**Protein purification and analysis**

1. Initial steps: size, charge/solubility
2. Assays: activity and protein; specific activity
3. Separation/purification techniques

**Initial steps**

1. Acquire/prepare tissue (1 g = $10^6$ plant cell, $10^9$ animal cells, $10^{12}$ bacteria)

2. Suspend in buffer
   - pH stabilizers: phosphate, Tris, “Good” buffers, e.g. HEPES
   - Solubilizers/detergents: digitonin, Triton X-100
   - Protease inhibitors: cold, PMSF, leupeptin, chymostatin

3. Lyse cells (Blender, sonicator, French press, mortar and pestle)

4. Centrifuge (e.g. 12 kg x 30 min)

5. (NH$_4$)$_2$SO$_4$ or pH precipitations: (“salting out”)
   - Salt competes for water of solvation, precipitating proteins
   - Different proteins precipitate at different concentrations of (NH$_4$)$_2$SO$_4$ (e.g., 20%, 50%, 80% of saturation) or different pHs; 2-3-fold purification

**Assays: measures of specific identity (e.g. enzyme activity) and [protein]**

Spectrophotometry (for activity, protein concentration, etc.)
\[ A = -\log_{10} \left( \frac{I}{I_0} \right) \]

\[ A = a \times c \times l \]

- \( a \) = absorbance (specific for compound);
- \( c \) = concentration;
- \( l \) is cuvette thickness (usually 1 cm)
- If you measure \( A \) and know \( a \), you know \( c \)

Chromophore detection (e.g., of protein (\( A_{280} \), peroxidase, with heme, \( A_{430} \))

\[ A(\lambda) \]

Wavelength (\( \lambda \))

Use spectrophotometry to measure concentration of protein
- Tyr, trp, phe absorb UV at ca. 280 nm
- For many proteins, \( a = 1 \) (i.e. \( I/I_0 = 0.1 \)) means [protein] = 1 mg/ml
- Alternatively, add reagent that forms color with protein: Lowry/BCA (Cu reacting with peptide bonds), Bradford (dye binding with absorption in hydrophobic environment) (need standard: BSA)

Use spectrophotometry to measure enzyme activity
- Some substrates and/or products of a reaction absorb UV or visible light
- Some substrates/products will react with a compound that absorbs UV/visible light

Enzyme: \( A + B \rightarrow C + D \): detect increase of \( C \) (or decrease of \( A \)) as function of time (ideally, linear with time and amount of extract)

\[ \frac{dI}{I} = -ec \, dl \]
\[ \int \frac{dI}{I} = -ecl \]
\[ \ln_e(I/I_0) = -ecl \]
\[ a = e/2.3 \]
\[ \log_{10}(I/I_0) = -acl \]
\[ A = -acl = -\log_{10}(I/I_0) \]
Specific activity: measure of purification

sp. act. = enzyme activity (chromophore, etc) / protein

1 IU (international unit) = 1 µmol substrate used (product formed)/min-mg protein

purification (fold purification) = sp. act. (step x)/sp. act. of crude extract

one measure of “purity” is constant sp. act.

Note: if the protein of interest is 0.2% of the total in the crude extract, then you require 500-fold purification for purity; if each step gives 3-fold purification, then you need 5-6 steps ($3^5 = 243$); if each step causes some loss of the protein of interest (e.g. 50%), you need to start with 30-60-times more enzyme than you will recover ($2^5 = 32$).

Purification techniques: size, charge, isoelectric point, affinity

Size

Exclusion chromatography (Sephadex dextran; Biogel acrylamide)

Porous beads in column: $V_o =$ volume outside beads; $V_i =$ volume outside+inside beads

Excluded (large) Partially included Included (salt)

[Protein]

$V_o$ $V_i$

Volume of eluate

Other size techniques: zonal untracentrifugation, ultrafiltration

Charge

Electrophoresis: $F = Eq/d - 6πηνv$;
$v(velocity) = E(\text{voltage}) q(\text{charge}) / 6π(x-\text{section}) \ η(\text{viscosity}) d(\text{electrode separation})$

negative electrode

starch gel, stabilized liquid, etc.

less negative protein (surface charge density)

more negative protein

positive electrode
SDS (sodium dodecylsulfate) - PAGE (polyacrylamide gel electrophoresis) (size again)/
SDS: charged detergent: denatures protein and gives uniform surface charge density
acrylamide gel: cross-links produce resistance to protein movement
negative electrode

larger protein

smaller protein

positive electrode

Isoelectric electric focusing (separate by pH at which proteins have zero charge)

use “ampholytes” to stabilize pH gradient in electrophoresis gel
protein moves to pI (isoelectric point, zero charge)

Note 2-D gels combine isoelectric focusing with SDS-electrophoresis

Biological specificity: affinity chromatography

1. Attach “ligand” to insoluble bead
2. Combine protein mixture with beads: enzyme binds to ligand
3. Load column; wash column
4. Elute enzyme with substrate or change in pH (to reduce binding)

What do you use for ligand: substrate analog; dyes simulate enzyme cofactors (often planar, aromatic)