Enzymology

- Rene Reaumur (1751) and Lazzaro Spallanzani (1780) laid a scientific foundation for the study of enzyme catalysis. Reaumur's experiments with digestion of meat represent the first systematic record of the activity due to an enzyme.
- Theodor Schwann used the word pepsin in 1836 for the proteolytic activity of the gastric mucosa. Schwann also coined the term metabolism

- Gottlieb Kirchhoff discovered plant amylase (later identified as α-amylase) activity while characterizing the hydrolysis of starch to sugar.
- Anselme Payen and Jean Persoz (1833) enriched (first attempts of enzyme purification!) the hydrolytic activity from malt gluten and termed it as diastase.
- The name diastase (Greek; diastasis – to make a breach) has significantly influenced the development of the field of enzymology
- Swedish chemist Berzelius (1779–1848) proposed the name catalysis (from the Greek kata, wholly, and lyein, to loosen) in 1836
- The suffix "ase" – arising out of diastase – was subsequently recommended for all enzyme names (by Duclaux in 1898).
- Berthelot's extraction of "ferment" (1860) from yeast cells marks the beginning of action of enzymes outside of a living cell.
- Willy Kuhne in 1867 extended this further to pancreatic digestion of proteins and called this activity trypsin in 1877.
- The word enzyme (in yeast) was first used by Kuhne in 1877.
- Trypsin was the first candidate "ferment" to be called an enzyme
- James Sumner, proving that urease is a protein, therefore assumes great significance (Sumner 1933).
- This view was further confirmed by purification and crystallization of three more enzymes – pepsin, trypsin, and chymotrypsin – by Northrop and Kunitz (between 1930 and 1935).
- All this was accomplished by just two simple purification techniques – fractional precipitation of proteins by ammonium sulfate and pH changes.

Table 2.1 Landmarks in enzyme studies (enzymology classics)

Author(s)	Year (Discovery/ Publication)	Contribution
R. Reaumur	1751	Gastric digestion in birds
L. Spallanzani	1780	Digestion of meat by gastric juice
A. Payen & J. Persoz	1833	Amylase (diastase) activity
J. Berzelius	1836	Catalysis as a concept
W. Kuhne	1867	"Enzyme" term defined
J. Takamine	1894	Patent on fungal diastase
E. Fischer	1894	Lock and key concept
G. Bertrand	1897	Co-ferment (coenzyme) conceived
P.E. Duclaux	1898	Enzyme names to end with suffix "ase"
V. Henri	1903	Hyperbolic rate equation
S.P.L. Sorensen	1909	pH scale and buffers
L. Michaelis & M. Menten	1913	Equilibrium treatment for *ES* complex
R.M. Willstatter	1922	Trager theory of enzyme action
G.E. Briggs & J.B.S. Haldane	1925	Steady-state treatment for *ES* complex
J.B. Sumner	1926	Urease – Purification and crystallization
H. Lineweaver & D. Burk	1934	Double reciprocal plot (1/v versus 1/[*S*])
K. Stern	1935	First *ES* complex observed
M. Doudoroff	1947	Radioisotope use in enzyme mechanisms
A.G. Ogston	1948	Asymmetric interaction with substrate
D.E. Koshland Jr.	1958	Induced fit hypothesis
C.H.W. Hirs et al.	1960	First enzyme sequenced – RNase A
Enzyme commission	1961	Enzyme classification and nomenclature
D.C. Phillips et al.	1962	First enzyme structure – lysozyme
W.W. Cleland	1963	Systematization of enzyme kinetic study
J. Monod et al.	1965	Model for allosteric transitions
R.B. Merrifield	1969	Chemical synthesis of RNase A
S. Altman & T.R. Cech	1981	Catalysis by RNA molecules

Enzyme Extraction and Purification techniques

- For the enzymes from cell sources, they need to be fractionated into components before purification.
- The first step usually involves homogenization of cells, which disrupt the cell wall to release the enzyme into the homogenate, along with other components.
- Depending on the cell type, the homogenization could be easy as in the case of mammalian tissue without rigid cell wall,
- Harsher conditions such as abrasion, freezing, and high pressure are allowed to disrupt the rigid cell wall of the plant tissue/ fungalor bacterial cell.
- Sometimes, additional hydrolytic enzymes or detergents are added for better extraction.
- The mixture is then fractionated by centrifugation.
- The supernatant could be again centrifuged at a greater force by differential centrifugation, yields several fractions of decreasing density, each still containing hundreds of different proteins, which are subsequently assayed for the activity being purified.
- The choice of temperature, pH, buffering salt, buffer strength, ionic strength, osmolarity, additives (EDTA, SDS, non-ionic detergents etc.), and homogenization technique are important of the success of purification

Homogenizer

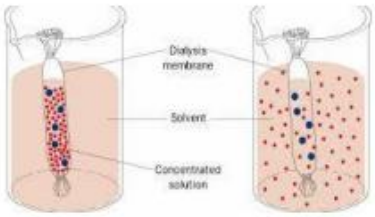
- Purification and separation of enzymes are generally based on solubility, size, polarity, and binding affinity.
- The properties of the enzymes should all be considered when choosing the proper separation method.

1. **Solubility based separation** The principle of the type of separation is that enzyme solubility changes drastically when the pH, ionic strength, or dielectric constant changes.
   - **Salting Out** most proteins are less soluble at high salt concentrations, an effect called salting out
   - The salt concentration at which a protein precipitates differs from one protein to another.

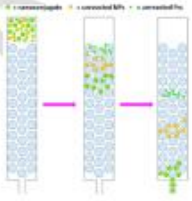
   Salting out is also useful for concentrating dilute solutions of proteins, including active fractions obtained from other purification steps.

2. **Size or mass based method** enzymes are relatively large molecules, separation based on the size or mass of molecules favours purification of enzymes, especially the ones with high molecular weight.

- **Dialysis** is a method, where semipermeable membranes are used to remove salts, small organic molecules, and peptides
- The process usually needs a large volume of dialysate, the buffer outside the dialysis dag, and a time period which varies from hours to days to reach the equilibrium.
- **Ultrafiltration membranes**, which are made from cellulose acetate or other porous materials, can be used to purify and concentrate an enzyme larger than certain molecular weight.
- The ultrafiltration process is usually carried out in a cartridge loaded with the enzyme to be purified. Centrifugal force or vacuum is applied to accelerate the process.



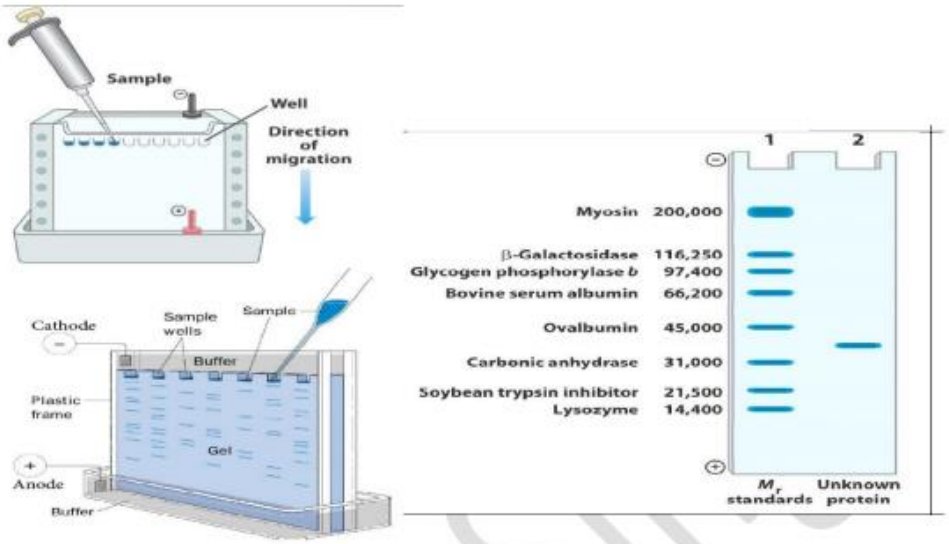
- **Size exclusion chromatography**, also known as gel-filtration chromatography, relies on polymer beads with defined pore sizes that let particles smaller than a certain size into the bead, thus retarding their movement from a column.
- The smaller the molecule, the slower it comes out of the column.
- Size exclusion resins are relatively "stiff" and can be used in high pressure columns at higher flow rates, which shortens the separation time.



3. **Polarity based separation**

- Enzymes can be separated on the basis of polarity, more specifically, their net charge, charge density, and hydrophobic interactions.
- **Ion-exchange chromatography,** a column of beads containing negatively or positively charged functional groups are used to separate enzymes. The cationic enzymes can be separated on anionic columns, and anionic enzymes on cationic column.
- **Electrophoresis** is a procedure that uses an electrical field to cause permeation of ions through a solid or semi-solid matrix or surface resulting in separations on constituents on the basis of charge density.
- **SDS-PAGE** The distance a protein migrates in SDS-PAGE is inversely proportional to the log of its molecular radius, which is roughly proportional to molecular weight. **Isoelectric focusing separation** A protein moves under the influence of an electrical field and stops upon reaching the pH which is the pI for the protein (net charge = 0).

- **HPLC** could be based on polarity, affinity, or both.



4. **Affinity or ligand-based purification** Affinity chromatography is another powerful and generally applicable means of purifying enzymes. This technique takes advantage of the high affinity of many enzymes for specific chemical groups. In general, affinity chromatography can be effectively used to isolate a protein that recognizes a certain group by (1) covalently attaching this group or a derivative of it to a column, (2) adding a mixture of proteins to this column, which is then washed with buffer to remove unbound proteins, and (3) eluting the desired protein by adding a high concentration of a soluble form of the affinity group or altering the conditions to decrease binding affinity.

- Affinity chromatography is most effective when the interaction of the enzyme and the molecule that is used as the bait is highly specific
- After purification, the enzymes need to be concentrated, and sometimes lyophilized to give the pure, stable form
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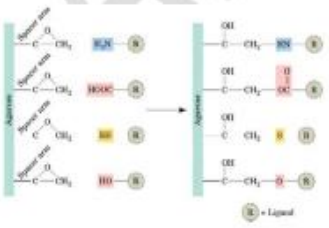


Protein Homogeneity Analysis

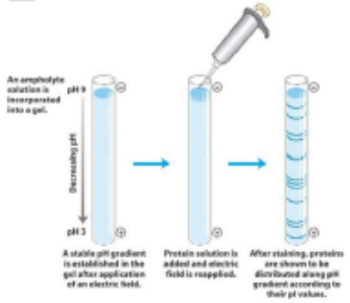
As final products in the biotechnological and pharmaceutical industry, the antibodies, protein-based therapeutics, and other recombinant proteins must have their purity and heterogeneity determined.

That's because contaminants and modified forms of the product during the production process will drastically alter the biological activity of these proteins. The contaminants and modified forms of the product should be removed to ensure the high purity and then the safety and efficiency of the protein drugs. To successfully evaluate each step of the production system, it is important to develop sensitive and accurate analytical methods for protein purity and homogeneity analysis.

The impurities can be categorized into two kinds:

1. **Modified forms or the product and contaminants coming from the host**, the culture medium, and the separation processes such as affinity chromatography. In the cell, the adverse culture conditions, the wrong proteolytic cleaving, incorrect folding, or incorrectly paired disulfide bonds all can introduce impurities.
2. **In the purification process,** contact with specific reagents may result in conformation change and the limited proteolytic degradation of the product may lead to impurities.
3. **During the production or storage**, chemical modifications such as deamination of asparagine and glutamine residues and oxidation of methionine to methionine sulfoxide can also occur.

- The purity and homogeneity of proteins are usually determined by SDS-PAGE, which separates proteins depending on their different sizes.
- Isoelectric focusing, IEF, is a more sensitive method for protein purity and homogeneity detection, based on charge differences.
- HPLC is a simple and fast analytical procedure for purity and homogeneity without any particular pre-treatment of the samples.
- Different determination methods are used in different stages for different purposes.



Isoelectric focusing