2.2 Why isolate enzymes?

- It is important to study enzymes in a simple system (only with small ions, buffer molecules, cofactors, etc.) for understanding its structure, kinetics, mechanisms, regulations, and role in a complex system.
- Also isolating pure enzyme is important to use it for medical and industrial purposes.
2.3.1 Objectives of enzyme purification

- Objectives: maximum possible yield + maximum catalytic activity + maximum possible purity
- Assay procedure (Chapter 4)
- History
  - Crystallization
  - Homogenization + large scale separation
  - Attach the affinity tag to enzyme using DNA recombinant technology (ex. (His)$_6$-tag)

2.3.2 Strategy
2.4 Choice of Source

- Classical approach involves choosing a source containing large quantity of enzymes
  - Acetyl CoA carboxylase (mammary gland)
  - Alkaline phosphatase (kidney)
- Modern approach with DNA recombinant technology
  - 3-phosphoshikimate-1-carboxyvinyl transferase in *E. coli* (1984)
  - 100-fold increase in productivity
- Prokaryotes as host organisms (*E. coli* and *Bacillus*)
  - Rapid growth and simple medium components
  - Disadvantages: lack of post-translational modification (glycosylation) and forming inclusion bodies

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2.4 Choice of Source

- Yeasts as enzyme source
  - *Saccharomyces cerevisiae* rarely forms inclusion bodies, but grow slowly and make hyperglycosylation
  - *Kluyveromyces lactis* and *Pichia pastoris* are also being developed
- Insect cell with baculovirus vector
  - It can employ many of the protein modification, processing, and transport system in higher eukaryotic cells
- ‘Fusion Protein’
  - Glutathione–S–transferase, maltose binding protein, or His–tag are popularly used
  - They greatly enhance the power of purification and sometimes solubility of protein
2.4 Choice of Source

- Production occurred in the strain known to make the enzyme of interest
- alkaline protease or $\alpha$-amylase $\rightarrow$ Bacillus licheniformis,
- glucoamylase $\rightarrow$ Aspergillus,
- acid cellulase $\rightarrow$ Trichoderma,
- glucose/xylose isomerase $\rightarrow$ Streptomyces

2.5 Methods of homogenization

- Mechanical methods
  - High pressure homogenizer* (55 MPa) : cooling is important
  - Wet grinding by mills or glass balls
- Non-mechanical methods
  - Drying
  - Lysis by osmotic shock, detergents, or enzymes
  - Ultrasound*
- Cooling and protease inhibition are important to recover the enzyme
2.5 Methods of homogenization

- **Animal cells (organs)**
  - It is easy to homogenize due to the lack of cell wall.
  - Fat and connective tissue must be removed before homogenization.

- **Bacteria and Fungi**
  - Cell wall must be digested by enzymes (Protoplasts can be made by treating lysozyme or chitinase/3-glucanase).

- **Plant**
  - Disruption of vacuole can damage enzymes.

- **Membrane proteins**
  - Usually detergent (anionic, cationic, or neutral) is added.
  - Detergent must be chosen by considering the choice of purification method, especially column chromatography.

2.6 Methods of separation

1. **Size and mass**

- **Ultracentrifugation** (300,000g)
  - $M_r$ is the major factor for separation.
  - Not very efficient to separate a enzyme from enzyme pool: Usually used to remove impurities.

- **Gel filtration** ($M_r$ ~ hundreds of thousands)
  - Usually in later stage of purification.

- **Dialysis** ($M_r$ ~ tens of thousands)
  - Usually used for removing salts, organic solvents, etc..

- **Ultrafiltration**
  - Small molecules are filtered out by pressure.
  - Used for concentrating proteins.
  - Alternatively, centrifugation with dialysis membrane.
2.6 Methods of separation

2.6.1 Polarity

- Ion-exchange chromatography
  - Electrostatic property
  - Flow through in low salt and at appropriate pH
  - Desorption by changing salt conc’ and pH
  - Enzymes can be separated by gradient condition
  - Large scale is possible
  - Usually 10-fold increase in purity
2.6 Methods of separation

2. Polarity

- **Electrophoresis**
  - Separation by movement of charged molecules
  - Capillary electrophoresis (cross section less than 100 μm)

- **Isoelectric focusing**

![Diagram of pH gradient]

2.6 Methods of separation

2. Polarity

- **Hydrophobic interaction chromatography**
  - Depending on the nonpolar amino acid on the surface of enzyme
  - Octyl- or phenyl-Sepharose with high ionic strength
  - Desorption by lowering ionic strength or adding organic solvents (or detergents)
2.6 Methods of separation

3. Solubility

- Change in pH
  - Enzymes are least soluble at pI because there is no repulsive force between enzymes
  - Enzyme must not be inactivated in a range of pH
- Change in ionic strength
  - Large charged molecules are only slightly soluble in pure water: Addition of ion promotes solubility (Salting in)
  - Beyond a certain ionic strength, the charged molecules are quickly precipitated (Salting out)
  - Ammonium sulfate is popularly used
  - 10–fold increase in purity
  - Fructose-bisphosphate aldolase from rabbit muscle can be purified in high purity by ammonium sulfate

- Decrease in dielectric constant
  - Addition of water–miscible organic solvent (ethanol or acetone)
  - Decrease dielectric constant
  - Sometimes deactivate the enzyme
  - Work at low temperature
  - PEG (poly ethylene glycol) ~ M, 4000 to 6000 is commonly used
2.6 Methods of separation

4. Specific binding sites

- Affinity chromatography

- Substrate or inhibitor is linked to a matrix
- Desorbed by a pulse of substrate or changed pH, ionic strength

- Staphylococcal nuclease
2.6 Methods of separation

4.1 Affinity chromatography

Problems
- Attaching a suitable substrate or inhibitor to the matrix can be difficult
- Linking b/n substrate and matrix itself may inhibit the binding b/n enzyme and substrate; Spacer arm (diaminehexane) may be needed
- Binding affinity b/n enzyme and substrate must be in a proper range
- Special attention is necessary to separate the enzymes using same substrate or using more than one substrate

Fusing proteins to solve the problems
- Glutathione–S–transferase : glutathione
- Maltose binding protein : maltose
- Hexahisitidine : Ni²⁺ (Elution by imidazole or thrombine cleavage site is added after the tag)

4. Other chromatographies

Affinity elution
- Affinity occur at desorption step
- Can solve some problems of affinity chromatography and easy to scale up

Dye–ligand chromatography
- Cibacron Blue F3G–A can bind to a number of dehydrogenases and kinases
- Procion Red HE–3B binds well with NADP⁺–dependent dehydrogenase

Immunoadsorption chromatography
- Immobilize the antibody to CNBr treated Sepharose
- Achieve much higher purity
2.6 Methods of separation
4. Other chromatographies

- Covalent chromatography
  - Separation of cysteine containing protein using thiol-Sepharose 4B

2.6 Methods of separation
5. Choice of method

- Time/Large scale -> Precipitation by ethanol or ammonium sulfate or purification based on solubility
- Small scale/high purity -> Column chromatography or electrophoresis
- FPLC or HPLC -> Fast and high purity, expensive
2.7 How to know the success of purification

- Test for purity see Table 2.2
2.7 How to know the success of purification

- Tests for catalytic activity
  - By enzyme assay
  - Check cofactors and inhibitors
- Stabilizing factors
  - Neutral pH, storage in 50% glycerol may help
  - 2-mercaptoethanol or DTT (Dithiothreitol) *
  - Protease inhibitor PMSF (Phenylmethylsulfonyl flouride)
- Active site titrations
  - Checking the proportion of active enzyme in the purified enzyme

Inactivation of protein by oxygen
2.8 Examples of purification

- Adenylate kinase from pig muscle
- Adenylate kinase is stable at low pH (Step 2)
- High affinity with AMP (Step 3)
- Purification using size (Step 4)

<table>
<thead>
<tr>
<th>Step</th>
<th>Total volume (mL)</th>
<th>Total protein (mg)</th>
<th>Total activity (Katal)</th>
<th>Specific activity (Katal mg⁻¹)</th>
<th>Yield (%)</th>
<th>Purification factor per step</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 (extraction)</td>
<td>16600</td>
<td>435000</td>
<td>9.0413</td>
<td>0.095</td>
<td>1000</td>
<td>1.0</td>
</tr>
<tr>
<td>2 (pH)</td>
<td>15700</td>
<td>112000</td>
<td>9.0985</td>
<td>0.825</td>
<td>88.1</td>
<td>3.42</td>
</tr>
<tr>
<td>3 (phosphocellulose)</td>
<td>1380</td>
<td>1716</td>
<td>9.0233</td>
<td>5.02</td>
<td>54.0</td>
<td>40.0</td>
</tr>
<tr>
<td>4 (gel filtration)</td>
<td>211</td>
<td>462</td>
<td>0.0202</td>
<td>43.17</td>
<td>48.4</td>
<td>3.32</td>
</tr>
<tr>
<td>5 (crystallization)</td>
<td>—</td>
<td>344</td>
<td>0.0150</td>
<td>46.5</td>
<td>38.7</td>
<td>1.08</td>
</tr>
</tbody>
</table>

Minced Muscle

Step 1 extract with 0.01 mol dm⁻³ KCl, strain through cheese cloth

Extract

Step 2 incubate at pH 3.5; then at pH 7.0; centrifuge

Supernatant

Step 3 load onto phosphocellulose column, elute with pulse of AMP (5 mmol dm⁻³)

Pooled fractions containing activity

Step 4 concentrate by (NH₄)₂SO₄ precipitation; gel filtration (Sephadex G-75)

Pooled fractions containing activity

Crystalline enzyme
2.8 Examples of purification

- Ribulosebisphosphate carboxylase from spinach
  - 95% purity
  - Two subunits confirmed in electrophoresis
  - Assembly of two units is difficult due to the chaperon bound to large subunit

- RNA polymerase from *E. coli*
  - Bacterial cell extract: highly viscous →
    - Deoxyribonuclease
  - Oligonucleotide will be eliminated at step 4
2.8 Examples of purification

- Arom multienzyme from *Neurospora*
  - Fungi contains large amount of proteases (add PMSF)

- Recombinant Adenylyl cyclase from baculovirus
  - Forskolin : Activator of the enzyme