Isolation and purification of enzymes

Introduction

To conduct a structural study, the protein must be produced in its native form, with a high degree of purity and a good yield in order to obtain the necessary quantities. Purification methods depend primarily on the raw material (animal, plant, microbial, etc.) and the location of the protein (intra- or extracellular, soluble or membrane-bound).

To avoid losing protein activity and denaturing it, work must be carried out at low temperatures (4 to 10°C), using buffered media with the addition of more or less specific protective agents (antiproteases, sucrose, etc.) and as quickly as possible, with the utmost care.

1. Sources of enzymes :

Enzymes generally originate from animal or plant tissue or microbial cells (bacteria, yeast, fungi, etc.). Examples include amylase, which is extracted from cereals and also from animal saliva, and invertase, which is extracted from yeast.

2. Enzyme localization :

There is :

Extracellular enzymes (exoenzymes) : synthesized inside the cell, then secreted into the extracellular space.

Intracellular enzymes (endoenzymes) : synthesized and used entirely within the cell, where they are generally bound to subcellular particles or intracellular membranes, making their extraction more difficult.

3. The steps of purification :

The process of purifying enzymes or proteins generally involves three main steps : Extraction, fractioning, and final purification.

The table below summarizes the purification protocol :

Origins of biological material	Step	Molecules	Extract
Animal	Extraction	Mixture of soluble molecules	Total
Plant	fractioning	Mixture of molecule families	Raw
Microbial	Purification	Identical molecules	Pure

3.1. Extraction methods

They involve releasing proteins from cells or cellular components, which may require breaking down cell walls and membranes using various processes.

Definition

Extraction involves transferring a compound from one phase to another :

- From a liquid phase to another liquid phase.
- From a solid phase to a liquid phase.

The purpose of extraction is therefore to release enzymes from the cells or subcellular structures in which they are found. This requires destroying the cell wall, cell membrane, and subcellular structures, as appropriate, using effective physical or chemical means that do not denature them.

Choice of extraction technique :

The choice of technique depends on :

- ✓ The type of cells used ;
- ✓ The location of the enzyme ;
- ✓ The purification conditions (temperature, pH, salts, solvents, etc.) ;
- ✓ The equipment available.

Mechanical processes

- ✓ These methods are based on grinding the raw material, usually in an aqueous, buffered, saline medium to ensure protein solubility.
- ✓ The grinder used may be mechanical, electric, or even manual, such as Potter's apparatus.
- ✓ The homogenate obtained is more or less coarse, depending on the nature of the raw material. It contains molecules of various types, but which are soluble under these conditions.

Physical processes

They are mainly applied to isolated cells, whether microbial or from a culture, and to previously separated cell components. The most commonly used processes are :

Freezing/thawing cycles

Create microcrystals and break down cell structures during sudden phase changes (e.g., back and forth from -80°C to +37°C or from liquid nitrogen to room temperature).

Sonication

Destabilizes membrane structures using ultrasound (sound waves).

High-pressure homogenization

Used mainly for bacterial suspensions, creates excess pressure by passing the frozen suspension through a restricted orifice.

Osmotic shock

Consists of incubating fragile cells in a hypoosmotic solution, which allows water to enter the cell, causing it to swell until the lipid membranes rupture and release their contents into the medium. The bursting of organelles is the disadvantage of this technique.

Cold precipitation (cryoprecipitation)

The solubility of most proteins increases with temperature up to a certain limit (40 and 50°C). Beyond this limit, proteins irreversibly precipitate in the form of a coagulum. They therefore lose their solubility at low temperatures (+4°C) in different ways depending on the protein.

Chemical processes

Regroup methods :

Membrane permeabilization :

Either by alkaline lysis if the protein to be purified is stable under these conditions, or by enzymatic lysis (using lysozyme, trypsin, or phospholipase C).

Extraction using organic solvents :

It is limited to the use of polar solvents, such as alcohols and acetone, as they must not denature the protein to be purified.

Extraction with detergents :

Under certain pH and ionic strength conditions, detergents combine with membrane lipids and proteins to form micelles, ensuring the extraction of anchored proteins while maintaining their stability.

3.2. Fractioning methods

These methods are based on the physicochemical or biological differences between the molecules to be separated. They use the characteristic properties of proteins such as size, density, charge, solubility, hydrophobicity, and certain added markers.

- ✓ *Selective precipitation :*
  - ***Isoelectric precipitation.*** Proteins precipitate at the isoelectric point (pI or pHi) because, at this pH value, they have no net electrical charge (zwitterionic form)**.**

- ***Affinity precipitation.*** is based on the formation of intermolecular networks created by binding multifunctional ligands to purified proteins. When the network is large enough, it precipitates (IgG antibody, substrate, or inhibitor).

✓ *Dialysis :*

It allows substances to be separated using their ability to pass through the pores of a semi-permeable membrane called a dialysis membrane.

✓ *Electrodialysis :*

It allows the elimination of mineral salts by establishing a direct electric current of a few milliamps.

✓ *Filtration :*

It consists of separating solid particles suspended in a liquid using filters. The filter medium is made of paper or a granular material that forms narrow channels through which the liquid flows (sintered glass).

✓ *Ultrafiltration :*

It allows substances to be separated according to their molecular size, i.e., their molecular weight, using selective permeability membranes. The force enabling ultrafiltration is pressure exerted on the liquid to be filtered by nitrogen or compressed air.

✓ *Centrifugation*

It allows the components of a mixture to be separated based on their difference in density in a solvent and using centrifugal force. It can be differential or gradient.

✓ *Preparative ultracentrifugation :*

It allows high accelerations (≥ 100,000 g) to be achieved. Its purpose is to obtain purified preparations of particles present in a liquid medium. It can be differential or gradient.

✓ *Chromatography :*

- It allows the elements of a more or less complex solution mixture to be separated.
- The basic principle is the distribution of molecules in a mixture (analyte) between two immiscible phases according to a partition coefficient Kd.
- One of these phases, called the stationary phase, can be solid or liquid. The second, the mobile phase, can be liquid or gaseous.
- The molecules are retained to a greater or lesser extent depending on the extent of their interaction with the stationary phase. They are then carried along by the mobile

phase at varying speeds depending on their physicochemical properties, which allows them to be separated.

- Chromatography can be analytical (qualitative or quantitative) or preparative.
- Chromatographic techniques are classified according to the separation mechanism involved and the properties of the stationary phase.

- ***Molecular exclusion chromatography :***

Also known as gel filtration chromatography or molecular sieving, this technique is used to separate molecules according to their size using organic polymer compounds. These compounds are capable of hydrating and, due to their unique three-dimensional structure, forming a porous matrix called a “gel” which constitutes the stationary phase.

The largest particles are completely excluded, while small molecules are distributed between the mobile phase and the stationary phase, as they can diffuse into the pores of the gel.

- ***Ion exchange chromatography (anionic or cationic)***

This technique allows charged molecules to be separated using ion exchangers. The net charge therefore depends on the pKa of the substance and the pH of the solution. The analytes are bound according to their affinity.

- ***Hydroxyapatite adsorption chromatography***

In this technique, the stationary phase consists of a mineral such as calcium phosphate **[$Ca_{10}(PO_4)_6(OH)_2$]**. Protein retention and desorption depends on the isoelectric point, which is between 3.5 and 11. Acidic and basic proteins are eluted using salts.

- ***Hydrophobic interaction chromatography***

Hydrophobicity is defined as the repulsion that occurs between a nonpolar structure and a polar environment such as water.

In HIC chromatography, the stationary phase contains a hydrophilic matrix (gel) carrying nonpolar carbon chains or aromatic groups such as the phenyl group. The mobile phase consists of an aqueous solution loaded with salts.

- ***Affinity chromatography***

This chromatography technique exploits the properties of biological interactions to separate and purify substances. It was originally developed for the purification of enzymes. It requires that the material to be isolated be capable of reversibly binding to a specific ligand, which is attached to an insoluble matrix (cross-linked dextrans, agarose, polyacrylamide gels, etc.).

The elution of the purified molecule is carried out either specifically (with a competitor) or non-specifically (by changing the pH).

- ***High-pressure liquid chromatography (HPLC)***

In this technique, separation occurs in a column where the stationary phase is highly divided and can withstand pressures of up to 10 MPa without damage. The diversity of the stationary phase allows HPLC to bring into play all possible interactions (partition, ion exchange, molecular exclusion, affinity, and hydrophobic interactions).

3.3. Final purification methods

The final chromatography step may achieve the expected purity, but there is usually one last contaminant that needs to be removed.

4. Biochemical purity control techniques

There are methods that allow the purity of an extract to be checked after the final step :

- ✓ Polyacrylamide gel electrophoresis in denaturing medium (sodium dodecyl sulfate) SDS-PAGE :

  This is the most commonly used method for separating and determining the molecular weight of proteins. SDS, also known as lauryl sulfate, is an anionic detergent that binds strongly to proteins and denatures them.

- ✓ Isoelectrofocusing :

  This technique allows proteins and other amphoteric biological molecules to be separated in a pH gradient where the anodic region has a lower pH than the cathodic region. Thus, the analytes stop migrating at the isoelectric pH, which gives the zwitterion state.

- ✓ Nuclear magnetic resonance spectrum (NMR spectrum) :

  This technique is based on nuclear magnetism. It involves measuring the absorption of radio frequency radiation by an atomic nucleus in a strong magnetic field.

  It is one of the most powerful methods for determining the structure of both organic and inorganic molecules. Absorption depends on certain nuclei present in the molecule being studied.

5. Criteria for the homogeneity of a purified enzyme protein

The activity of an enzyme in a particular fraction is expressed by its specific activity, which is determined at each step of purification :

$$Zs = Z / [Pt]$$

Good fractionation allows a fraction with high specific activity to be obtained.

The main criteria for protein homogeneity are :

The degree of purification (enrichment E)

Also known as the purification factor, it corresponds to the ratio of specific activity after purification (Zs_2) to specific activity before purification (Zs_1) :

Purity degree (E) = Zs_2 / Zs_1

Purification efficiency (yield)

Also known as the recuperation percentage, it corresponds to the ratio of enzyme activity after purification (Z_2) to enzyme activity before purification (Z_1) :

Efficiency (%) = Z_2 / Z_1 . 100

Example of a purification table :

Enzyme	Total volume (ml)	Total activity (U)	Total Protein (mg)	Specific activity (U/mg)	Overall Yield %	Purity (fold)
Cell free supernatant	**100**	**1725**	**530**	**3.25**	**100**	**1**
Isopropanol precipitate	**1**	**1150**	**9.0**	**127.77**	**67**	**39.31**
CM-Cellulose column	**25**	**684**	**4.4**	**155.45**	**40**	**47.83**
DEAE-Cellulose column	**25**	**596**	**2.4**	**248.33**	**35**	**76.41**
Sephadex G-100 column	**20**	**248**	**0.78**	**317.9**	**15**	**97.8**