Purification of Enzymes

Enzyme Engineering

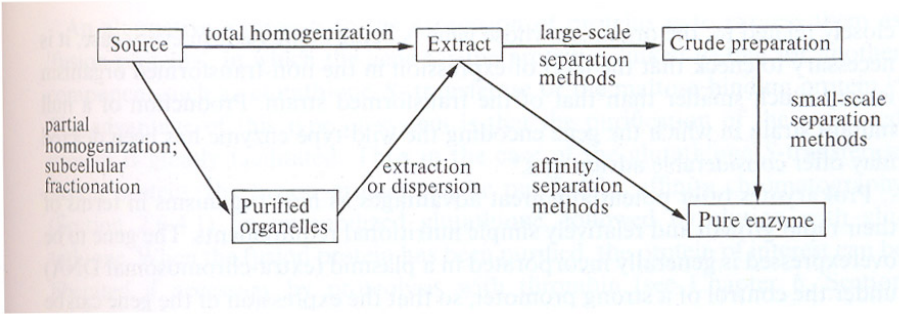
2.2 Why isolate enzymes?

- 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

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 α-amylase -> *Bacillus licheniformis*,
  - glucoamylase -> *Aspergillus*,
  - acid cellulase -> *Trichoderma*,
  - glucose/xylose isomerase -> *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 filteration (M_r ~ hundreds of thousands)
  - Sephadex, Bio-Gel P, Sephacryl, and Sepharose – expensive and time-consuming
  - Usually in later stage of purification
- Dialysis (M_r ~ tens of thousands)
  - Usually used for removing salts, organic solvents, etc..
- Ultrafilteration
  - Small molecules are filtered out by pressure
  - Used for concentrating proteins
  - Alternatively, centrifugation with dialysis membrane

2.6 Methods of separation

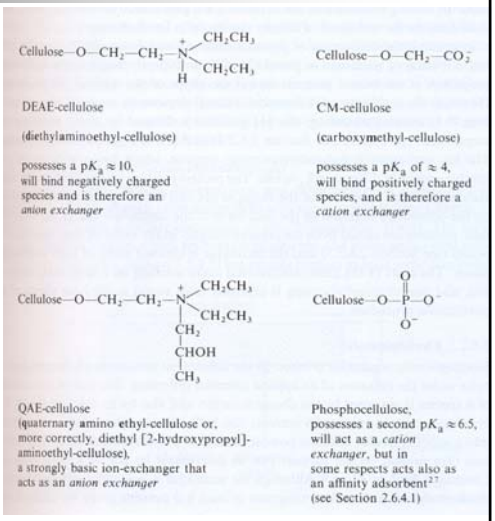
Table 2.1 Principal separation methods used in purification of enzymes

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

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.

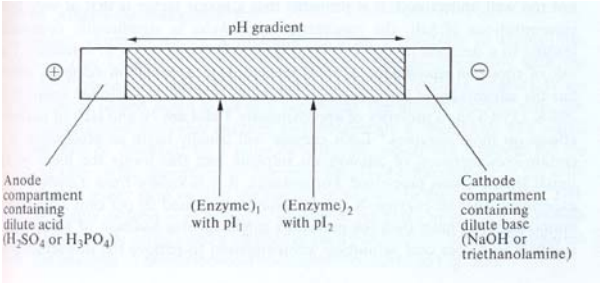
2.6 Methods of separation
2. Polarity

- 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 100μm)
- Isoelectric focusing



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

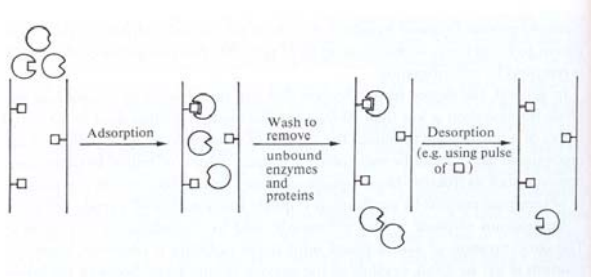
- Change in pH
  - Enzymes are least soluble at pI because there is no repulsive force between enzymes
  - Enzyme must not be inactivated in a range of pH
- Change in ionic strength
  - Large charged molecules are only slightly soluble in pure water; Addition of ion promotes solubility (**Salting in**)
  - 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

2.6 Methods of separation
3. Solubility

- 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_r 4000 to 6000 is commonly used

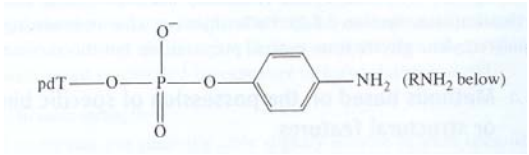
2.6 Methods of separation
4. Specific binding sites

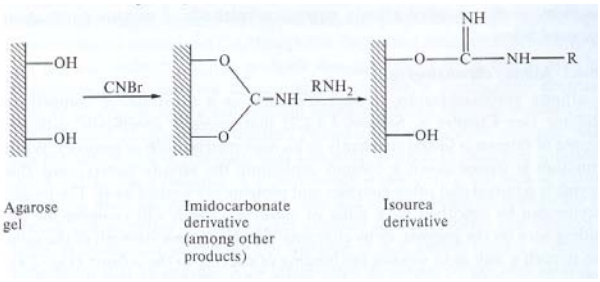
- Affinity chromatography



- 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 chromoatography

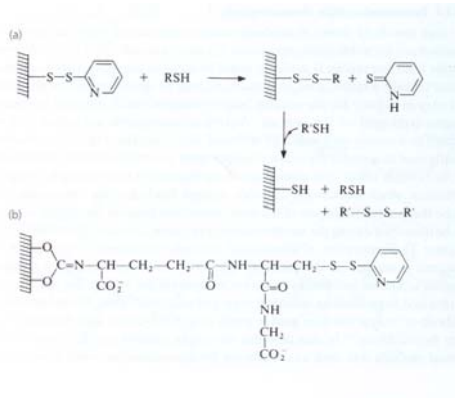
- Problems
  - Attaching a suitable substrate or inhibitor to the matrix can be difficult
  - Linking b/n substrate and matrix itself may inhibit the binding b/n enzyme and substrate: Spacer arm (diaminehexane) may be needed
  - Binding affinity b/n enzyme and substrate must be in a proper range
  - 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 : Ni^{2+} (Elution by imidazole or thrombine cleavage site is added after the tag)

2.6 Methods of separation
4. Other chromoatographies

- 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 chromoatographies

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



2.6 Methods of separation
5. Choice of method

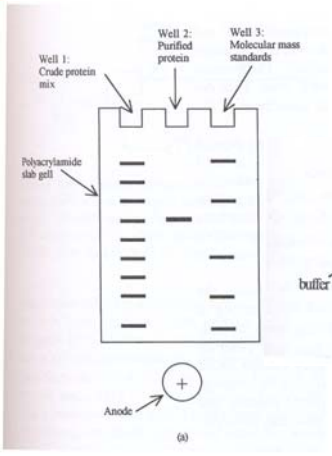
- Time/Large scale -> Precipitation by ethanol or ammonium sulfate or purification based on solubility
- Small scale/high purity -> Column chromatography or electrophoresis
- FPLC or HPLC -> Fast and high purity, expensive

2.7 How to know the success of purification

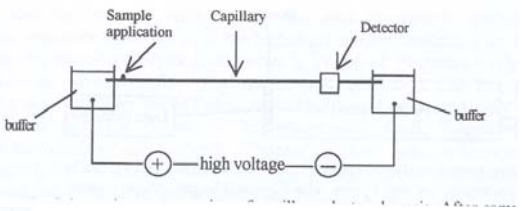
- Test for purity see Table 2.2

Table 2.2 Some commonly employed analytical methods to check the purity of enzyme preparations

Method	Comments
Ultracentrifugation (Chapter 3, Section 3.2.1)	Not very satisfactory for detecting impurities at the ≤5% level. Problems can arise from associating–dissociating systems (Chapter 3, Section 3.2.5)
Electrophoresis (Section 2.6.2.2)	A good method for examining enzymes composed of non-identical subunits (Chapter 3, Section 3.5.1.5)
Electrophoresis in the presence of sodium dodecylsulphate (Chapter 3, Section 3.2.3)	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)
Capillary electrophoresis (Section 2.6.2.2)	A powerful analytical technique which can be used in a variety of modes, including isoelectric focusing. Equipment required is specialized and relatively expensive
Isoelectric focusing (Section 2.6.2.3)	A very sensitive method for detecting impurities. Artefacts can arise suggesting apparent heterogeneity[85–87]
N-terminal analysis (Chapter 3, Section 3.3.2.4)	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)
Mass spectrometry (Chapter 3, Section 3.2.4)	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 3.3.2.10). Post-translational modifications can be identified



Electrophoresis

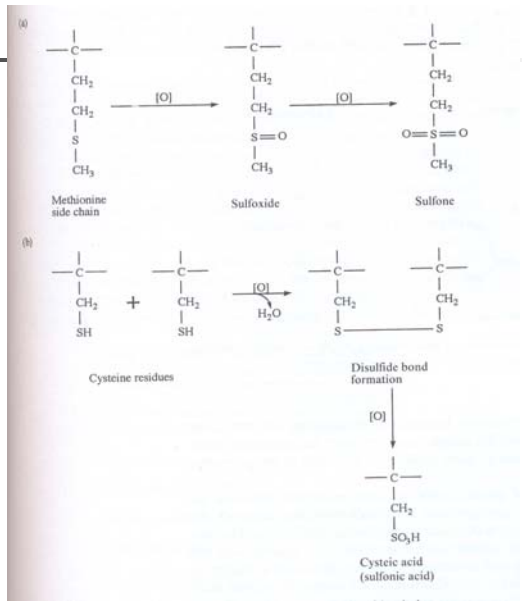


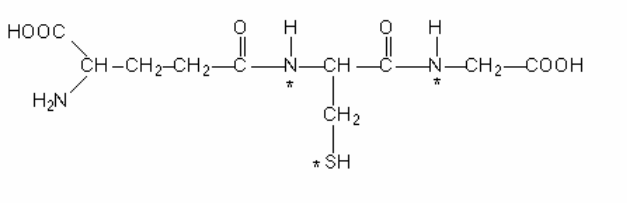
Capillary Electrophoresis

2.7 How to know the success of purification

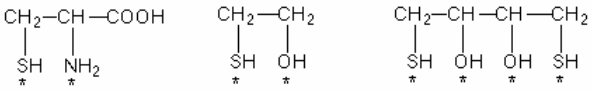
- Tests for catalytic activity
  - By enzyme assay
  - Check cofactors and inhibitors
- Stabilizing factors
  - Neutral pH, storage in 50% glycerol may help
  - 2-mercaptoethanol or DTT(Dithiothreitol)*
  - Protease inhibitor PMSF (Phenylmethylsulfonyl flouride)
- Active site titrations
  - Checking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

Inactivation of protein by oxygen





Glutathione

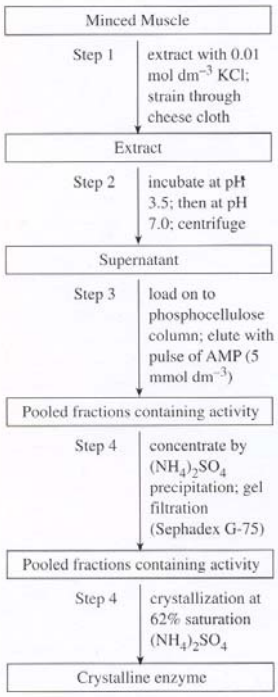


Cysteine β-mercaptoethanol Dithiothreitol

2.8 Examples of purification

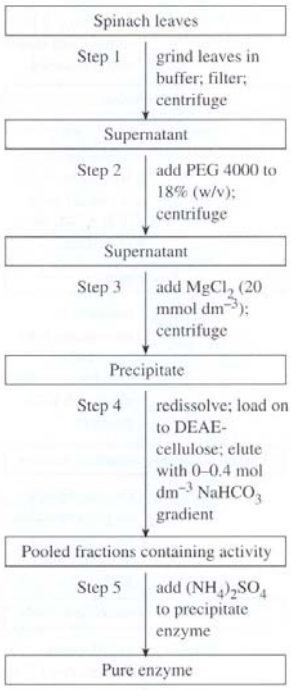
- Adenylate kinase from pig muscle
  - Adenylate kinase is stable at low pH (Step 2)
  - High affinity with AMP (Step 3)
  - Purification using size (Step 4)

Step	Total volume (cm^3)	Total protein (mg)	Total activity (katal)	Specific activity (katal kg^{-1})	Yield (%)	Purification factor per step
1 (extraction)	16600	435000	0.0413	0.095	(100)	(1.0)
2 (pH)	15700	112000	0.0365	0.325	88.3	3.42
3 (phosphocellulose)	1380	1716	0.0223	13.02	54.0	40.0
4 (gel filtration)	211	462	0.0200	43.17	48.4	3.32
5 (crystallization)	—	344	0.0160	46.5	38.7	1.08



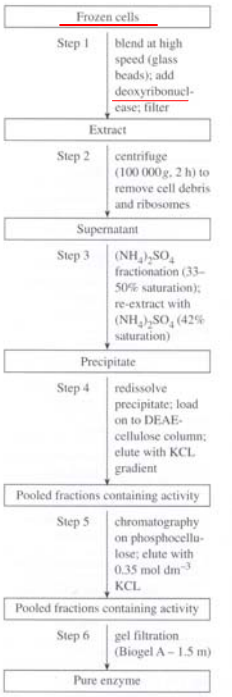
2.8 Examples of purification

- 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



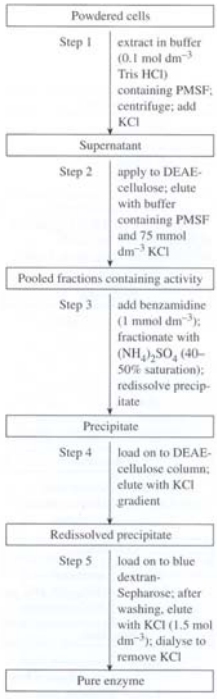
2.8 Examples of purification

- 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)



2.8 Examples of purification

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