Purification Strategy

Selection and combination of purification techniques

Guidelines for Protein Purification

Examples
PURIFICATION STRATEGY

- **General approach**: Protocol Development
- Commonly confronted decisions
- **DoE**: Design of Experiment
- Linking Chromatography Techniques
- Sample concentration - Storage
- Examples
Applications of Protein Purification

- In vitro Activity assays
- Antibody development / production
- Protein:protein interaction assays
- Cell-based activity assays
- Ligand-binding assays
- Mass-spectrometric analysis
- Structural analysis
- In vivo activity assay
- Post-translational modification tests
- N-terminal sequencing
- Electromobility shift assay (band shift)
- DNA footprinting
- Protein cross-linking studies
- Vaccine development/production
- Probes for protein arrays/chips
- Expression library screening
- Other

For each application you need:
✓ different quantities
✓ different protein purity
✓ start material is different, etc
✓ different strategy

Each purification project must be adapted to your start material and your final needs

Don’t waste clear thinking on dirty or not healthy proteins!!!!
Protein Purification - Aims

- Satisfactory
  - expression levels
  - protein activity
  - purity
  - homogeneity
  - stability

- Economical use of reagents/equipment/time

**Goal to Success:**

- Selection or optimization of the best source or best expression conditions
- A good understanding of the protein needs
- Selection & optimization of the most appropriate technique for each step
- A rapid and reliable assay for the target protein: biological assay, enzymatic, SDS-PAGE, Western, etc
Commonly confronted decisions

- Which is the best natural source?
- How much do we need?
- Active? Which assay?
- Purification grade?
- Which hosts: bacteria, yeast, insect cells or in human cells?
- Which expression vector should be used? Which strain(s) should be chosen?
- Should the protein be tagged? Which affinity tag is the best?
- Which is the best purification strategy?
- Which buffers should I use?
- Optimization of each purification step, where to stop?
- How much can I concentrate my sample?
- How to keep activity, solubility and homogeneity of my sample?
Protein Production Pipeline

- Target Selection
- Target Optimization
- Gene Cloning
  - Selection of Expression Vector
  - Selection of Expression Host
- Expression Analysis
- Solubility Analysis
- Scaling Up
- Fermentation
- Purification
  - Purification Optimization
- Characterization
- Concentration & Storage

Pharmaceutical Studies

Biochemical Studies

Structural Studies: Crystallization – NMR- etc
Interpreting the Language of Proteins

Proteins Have a Body Language That Depends on Post-Translational Modifications

- **Hydroxylation**: Attaches a hydroxyl group (-OH) to a side chain of a protein.
- **Phosphorylation**: Adds a phosphate to serine, threonine, or tyrosine.
- **Methylation**: Adds a methyl group, usually at lysine or arginine residues.
- **Glycosylation**: Attaches a sugar, usually to an “N” or “O” in an amino acid side chain.
- **Lipidation**: Attaches a lipid, such as a fatty acid, to a protein chain.
- **Ubiquitination**: Adds ubiquitin to lysine residue of a target protein for degradation.
- **Acetylation**: Adds an acetyl group to an N-terminus of a protein or at lysine residues.
- **SUMOylation**: Adds a small protein SUMO (small ubiquitin-like modifier) to a target protein.
- **Disulfide Bond**: Covalently links the “S” atoms of two different cysteine residues.
The Trip Adviser guide to the protein science world: a proposal to improve the awareness concerning the quality of recombinant proteins

Mario Lebendiker, Tsafi Danieli and Ario de Marco

Purification protocol development - step by step

Define the purification objectives

Consider what’s known about the target protein

Select purification tools

Develop analytical assays

Homogeneity and solubility Buffer optimization

Scale-Up

Storage optimization

Reproducibility & lot to lot consistency assessment

QC

Purity

Integrity

Homogeneity

Identity

Develop the individual purification steps
Design of Experiments (DoE)

✓ A statistical technique for planning and analyzing experiments
✓ Create a mathematical model of the process
✓ Used to understand the influence of experimental parameters and to find an optimum for the process
✓ Minimum number of experiments – maximum information

DoE measures the effect of each factor individually and in combination, allowing detection of interaction to give you more precise results quicker

Reach the same result in 16-32 experiments instead of 128 experiments using the traditional approach!
Integrated experimental design for quicker results

DoE could also be: "Design of Experience"

“I know that pH and conductivity are critical for my purification, how can I optimize the yield?”

DoE method scheme

Scouting run

Decision using model

Model validation

Model creation
Scouting runs for method optimization

Responses (Y’s):
External data:
- Capacity
- DBC (Frontal analysis)
- Yield
- Purity/Selectivity
- Molecular weight
- Activity
- HCP
- DNA
- Aggregates
- Protein A

Peak Data:
- Area
- Concentration
- Amount
- Resolution
- Asymmetry
- Plates per meter

Factors (X’s):
- Load pH
- Load conductivity
- Load concentration
- Mass load
- Wash volume
- Wash pH
- Wash conductivity
- Elution pH
- Gradient elution
- Step elution level
- Cut OD
- Elution Additive
- Media type
- Column size
- Bed Height
- Flow rate
- Residence time
PURIFICATION STRATEGY

- **General approach**: Protocol Development
- Commonly confronted decisions
- **DoE**: Design of Experiment
- **Linking Chromatography Techniques**
- **Sample concentration** - Storage
- Examples
Three Phase Strategy

1. Capture
   - Isolate product, concentrate, stabilize
   - Remove bulk impurities

2. Intermediate purification

3. Polishing
   - Achieve final purity, remove trace impurities, structural variants, aggregates etc.
Which type of chromatography resin provides the desired performance? Selection and combination of purification techniques

- **Objective: High resolution**
  Small, uniformly sized beads (e.g., 5-45 μm bead diameter)

- **Objective: Speed**
  Large, rigid and uniformly sized beads provide the highest speed (e.g., 50-90 μm, highly cross-linked agarose)

- **Objective: High binding capacity**
  Porous beads with high ligand density and directed ligand coupling

- **Objective: High recovery**
  Recovery is mostly dependent on buffer conditions and on how peaks are cut

- Every technique offers a balance between resolution, capacity, speed and recovery

- So, resins should be selected to meet the objectives of the purification step

- **GOAL: Fastest route to get a product of required purity**
Capture

- **GOAL**: Initial purification of the target molecule from clarified source material.
- Rapid isolation, and concentration (volume reduction) of the target protein
- **BONUS**: Concentration (smaller and faster columns). Stabilization (removal of proteases)
- **OPTIMIZATION**: Speed and Capacity: Use Macroporous and Highly Substituted matrix
- Most suitable techniques: Protein A/G/IEX / HIC / (Industry)
  or IMAC or similar Affinity / IEX / HIC (Academics)
- Maximize binding of the target proteins and minimize binding of contaminants during loading
- Maximize protein purity during wash & elution
- Higher speed that do not affect considerably the dynamic capacity of the column
- **Goal:** Removal of major impurities
- Focus mainly on resolution
- Continuous gradient or multi-step elution
- Most suitable techniques: IEX / HIC or expensive affinity
- For good resolution use around 20% of column capacity with HIC or IEX
- Use a different technique (EIX, HIC, GF, Affinity),
- Or change the selectivity (same IEX at different pH, different ligands or salts concentr for HIC, etc.):
  - **Selectivity optimization**
  - Increase efficiency by using non-porous smaller beads
Polishing

- Final removal of trace contaminants, or separation of closely related substances, like structural variants of the target protein and aggregates.
- End product of required high level purity and homogeneity (oligomeric conformation, post-translational modifications, phosphorylation, etc)
- Suitable techniques: GF/IEX/HIC (RPC for suitable proteins)
# Properties of each chromatographic techniques

<table>
<thead>
<tr>
<th>Chromatographic Method</th>
<th>Selectivity</th>
<th>Capacity</th>
<th>Capture</th>
<th>Interm</th>
<th>Polishing</th>
<th>Sample start conditions</th>
<th>Sample end conditions</th>
</tr>
</thead>
<tbody>
<tr>
<td>IEX</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>Low ionic strength; pH depends on protein and IEX type</td>
<td>High ionic strength and/or pH changed</td>
</tr>
<tr>
<td>HIC</td>
<td>+++</td>
<td>++</td>
<td>++</td>
<td>+++</td>
<td>+++</td>
<td>High ionic strength; addition of salt required</td>
<td>Lower ionic strength</td>
</tr>
<tr>
<td>MMC</td>
<td>+++</td>
<td>++</td>
<td>+++</td>
<td>++</td>
<td>+</td>
<td>pH depends on protein and type of ligand; salt tolerance of binding</td>
<td>pH and ionic strength depends on protein and ligand type</td>
</tr>
<tr>
<td>SEC</td>
<td>+++</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+++</td>
<td>Most buffer conditions</td>
<td>Buffer exchange Diluted sample Limited flow range Low resolution</td>
</tr>
<tr>
<td>AC</td>
<td>++++</td>
<td>+++</td>
<td>+++</td>
<td>++</td>
<td>+</td>
<td>Various binding conditions. Avoid specific interferents.</td>
<td>Specific elution conditions Sometimes harsh conditions</td>
</tr>
<tr>
<td>IMAC</td>
<td>+++</td>
<td>++</td>
<td>+++</td>
<td>++</td>
<td>+</td>
<td>Low concentration of imidazole. No chelants, divalent cations, etc</td>
<td>High concentration of imidazole</td>
</tr>
<tr>
<td>RPC</td>
<td>+++(+)</td>
<td>++</td>
<td>(+)</td>
<td>+</td>
<td>+++</td>
<td>Ion-pair reagents and organic modifiers may be required. Harsh conditions</td>
<td>Organic solvents (risk for loss of biological activity) Harsh conditions</td>
</tr>
</tbody>
</table>
Before or between chromatography

- Minimize sample handling between purification steps (concentration, dialysis, long assays, non-working days, etc.)
- Adjust conductivity and pH: dilution, pH titration, dialysis, desalting (SEC), others
- Concentrate sample by: ultrafiltration, AS precipitation, lyophilization, others (protein stability problems). Very important before SEC
- SEC equilibration buffer according to next step (next chromatography or storing buffer)

Ultrafiltration in large scale

- Rapid concentration + Quick adjusting of conductivity and pH
- Get rid of low MW molecules. Very useful for enrichment of huge proteins, complexes, virus, etc

Avoid Protease cleavage in crude materials

- Use PI, work fast & at low temperature
Linking Chromatography Techniques (2)

Classical Strategy for native or non-tag proteins

- IEX
- HIC
- SEC

- IEX Different selectivity

Classical Tag Affinity (IMAC, GST, Strept, MBP, etc)

- AC
- SEC
- IEX

Protein A/G for Industry and Academics

- AC
- SEC Academics
- IEX
- HIC
- MMC

Problems using Affinity for capture (presence of interferents, high cost, etc.)

- AC
- SEC
- IEX

Very colloidal crude material (high lipids, tissues, plants, etc)

- Ammonium Sulfate Precipitation
- HIC
- IEX
- SEC
Linking Chromatography Techniques (3)

Low MW toxins (venoms)
- SEC
  - Desalting, Eliminate higher MW proteins
- RPC
- IEX

Overexpression of peptides with Fusion Proteins (SUMO, GST, MBP, etc)
- SEC
  - Protease cleavage
- RPC
- IEX

Fusion Proteins (His, SUMO, GST, MBP, etc)
- AC
  - Protease cleavage
- Negative AC
- SEC
- SEC
  - Protease cleavage
- Negative AC
- SEC
  - (MW different)
- SEC
- SEC
  - (pl different)
- Negative AC

Salt dependent solubility
- MMC
- HIC
- SEC
Protein Purification Contaminants

- **Particulates**: Include cells and cell debris. Removed using centrifugation or filtration
- **Host Cell Contaminating Proteins**: Gross contaminants removed using chromatography techniques
- **Modified target protein**: Target protein modified through altered amino acid sequence, glycosylation, denaturation, etc. Removed using chromatography
- **Aggregates**: Natural aggregates or as a consequence of partial inclusion bodies solubilization. May be removed using gel filtration chromatography
- **Lipids, lipoproteins**: May be derived from host cells (membranes) or added to a fermentation (antifoams). Removed using chromatography
- **Small molecules**: Include salts, sugars and reagents added to a purification. Typically removed using gel filtration or diafiltration
- **Polyphenols**: Coloured compounds often derived from plant sources or in the fermentation ingredients. Removed by precipitation or chromatography
- **Nucleic acids**: Released during cell lysis. Remove using ion exchange, precipitation techniques such as protamine sulfate, or through hydrolysis with nucleases
- **Pyrogens**: Usually lipopolysaccharides derived from Gram negative bacterial cell walls. Removed using anion exchange chromatography and other methods
- **Virus**
Methods:

Ultrafiltration (gas pressure, centrifugal force)
Ammonium Sulfate or other precipitants
Lyophilization

Concentration by dialysis vs hygroscopic molecules: Sephadex, Ethylene Glycol, etc

Parameters to change to avoid aggregation:

Temperature
Buffer / pH 10-20mM buffer at relatively neutral pH.
(Avoid buffers like phosphate that forms crystal salts)
Salts (75-200mM NaCl to keep the protein in solution)
Additives: amino acids, osmolytes, reducing agents, PI, NaAzide, etc
Detergents (problematic)
Thermofluor-based high-throughput stability test

Set up concentration limit and best stability/storage conditions

- **Set-up concentration limit**
  - Select the proper molecular weight cut off (MWCO) for ultrafiltration
  - Start concentration of part of your stock, and check OD 280nm using ultrafiltrate as blank (experiment could be done with protein in different conductivity and pH buffers)
  - Take aside samples at different concentrations, ~1.0 2.0 4.0, 6.0, etc OD280nm/ml. Check presence of soluble aggregates same day and after ON at 4°C (PAGE-SDS, analytical SEC, others):
  - Stop concentration if there is aggregation, precipitation or difficulties in the concentration
  - Check absence of soluble aggregates and correct oligomeric state by analytical SEC (start with more concentrated)
  - Optional: confirm yield by PAGE-SDS (spin previously 10min 15000rpm to eliminate aggregates)
  - Next day: check absence of insoluble aggregates (by eye) and repeat analytical SEC and PAGE-SDS of selected samples

- **Set-up storage conditions**
  - Check absence of insoluble aggregates (by eye) and soluble aggregates (analytical SEC) in fractions keep at -80°C after freezing and thawing or at 4°C after several days
Storage of biological samples

General recommendations for purified proteins

According to the website of The Protein Purification Service of The EMBL (European Molecular Biology Laboratory)

General advice - Cannot be applied to every biological sample. Consider properties of sample and its intended use before following any of these recommendations

- For short term storage [up to 24 h], most proteins can be kept at 4°C.

- For storage times longer than 24 h at 4°C, it may be necessary to filter sterilize the protein preparation [through a 0.22 µm filter] or to add a bacteriostatic agent [e.g. 0.02% sodium azide] to avoid bacterial growth. **Note that not all proteins are stable at 4°C for longer periods.**

- For long term storage [more than a week]:

  It becomes necessary to freeze the protein preparation.

  Freeze it rapidly using liquid nitrogen to avoid denaturation.

  Freeze the solution in small aliquots to avoid repeated freezing and thawing which may reduce the biological activity or affect the structure.

  Several stabilizing agents can be added, such as glycerol [5-50% [w/v]], serum albumin [10 mg/ml], reducing agents [such as 1 mM DTT], and ligands [the nature and concentration depending on the nature and concentration of the target protein].
Storage of biological samples

General recommendations for purified proteins

According to the website of The Protein Purification Service of The EMBL (European Molecular Biology Laboratory)

Before anyone of these procedures, check stability of the protein with a little sample.
Aliquot before freezing

- **For several months storage at -20°C:** At this temperature it is recommended to add 50% glycerol to the solution to avoid freezing (or dialyze vs. buffer with 50% glycerol).

- **For longer periods storage [months to years],** freeze it at -70°C or even in liquid nitrogen. Although it is not really necessary to add glycerol at these temperatures, the addition of 5-50% glycerol could help to keep the protein stable.

- **Alternative methods are:**

  Storage of the protein at 4°C as an ammonium sulfate precipitate (4M).

  Storage of the protein at 4°C or lower in a lyophilized form (the protein could be dissolved in a volatile buffer [such as trimethylamine/HCl; pH range 6.8-8.8]. *Note that not all proteins are stable during the freeze-drying process.*

- **Proteins sensitive to temperature:** should not be stored at 4°C as they precipitate or lost ativity at this temperature. Keep at room temperature in the presence of a preserving agent.
Purification protocol development - step by step

1. Define the purification objectives
2. Consider what's known about the target protein
3. Select purification tools
4. Develop analytical assays
5. Develop the individual purification steps
6. Homogeneity and solubility
   - Buffer optimization
7. Reproducibility & lot to lot consistency assessment
8. Scale-Up
9. Storage optimization
10. QC
   - Purity
   - Integrity
   - Homogeneity
   - Identity
11. Develop the individual purification steps
Recent Advances in Endotoxin Removal
An Upgrade to a Traditional Method and a New Adsorption Chemistry

Pete Gagnon BioProcess International 15(9) October 2017

• In many cases, MULTIMODALS support more effective endotoxin removal than traditional anion exchangers, but they have two limitations: they bind many proteins, and their effectivity is dramatically altered by variations in pH and conductivity.

• The only method that effectively exploits hydrophobicity of lipid A at present involves addition of the Triton X114 surfactant (17). Incubation at room temperature to promote association with lipid A is followed by refrigeration that causes the detergent to gelatinize, facilitating its removal with still-associated endotoxin.

Combination of allantoin & VEAX as a ROUTINE PLATFORM approach for purification of low endotoxin research antibodies

Anion exchange in void-exclusion mode (VEAX)
Optimize purification parameters: buffers, resins, strategies, more purification steps, etc

Co-expression of interacting proteins

Ligand supplementation

Co-expression of chaperones

Change, move or eliminate tag

Change expression conditions

Change expression system

Crystallization of the “main domain”:

Hypothesize domain according to bioinformatics, literature, sequence alignment, etc.

Check domain boundaries by mild proteolysis digestion

http://wolfson.huji.ac.il/purification/Protocols/Partial_Proteolytic_Digestion.html

Generate a series of protein constructs of slightly different lengths (random incremental truncation)
Alternative strategies to rescue difficult projects - II

- Mutation of non-essential cysteine residues
- Site specific mutagenesis of hydrophobic residues
- Removal of flexible regions: truncations or loop deletions
- Thermostabilization of protein by mutations
- Deglycosilation of the protein by mutation, by inhibiting glycosilation during expression or by enzymatic removal during purification
- Design expression fusion construct with a well known crystallizable protein as Maltose binding protein (MBP) or insertion of T4-lysozyme
- Complexation with antibody fragments
- Additives (lipids, etc)
- In Situ proteolysis: adding a protease (chymotrypsin or trypsin) step to crystallization trials

DO NOT LOSE HOPE AND TRY HARDER!!!!