Protein purification strategies

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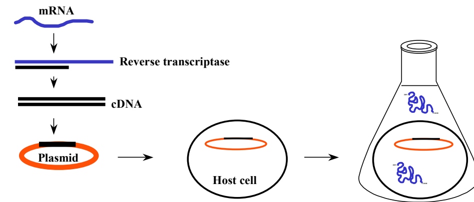
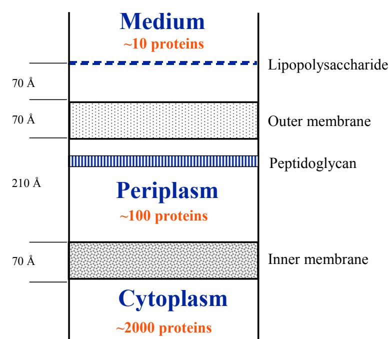
Purification of recombinant proteins

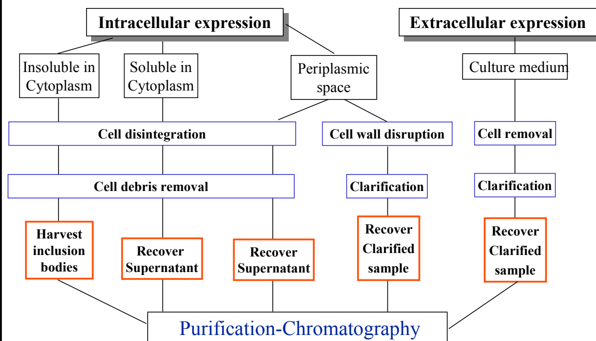
First stage: Expression!



Location of proteins in *E. coli*



Sample preparation



Protein Purification Strategies

General Advice

1. Develop a simple and reliable assay of activity.
2. Collect as much information as possible about the characteristics of the protein and preferably also about the most important impurities. E.g. Mw, pI, carbohydrate, hydrophobicity, free -SH etc..
3. Stability criteria should be established regarding pH, temperature, oxygen (air), org. solvents, heavy metals, proteolytic sensitivity, mechanical shear etc..

SEPARATION PARAMETERS IN PROTEIN PURIFICATION (1/2)

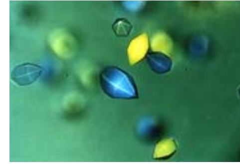
- Temperature stability — Heat denaturation
- Solubility — Salt precipitation; Solvent precipitation; Polymer precipitation; Isoelectric precipitation; Partitioning in aqueous polymer 2-phase systems; Partition chromatography
- Size and shape — Size exclusion chromatography (gel filtration); Ultrafiltration; Gel electrophoresis
- Net charge — Free zone electrophoresis (e.g. capillary electrophoresis); Zone electrophoresis in convection stabilizing media; Isotachophoresis; Ion exchange chromatography
- Isoelectric point — Isoelectric focusing; Chromatofocusing

SEPARATION PARAMETERS IN PROTEIN PURIFICATION (2/2)

• Hydrophobicity	Hydrophobic interaction chromatography Reversed phase chromatography
• Function	Affinity chromatography
• Antigenicity	Immunosorption
• Carbohydrate content	Lectin affinity chromatography
• Content of free -SH	Chemisorption ("covalent chromatography")
• Metal ion binding	Immobilized metal ion affinity chromatography
• Miscellaneous	Hydroxyapatite chromatography Dye affinity chromatography

Some applications demand high purity

Structural studies

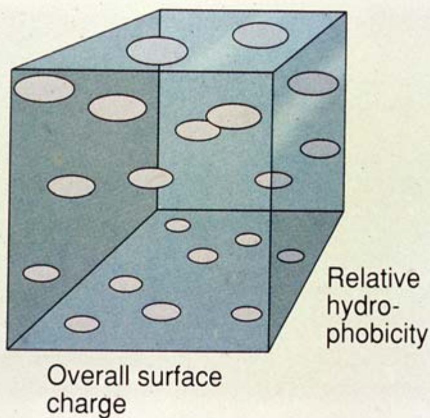


	Purity
Antibody production:	> 90-95%
Crystallization:	> 99%
Characterization:	> 99%

Biopharmaceutical production

Authority regulated e.g. FDA
Impurities to check:
- DNA
- Endotoxins
- Host cell proteins
- Modified forms
- Dimers
- Misfolded forms

Need economical and robust processes.
Validation is important.



Protein Purification Strategies

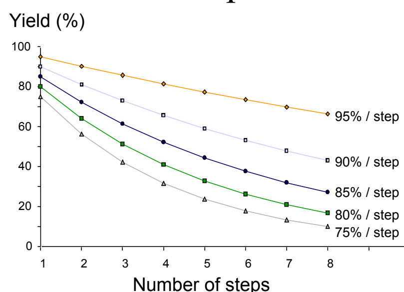
The Order of the Chromatographic Steps

There are practical rather than theoretical reasons why certain chromatographic techniques are better suited for the early steps and others for the final steps of a protein purification process. The choice is primarily governed by the following parameters:

-The sample volume.
-The protein concentration and viscosity of sample solution.
-The degree of purity of the protein product.
-The presence of nucleic acids, endotoxins and proteolytic activity.
-The ease of adsorbent regeneration and cleaning.

It is often advisable to start with methods which allow the application of large volumes of crude extract and which have the highest capacities, e.g. ion exchange chromatography and hydrophobic interaction chromatography. Buffer exchange and concentration between steps should be avoided. Gel filtration (size exclusion chromatography) is often chosen as a final, polishing step.

Always Minimize the Number of Steps



PURIFICATION PROCESS

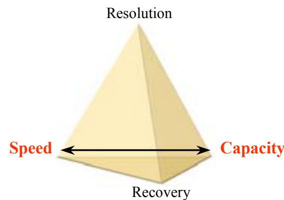
- **Capturing** (to concentrate and isolate)
- **Purification** (removal of gross contaminants)
- **Polishing** (to remove the last traces of microheterogeneity)

1. Capture

Goals

- **Initial purification of crude sample**
- **Rapid concentration (volume reduction) and stabilization (elimination of proteases)**

Most suitable techniques: IEX / HIC



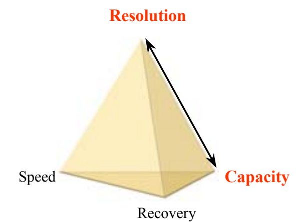
2. Purification

Goal

- **Remove major impurities**

Most suitable techniques: IEX / HIC/AC

HiLoad™ HIC columns

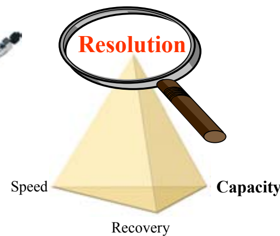


3. Polishing

Goal

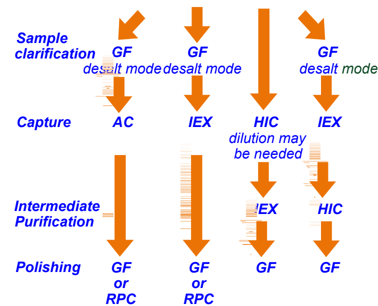
- **Achieve final purity (removal of aggregates, structural variants)**

Suitable techniques: GF/IEX/HIC/RPC



Logical Combinations of Techniques

Crude sample or sample in high salt concentration



MAIN TYPES OF IMPURITIES

- **Other proteins, nucleic acids, polysaccharides, lipids, low molecular weight substances (often called gross contaminants)**
- **Variants of the protein itself (often called micro heterogeneity)**

Removal of "gross contaminants" usually no big problem.

The real problem is that of microheterogeneity, i.e. the presence of traces of modified versions of the protein itself!

CAUSES OF MICROHETEROGENEITY

- Post-translational modifications
- Covalent modification during processing

Examples of post-translational modifications

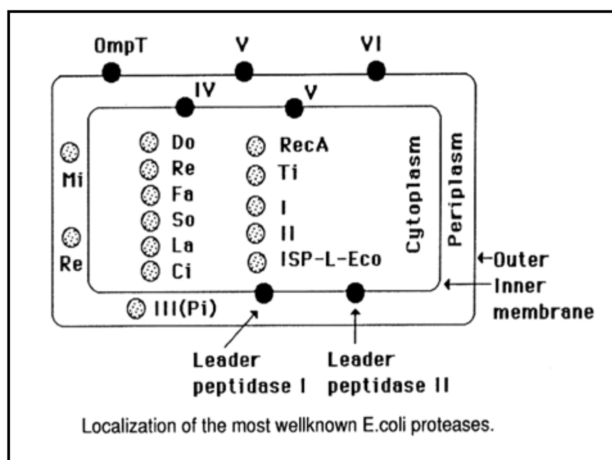
- Incomplete removal of signal peptide.
- Incomplete removal of initiator methionine or deformylation of N-formyl methionine
- Incomplete or mixed disulphide formation.
- Unexpected glycosylation pattern.
- Incomplete or inappropriate acylation.
- Phosphorylation

Examples of covalent modification during processing

- Deamidation (avoid pH extremes)
- Proteolysis
- Oxidation
- Mixed disulphides (refolding of aggregated proteins)
- Carbamylation (when using urea buffers at high pH)

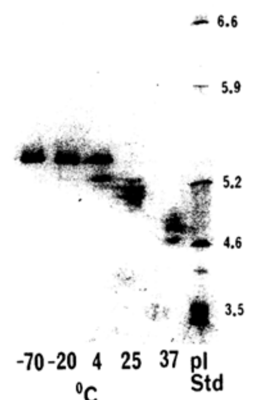
WHEN INTRACELLULAR PROTEINS ARE RELEASED AND DILUTED WITH BUFFER SOLUTIONS, THEY ARE SUBJECT TO FOUR THREATS:

1. Proteolytic degradation
2. Denaturation (loss of tertiary structure)
3. Inactivation of active sites
4. Adsorption to surfaces



Localization of the most wellknown E.coli proteases.

IEF analysis of recombinant Tumour Necrosis Factor (TNF, liquid formulation) after 6 months in storage at shelf temperatures ranging from -70°C to 37°C. (J. Geigert et al., J.Parent. Sci. & Technol., Vol. 43, No.5, Sept.- Oct. 1989, 220-224

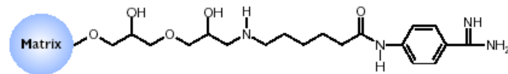


REDUCTION OF PROTEOLYTIC ACTIVITY

ALTERNATIVES

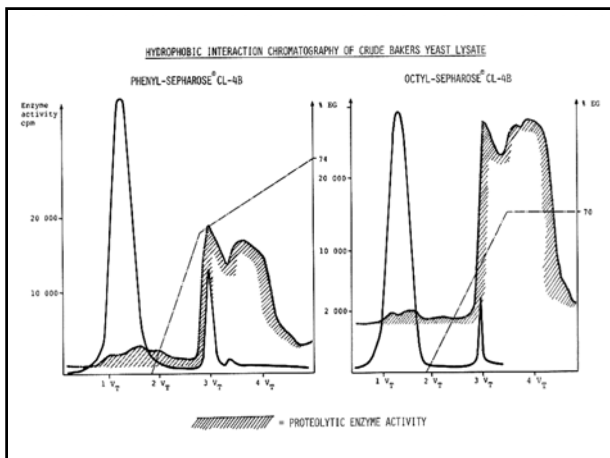
- WORK FAST AT LOW TEMPERATURES
- ADD INHIBITORS
- REMOVE THE PROTEOLYTIC ENZYMES

Benzamidine Sepharose™ 4 Fast Flow



Mean particle size:	90 µm
Bead structure:	Macroporous cross-linked 4% agarose
Ligand density:	>12 µmol/ml gel
Binding capacity:	>35 mg trypsin/ml gel
Flow:	150–250 cm/h 25 cm bed height, 0.1 MPa, 25 °C, distilled water in an XK 50 column

Benzamidine Sepharose 4 Fast Flow and HiTrap Benzamidine FF (high sub) can be used for either rapid removal of proteolytic activity or for purifying trypsin like serine proteases.

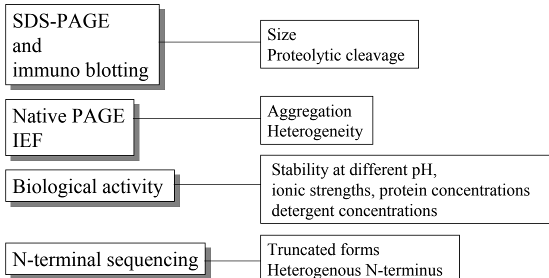


Other problems to be aware of:

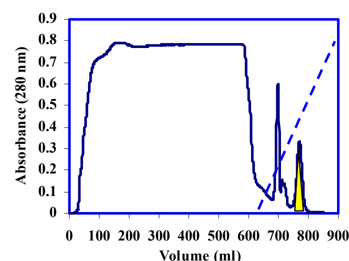
- **Lyophilization and freezing may cause some protein denaturation.**
- **Heavy metals from dialysis tubing, containers, water, bulk chemicals.**

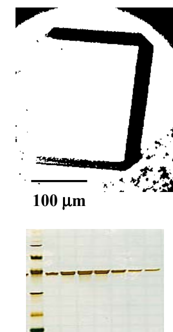
Analytical tools

Is the recombinant protein correctly expressed?



One-step purification of recombinant mannanase directly from *P. pastoris* cell culture supernatant (expressed without tags)





Immobilized Metal ion Affinity Chromatography on Chelating Sepharose FF saturated with Ni^{2+}. The elution was done by applying an imidazole gradient from 0-0.5 M in 10 column volumes.

A Systematic Approach to Purification Development - Summary

- Develop assay methods
- Set the aims (purity and quantity)
- Characterize the target protein
- Use different separation principles
- Use few steps
- Limit sample handling between purification steps
- Start with high selectivity - increase efficiency
- Remove proteases quickly
- Reduce volume in early step
- **Keep it simple!**

Shortcuts - Rapid Establishment of Milligram Scale Purification Protocols

- If a biospecific ligand is available:
  use AC as the main purification step.
- If the purification is not intended to be scaled up:
  use high performance media (e.g. MonoBeads) throughout.
- For "one-of-a-kind" purification of a protein e.g. for sequencing before gene isolation:
  sacrifice yield for purity by making narrow cuts.
- If nothing is known about target protein and contaminants properties:
  try the IEX ⇨ HIC ⇨ GF combination.
- **Establish a fast and reliable assay for the target protein.**

Trends In Affinity Ligand Design For Protein Separation

- "Rational ligand design", i.e. computer aided design based on protein docking algorithms. (Requires structural information)
- Chemical synthesis of libraries (peptidomimetic etc.).
- Phage display peptide libraries (constrained peptides better).
- Phage display of hypervariable CDRs (antibody engineering) ("Coliclonal" antibodies).
- Poly- and oligonucleotide libraries (e.g. RNA aptamers).
- Molecular imprinting (template polymerization). Of particular promise for protein separation are metal complexing polymers.

Thank you very much

for your attention!