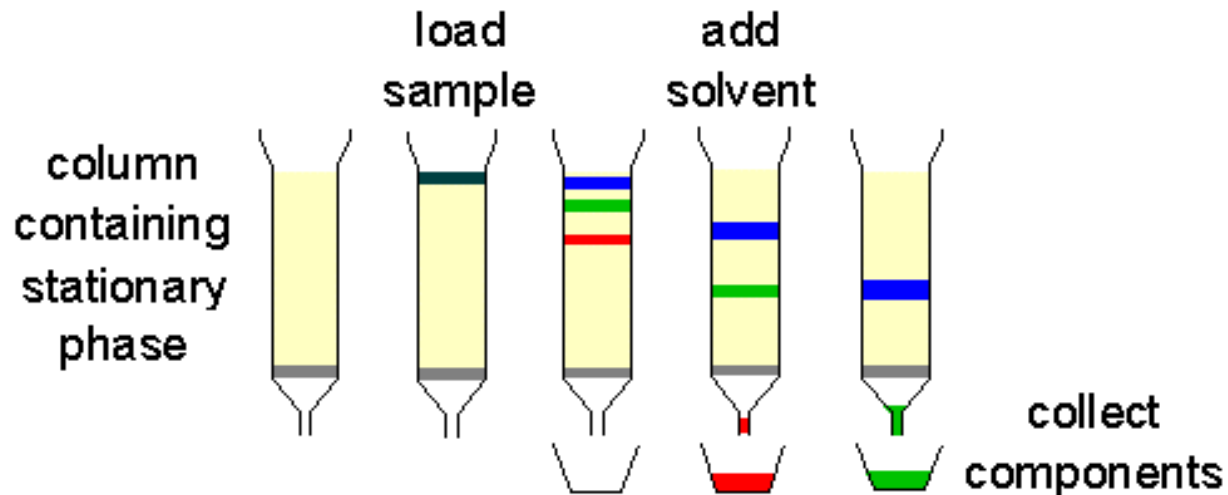
Column Chromatography

Pass a solution through a medium that shows selective absorption for different solutes.

Solute is added to the top of column and the column is eluted with solvent.

Fractions are collected. Molecules that do not absorb to the column elute first followed by molecules that absorb more strongly to the column.

Sometimes the composition of the elution solvent must be changed to remove more tightly bound molecules.



Tools of Biochemistry

Column Chromatography

Ion-Exchange Chromatography

Separate molecules on the basis of their electrical charge.

Ion-exchange resins are either polyanions or polycations.

How can three molecules with different charges be separated?:

Molecule A: negatively charged

Molecule B: weakly positively charged

Molecule C: strongly positively charged

A negatively charged (anionic) resin is used.

Molecule A would not bind to the resin and would therefore appear in early fractions.

Molecule B and **C** would bind, therefore a gradient of salt would be used to separate these molecules. **Molecule B** would elute before **Molecule C**.

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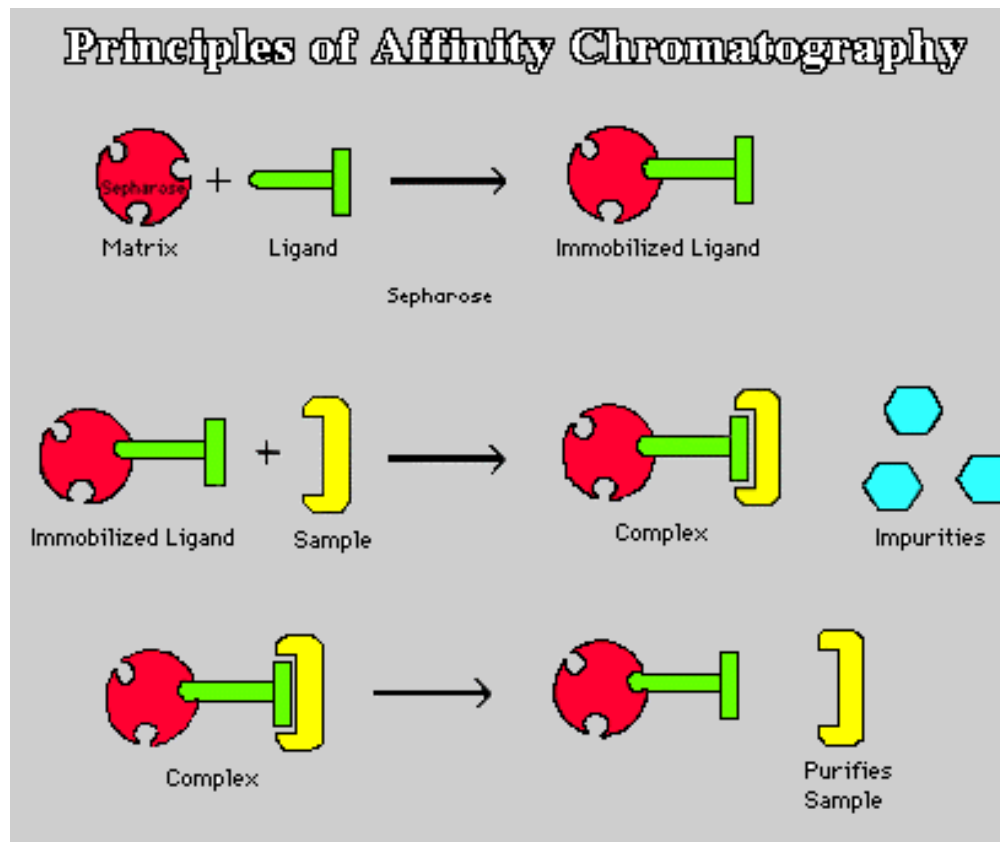
Column Chromatography

Affinity Chromatography

Successful separation by affinity chromatography requires that a biospecific ligand is available and that it can be covalently attached to a chromatographic bed material called a matrix.

It is important that the biospecific ligand retains its specific binding affinity for the substance of interest.

Methods must also exist for removing the bound material in active form with low pH, high pH, or high salt.



Tools of Biochemistry

Column Chromatography

Affinity Chromatography

Examples:

Substance to purify

Enzyme

Antibody

Nucleic Acid

Ligand bound to matrix

Substrate, inhibitor, cofactor

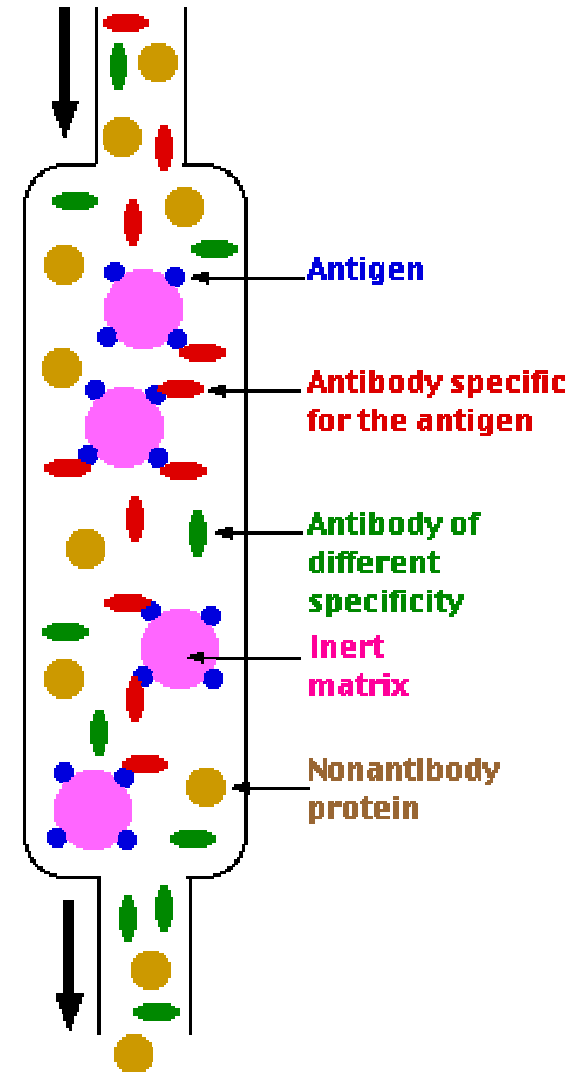
Antigen, virus

Complementary base sequence

An immunoabsorbent is prepared. This consists of a solid matrix to which the antigen (shown in blue) has been coupled (usually covalently).

The serum is passed over the immunoabsorbent. Those antibodies in the mixture specific for the antigen (shown in red) will bind (noncovalently) and be retained. Antibodies of other specificities (green) and other serum proteins (yellow) will pass through unimpeded.

A reagent (such as a soluble form of the antigen) is then passed into the column to release the antibodies from the immunoabsorbent. These compete with the immunoabsorbent for the antigen-binding sites of the antibodies and release the antibodies to the fluid phase.



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Column Chromatography

Affinity Chromatography with HIS-tagged proteins

Affinity chromatography can be performed using protein tags.

For example, poly-histidine tagged proteins.

The histidine tag is usually about 10 His residues long and should not alter the conformation of the tagged protein

The poly-His tag binds to a nickel chelate affinity column.

The protein is eluted from the column with free imidazole (histidine side chain).

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Column Chromatography

Gel Filtration

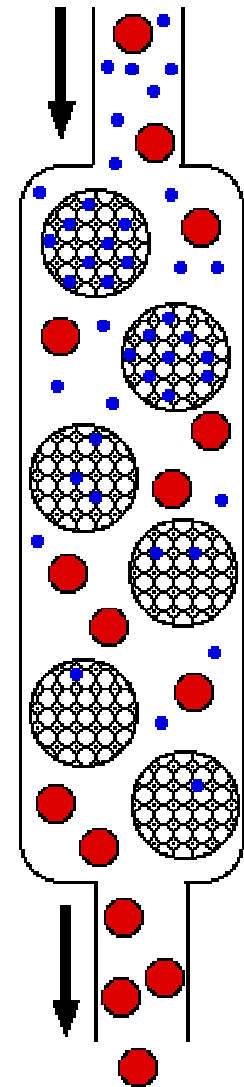
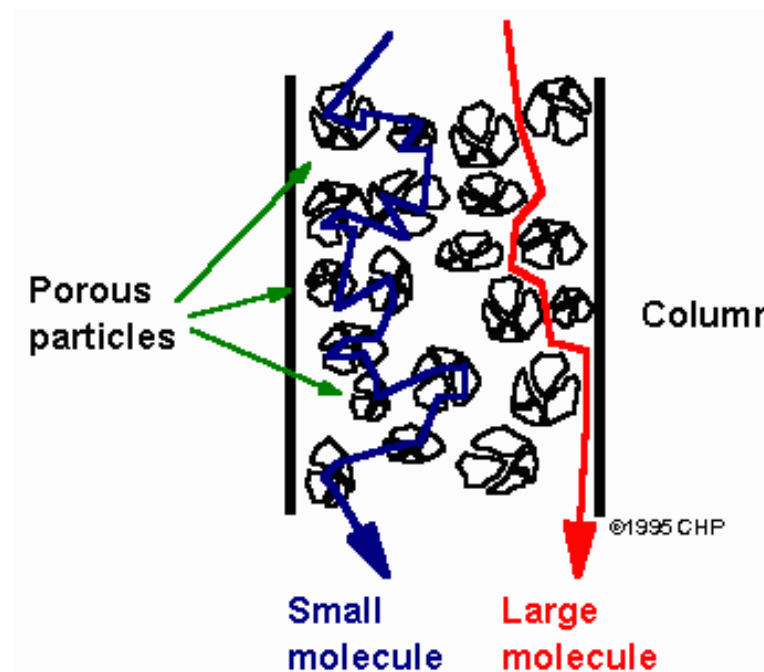
Basis of separation is molecular size.

The column is packed with porous gel beads (e.g., polysaccharide)

Small molecules penetrate the beads, whereas larger molecules cannot.

Large molecules elute first followed by smaller molecules.

Columns calibrated with protein of know molecular weight, can be used to determine molecular weights of unknowns.



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Column Chromatography

High-Performance Liquid Chromatography (HPLC)

Pumps that provide up to 10,000 psi of pressure are used to push solvents through metal columns.

This speeds up the process of separation and prevents spreading of solutes which may occur in gravity column chromatography.

Typical separations require minutes instead of hours and separation resolution is far superior.

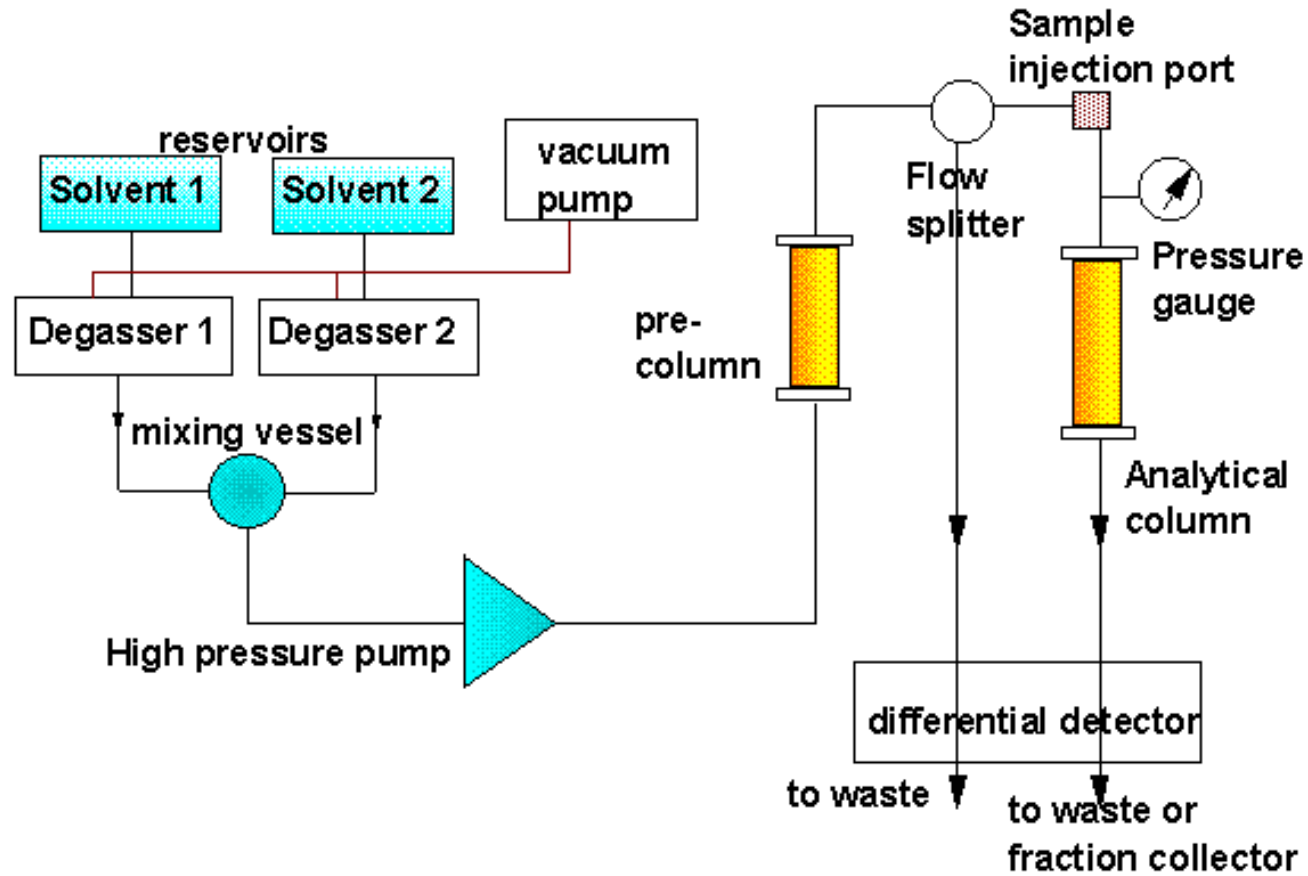
Using reversed phase chromatography two 100 amino acid proteins with a single amino acid difference can be separated.

Reversed phase columns are hydrophobic resins that separate molecules by their hydrophobicity. A water:acetonitrile gradient is used to elute molecules. Hydrophobic molecules bind to the column and elute later at higher concentrations of acetonitrile.

Tools of Biochemistry

Column Chromatography

High-Performance Liquid Chromatography (HPLC)



Tools of Biochemistry

Column Chromatography

High-Performance Liquid Chromatography (HPLC)



Detector

Column

Pump

Computer
(Data Collection)



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Amino Acid Analysis of Proteins

1. **Hydrolysis** of the protein to its constituent amino acids.
2. **Separation** of the amino acids in the mixture.
3. **Quantitation** of the individual amino acids.

The purified protein is dissolved in 6 M HCl and heated at 110° in a sealed ampoule for 24-72 hours.

The peptide bonds are completely hydrolyzed.

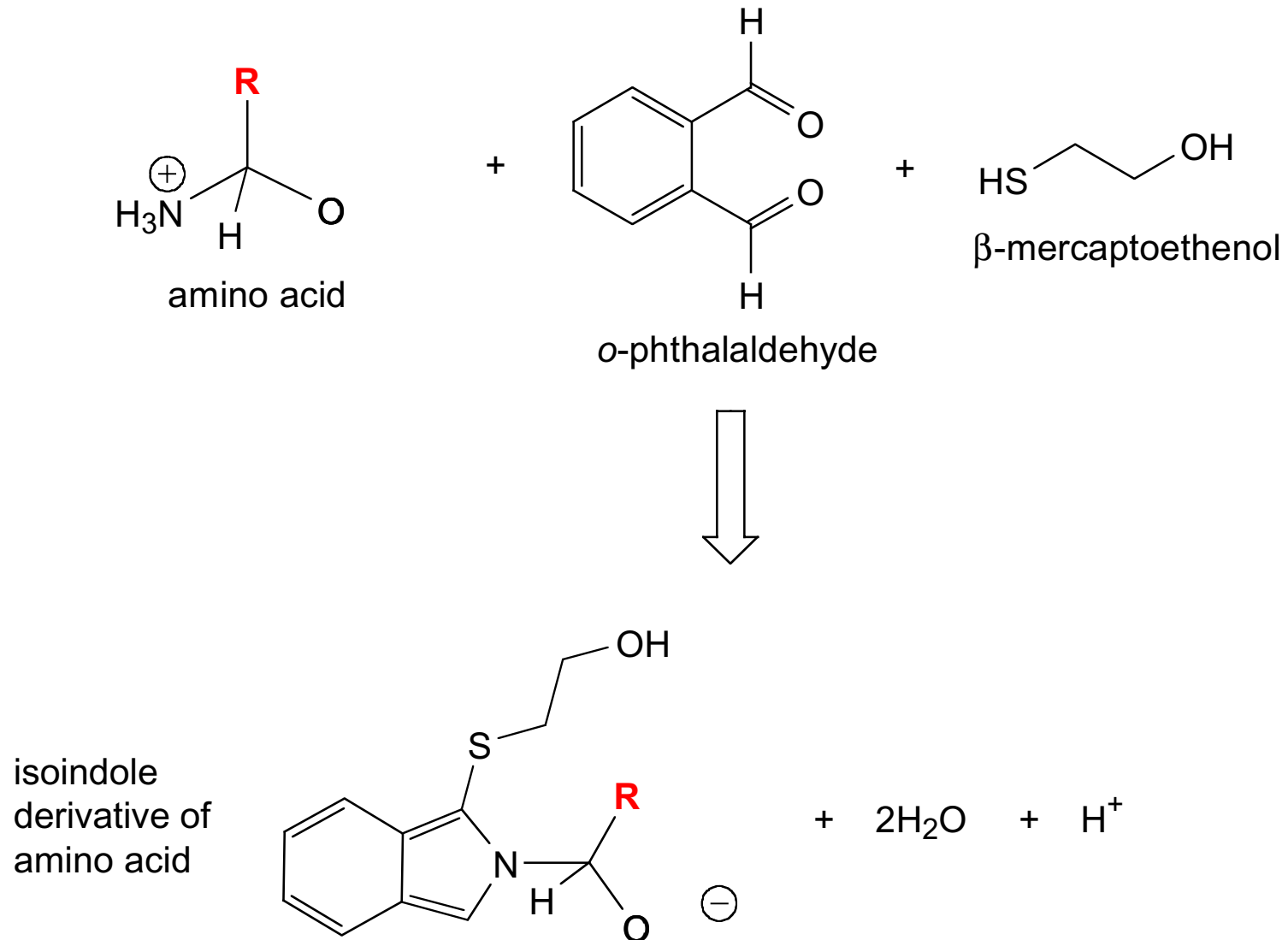
Certain amino acids can be totally destroyed by hydrolysis if not protected. Tryptophan and tyrosine need to be protected from chlorine by use of thiol reagents or phenols as scavengers. Cysteine becomes oxidized on hydrolysis and it is necessary to pre-oxidize to cysteic acid before hydrolysis. A particular problem is that of deamination of amides - these are deamidated to the corresponding acidic residues. In this case determination of the amount of liberated ammonia is needed to quantify the amines.

The amino acids are modified with a fluorescent tag to enhance detectability.

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Amino Acid Analysis of Proteins

The amino acids are modified with a fluorescent tag to enhance detectability:

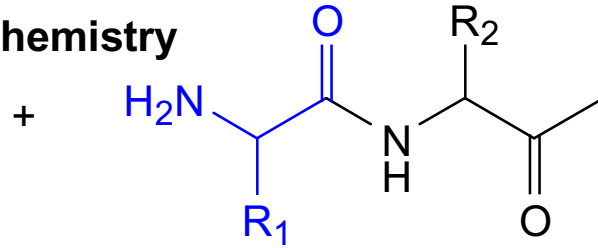
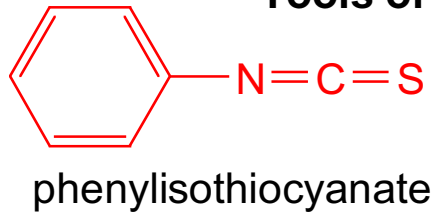


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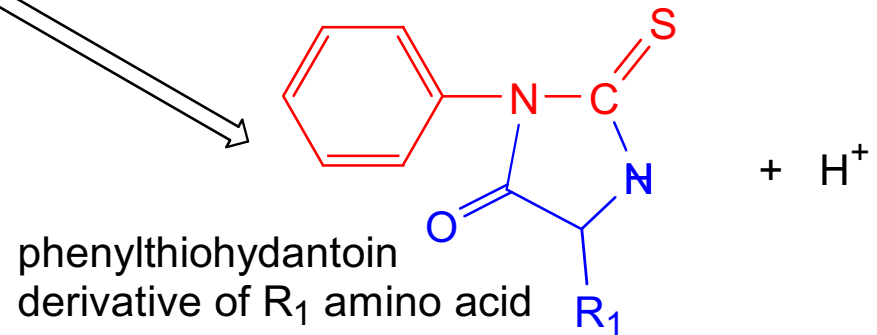
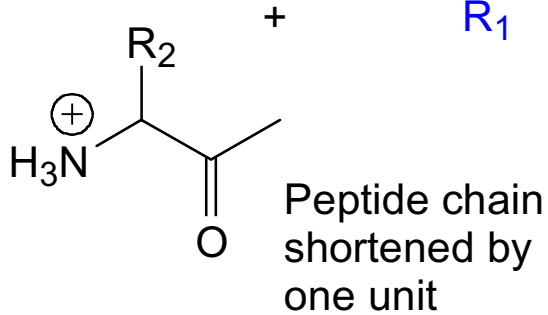
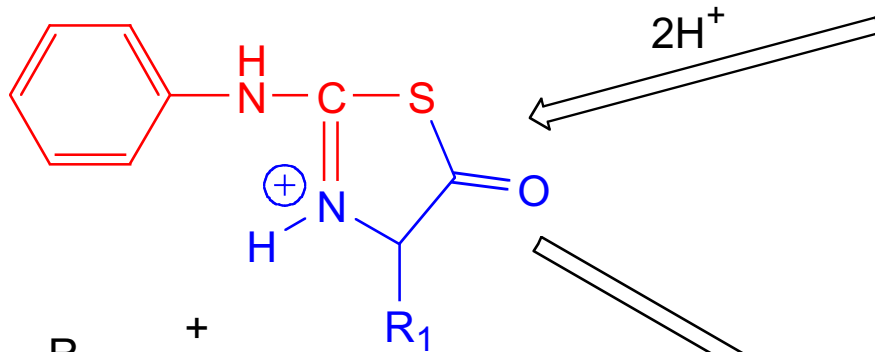
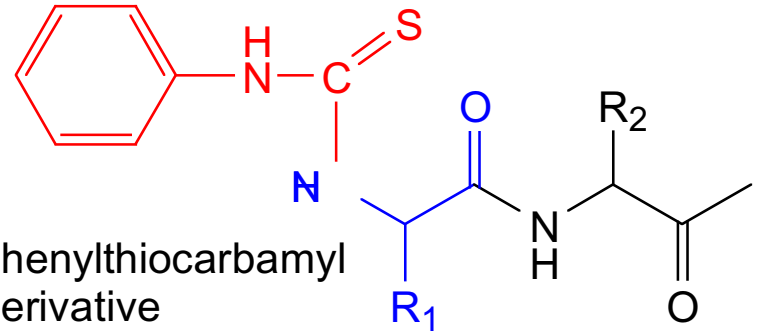
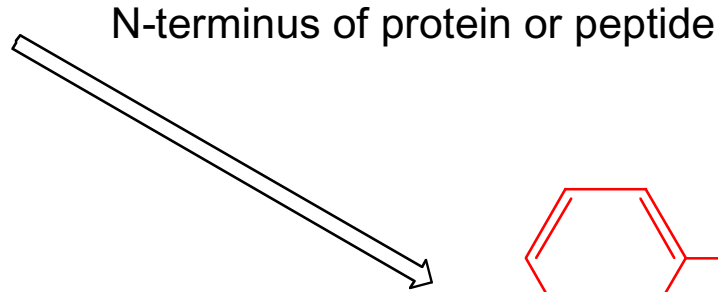
Amino Acid Analysis of Proteins



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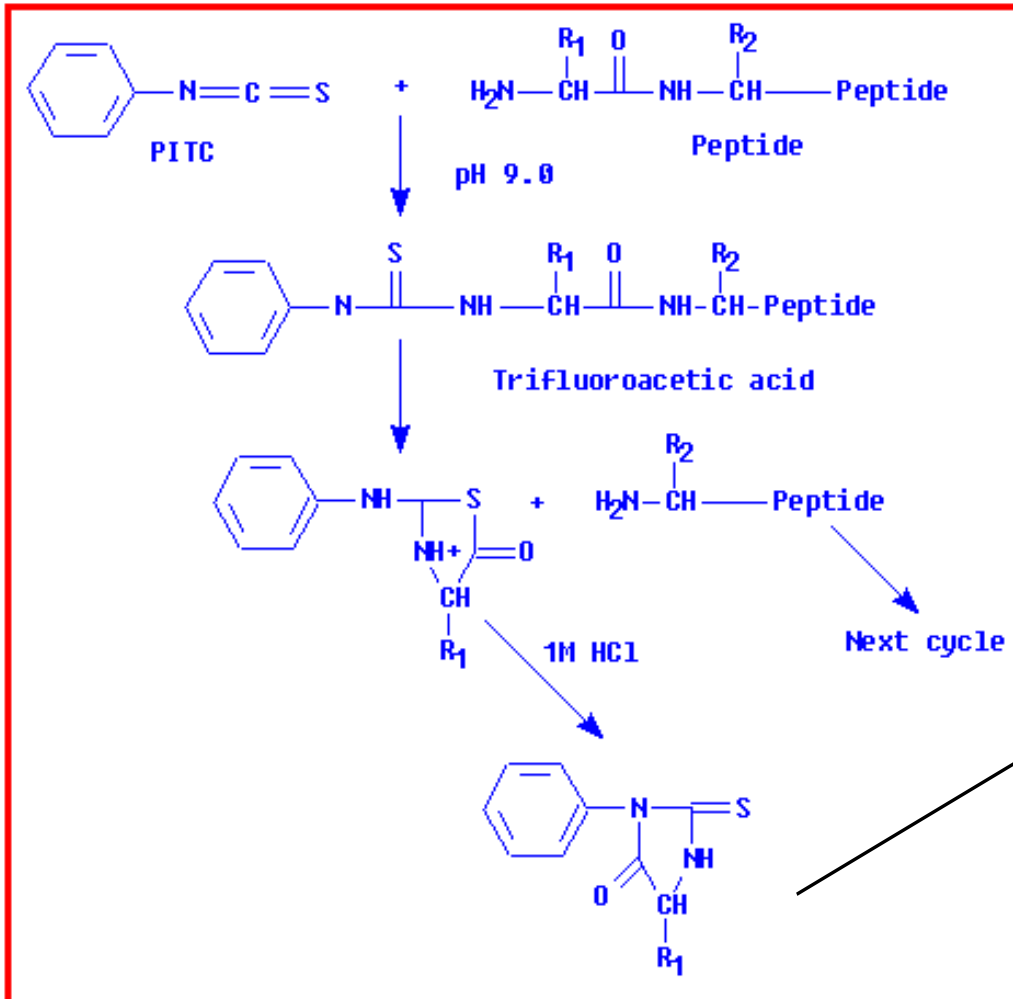


Protein Sequencing by Edman Degradation

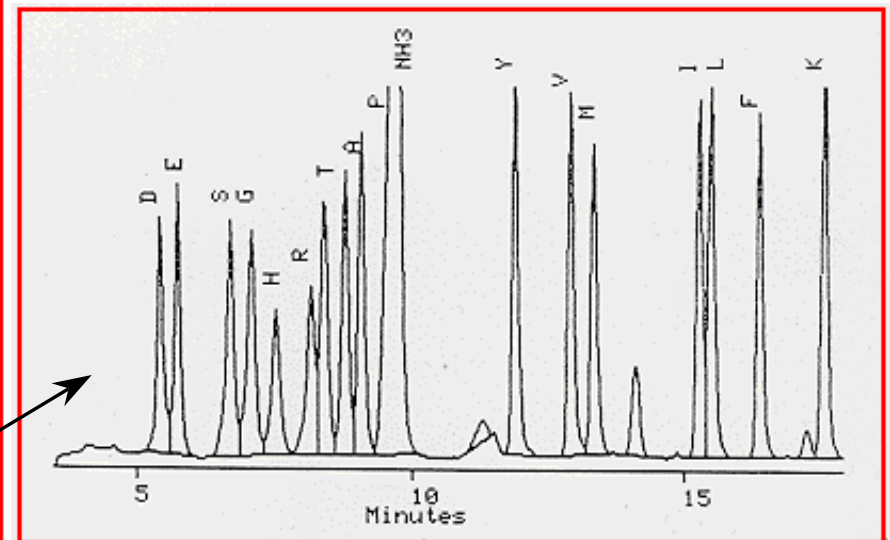


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Protein Sequencing by Edman Degradation



Chromatogram of phenylthiohydantoin derivatives of amino acid standards

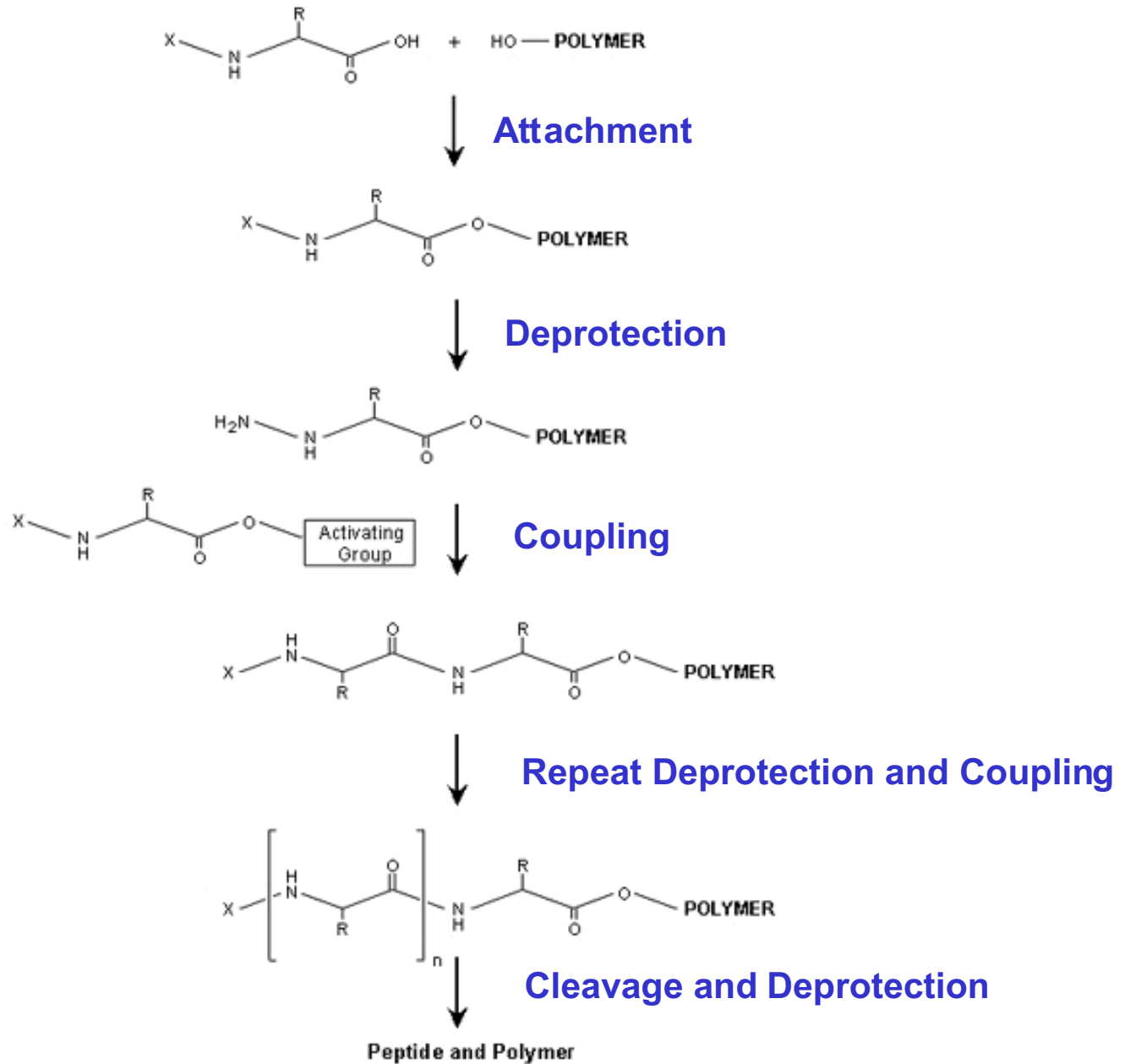


Error accumulation and side reactions limit the number of cycles.

The reaction has been automated allowing sequencing of up to 70 residues with only 5-10 picomoles of protein.

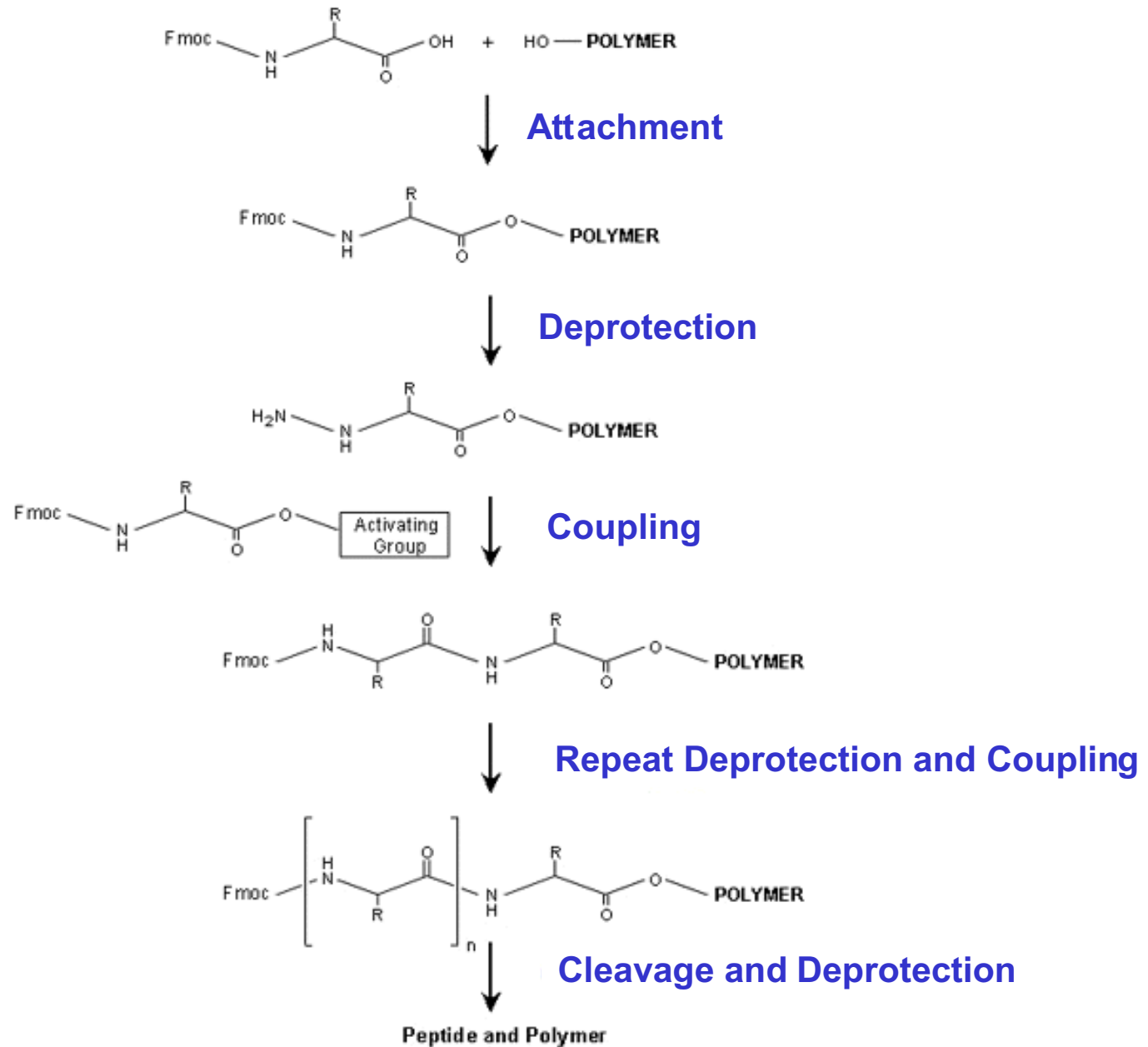
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Solid-Phase Peptide Synthesis



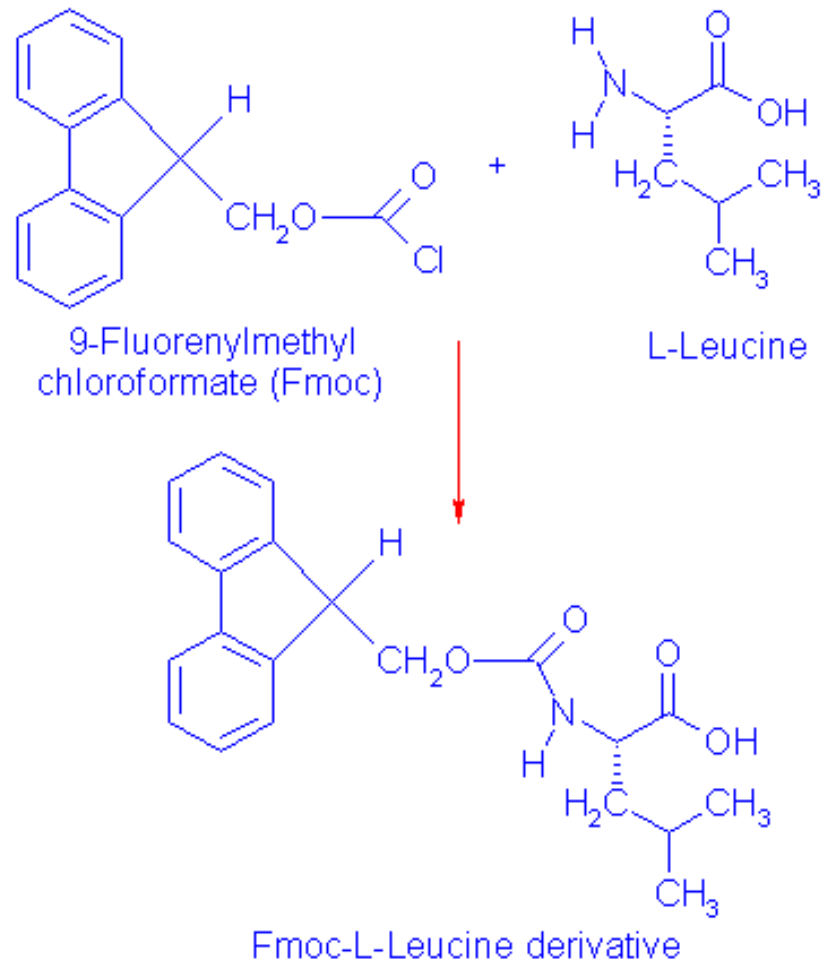
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Solid-Phase Peptide Synthesis using Fmoc protected Amino Acids



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Solid-Phase Peptide Synthesis



Commercial Solid-Phase Peptidizer Instruments

