Chap 2. The Purification of Enzymes

- To understand the behaviors of an enzyme in a complex system, it should be understood in a simple system
The Aim of a Purification Procedure

- The maximum possible yield:
  - recovered activity
- The maximum catalytic activity:
  - no degraded or other inactivated enzymes
- The maximum possible purity:
  - no other enzymes or large molecules

- A suitable assay procedure needed
- Unit (u): μmol/min
- Specific activity: u/mg
Strategy in Enzyme Purification

- Source of enzyme
- Methods of homogenization
- Methods of separation
Choice of Source

- A source in which the required enzyme occurs in large amounts
- A suitable expression system for the recombinant enzyme

- Prokaryotes
- Eukaryotes: yeasts, insect cells
Prokaryotic Expression Systems

**Prokaryotes: E. coli**

- **Advantages:**
  - rapid growth
  - relatively simple nutritional requirements,
  - strong promoters available
  - controllable expression by inducers or change in the culture medium

- **Disadvantages:**
  - lack of the correct machinery for post-translational modifications
  - often insoluble inclusion bodies formed
Candida antarctica Lipase B

1TCA
Eukaryotic Expression Systems

*Low eukaryotes: yeasts*

- *Saccharomyces cerevisiae:*
  - good growth rates on simple media
  - well understood at a genetic level
  - efficient secretion available by suitable signal sequences
  - difficult to grow to high cell densities in continuous culture
  - a tendency to hyperglycosylate proteins
- *Kluyveromyces lactis*
- *Pichia pastrois:*
  - high level of expression available using the promoter from the tightly regulated *AOX1* gene
Methods of homogenization

• Mammalian tissue:
  – lack of a rigid cell wall
• easy homogenization
• Plant, fungal, and bacterial material:
  – rigid cell wall harsher methods required: grinding with abrasives, freezing and thawing, long periods of blending, addition of glass beads, a French press
  – using appropriate hydrolytic enzymes: lysozyme, chitinase and 3-glucanases
• Extraction of membrane-bound enzymes:
  – detergents required
Methods of separation

- Size, charge, solubility, and the possession of specific binding sites

Table 2.1 Principal separation methods used in purification of enzymes

<table>
<thead>
<tr>
<th>Property</th>
<th>Method</th>
<th>Scale</th>
</tr>
</thead>
<tbody>
<tr>
<td>Size or mass</td>
<td>Centrifugation</td>
<td>Large or small</td>
</tr>
<tr>
<td></td>
<td>Gel filtration</td>
<td>Generally small</td>
</tr>
<tr>
<td></td>
<td>Dialysis; ultrafiltration</td>
<td>Generally small</td>
</tr>
<tr>
<td>Polarity</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(a) Charge</td>
<td>Ion-exchange chromatography</td>
<td>Large or small</td>
</tr>
<tr>
<td></td>
<td>Chromatofocusing</td>
<td>Generally small</td>
</tr>
<tr>
<td></td>
<td>Electrophoresis</td>
<td>Generally small</td>
</tr>
<tr>
<td></td>
<td>Isoelectric focusing</td>
<td>Generally small</td>
</tr>
<tr>
<td>(b) Hydrophobic character</td>
<td>Hydrophobic chromatography</td>
<td>Generally small</td>
</tr>
<tr>
<td>Solubility</td>
<td>Change in pH</td>
<td>Generally large</td>
</tr>
<tr>
<td></td>
<td>Change in ionic strength</td>
<td>Large or small</td>
</tr>
<tr>
<td></td>
<td>Decrease in dielectric constant</td>
<td>Generally large</td>
</tr>
<tr>
<td>Specific binding sites or</td>
<td>Affinity chromatography</td>
<td>Generally small</td>
</tr>
<tr>
<td>structural features</td>
<td>Immobilized metal ion chromatography</td>
<td>Generally small</td>
</tr>
<tr>
<td></td>
<td>Affinity elution</td>
<td>Large or small</td>
</tr>
<tr>
<td></td>
<td>Dye–ligand chromatography</td>
<td>Large or small</td>
</tr>
<tr>
<td></td>
<td>Immunoabsorption</td>
<td>Generally small</td>
</tr>
<tr>
<td></td>
<td>Covalent chromatography</td>
<td>Generally small</td>
</tr>
</tbody>
</table>

The term ‘large-scale’ is used to indicate that amounts of protein greater than about 100 mg can be readily handled at that particular step in the purification procedure.
Choice of Methods

- Considering factors for choice of methods
  1. The scale of the preparation and the yield of enzyme required
  2. The time available for the preparation
  3. The equipment and expertise available in the lab.

- Earlier stages (or large scale): methods based on changes in solubility
- Latter stages (or small scale): column chromatography or electrophoresis
### Table 2.2  Some commonly employed analytical methods to check the purity of enzyme preparations

<table>
<thead>
<tr>
<th>Method</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ultracentrifugation (Chapter 3, Section 3.2.1)</td>
<td>Not very satisfactory for detecting impurities at the $\leq 5%$ level. Problems can arise from associating–dissociating systems (Chapter 3, Section 3.2.5)</td>
</tr>
<tr>
<td>Electrophoresis (Section 2.6.2.2)</td>
<td>A good method for examining enzymes composed of non-identical subunits (Chapter 3, Section 3.5.1.5)</td>
</tr>
<tr>
<td>Electrophoresis in the presence of sodium dodecylsulphate (Chapter 3, Section 3.2.3)</td>
<td>A good method for detecting impurities that differ in terms of subunit $M_r$; excellent for detecting proteolytic damage. Problems arise from enzymes composed of non-identical subunits, which give rise to multiple bands (Chapter 3, Section 3.5.1.5)</td>
</tr>
<tr>
<td>Capillary electrophoresis (Section 2.6.2.2)</td>
<td>A powerful analytical technique which can be used in a variety of modes, including isoelectric focusing. Equipment required is specialized and relatively expensive</td>
</tr>
<tr>
<td>Isoelectric focusing (Section 2.6.2.3)</td>
<td>A very sensitive method for detecting impurities. Artefacts can arise suggesting apparent heterogeneity$^{85-87}$</td>
</tr>
<tr>
<td>N-terminal analysis (Chapter 3, Section 3.3.2.4)</td>
<td>Should indicate the presence of a single polypeptide chain. Some enzymes have a blocked N-terminus (Chapter 3, Section 3.3.2.7); others consist of multiple polypeptide chains held together by disulphide bonds (e.g. chymotrypsin)</td>
</tr>
<tr>
<td>Mass spectrometry (Chapter 3, Section 3.2.4)</td>
<td>A very powerful but specialized technique. Subunit $M_r$ values can be obtained very precisely, confirming the authenticity of the primary structure (Chapter 3, Section 3.2.10). Post- translational modifications can be identified</td>
</tr>
</tbody>
</table>
Tests for Catalytic Activity

- Checking optimality of the assay conditions
- Investigating the enzyme stability during storage
Active Site Titrations

- Estimation of the amount of active enzyme

General scheme

\[ \text{E} + \text{S} \xrightarrow{k_1} \text{E}' + \text{P}_1 \]

\( k_2 \) (slow)

If \( k_2 = 0 \), the concentration of \( \text{P}_1 \) produced gives the concentration of active enzyme directly. If \( k_2 \) is less than \( k_1 \), but not equal to zero, it is possible to calculate the concentration of active enzyme (see reference 94)
Mechanism of *Pseudomonas fluorescens* Esterase
Purification Procedures of RNA Polymerase from *E. coli*

**Frozen cells**

**Step 1** blend at high speed (glass beads); add deoxyribonuclease; filter

**Extract**

**Step 2** centrifuge (100,000 g, 2 h) to remove cell debris and ribosomes

**Supernatant**

**Step 3** \((\text{NH}_4\text{)}_2\text{SO}_4\) fractionation (33–50% saturation); re-extract with \((\text{NH}_4\text{)}_2\text{SO}_4\) (42% saturation)

**Precipitate**

**Step 4** redissolve precipitate; load on to DEAE-cellulose column; elute with KCL gradient

**Pooled fractions containing activity**

**Step 5** chromatography on phosphocellulose; elute with 0.35 mol dm\(^{-3}\) KCL

**Pooled fractions containing activity**

**Step 6** gel filtration (Biogel A – 1.5 m)

**Pure enzyme**