Isolating proteins for mass spectrometry analysis

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Reasons to purify proteins

• To characterize them
  – e.g., for their enzyme activity, chaperone function
• To determine structure
  – How they assemble in complexes
• To make antibodies
  – For immunoaffinity purification
• Their pharmaceutical properties
Purification fundamentals

• How much do we need and should it be pure?
• Do we want it in the native form?
  – Many of the recombinant proteins are misfolded
• How are we going to measure it during the purification steps?
  – Enzyme or other biochemical assay
  – Using an antibody
  – Using SDS-PAGE gel

Where do we get the protein from?

• Used to be from tissue or body fluids
  – Some proteins are from animal parts obtained at slaughter houses
  – These can include liver, brain, kidneys
  – Some proteins are richly expressed in one of these tissues, e.g., tau in brain
• Since 1980, recombinant expression in bacteria and other systems have provided replenishable and unlimited amounts
  – But the proteins still have to be purified
Limitations of recombinant expression

• Poor folding in bacteria
  – Large amount of proteins in inclusion bodies
  – Possible to coincidentally overexpress chaperone proteins

• Most of the posttranslational mechanisms in eukaryotic systems do not occur in bacteria
  – Phosphorylation on a serine can be simulated by mutating the serine to aspartate

Assists using molecular biology approaches

• It is possible to add tags that assist in recovering the protein
  – 6xHis, HAT, maltose-binding protein, glutathione S-transferase
  – Biotinylation site

• But from a structural/functional point of view, is that a disadvantage?
  – Alterations in protein structure caused by the tag
  – Back to protein purification
What to consider

- Choice of biological source
- How to maximize the tissue recovery
- How to monitor the protein
- How to develop a purification strategy
- Techniques to be used
- How to integrate the techniques

Harvesting the protein

- Remember that whatever you do from this point on, you’re likely to have remnants of any buffer component in your final purified sample
  - Avoid detergents!!!!!!
  - The reality is that proteins that will be studied by mass spec have to be water soluble
  - Grind tissue in liquid nitrogen to minimize degradation
  - Use BugBuster™ for bacteria
For tissues choose the correct compartment

- Homogenize the tissue in an isotonic buffer
- Separate by differential centrifugation and with sucrose density gradients
  - Nuclear fraction (x800g pellet)
  - Lysosomes/plasma membrane (x10,000g pellet)
  - Mitochondria (20-35,000xg pellet)
  - Peroxisomes (Opticlear gradient)
  - Endoplasmic reticulum (100,000xg pellet)
  - Cytosol (100,000xg supernatant)
- For bacteria, the cytosol or the inclusion bodies

Principles of protein purification

- Proteins should remain at high concentrations
  - Proteins stick to surfaces (sic, ELISA assays)
  - Early stages can use large surface areas, but miniaturize the system as the purification proceeds
- Consider the chemical and physical properties of the protein
- Most proteins benefit from being kept cold
Characteristics of proteins that can be exploited

- Solubility in different solvents
- Balance of charged amino acids (Asp and Glu versus Arg and Lys)
- Molecular weight
- Thermal stability
- Specific binding regions
- Availability of immunoaffinity reagents

Purification techniques

- (NH₄)₂SO₄ precipitation
- Ion exchange (anion and cation)
- Chromatofocusing (isoelectric point)
- Hydroxyapatite
- Hydrophobic interaction chromatography
- Reverse-phase chromatography
- Small molecule affinity chromatography
- Immunoaffinity chromatography
- Gel filtration
The techniques

(NH$_4$)$_2$SO$_4$ precipitation

- High concentrations of (NH$_4$)$_2$SO$_4$ strip water from the hydration sphere of the protein and lead to its reversible precipitation
  - This occurs at different degrees of (NH$_4$)$_2$SO$_4$ saturation - for instance, a protein will not be precipitated at 30% saturation, but others will. However, the protein is precipitated at 40% saturation
- But is adding huge amounts of (NH$_4$)$_2$SO$_4$ such a good idea - depends on protein amount
**Anion exchange**

At low ionic strength, the -ve charge on proteins bind to the +ve beads on the matrix. Increasing the ionic strength with NaCl will lead to their elution.

**Gradient elution IEX**

Gradient elution involves equilibration, sample injection, gradient elution, wash, and re-equilibration. Unbound molecules elute before the gradient begins, with elution occurring in 5-20 CV steps. Tightly bound molecules elute in high salt wash conditions.
More on anion exchange

- The initial binding conditions are crucial
  - If you know the isoelectric point of the protein, use an equilibration buffer that is at least 1 pH unit higher - that will ensure that most of the protein is negatively charged
  - If the protein has a low number of charged amino acids, then use a low ionic strength buffer - and vice versa - a highly charged protein will stick to the anion exchange phase when others won’t
The nature of ion exchange resins

<table>
<thead>
<tr>
<th>Anion exchangers</th>
<th>Functional group</th>
</tr>
</thead>
<tbody>
<tr>
<td>Quaternary ammonium (Q)</td>
<td>strong</td>
</tr>
<tr>
<td>Diethylaminomethyl (DEAE)*</td>
<td>weak</td>
</tr>
<tr>
<td>Diethylaminopropyl (ANX)*</td>
<td>weak</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Cation exchangers</th>
<th>Functional group</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sulfopropyl (SP)</td>
<td>strong</td>
</tr>
<tr>
<td>Methyl sulfonate (M)</td>
<td>strong</td>
</tr>
<tr>
<td>Carboxymethyl (CM)</td>
<td>weak</td>
</tr>
</tbody>
</table>

Summary for ion exchange

- A high capacity technique
- Typically can achieve a 10-fold purification
- Very high recovery of protein and its activity/function
- Often used to clean up a cytosol mixture prior to an affinity column
- But the purified fraction(s) can have high concentrations of salts (NaCl/KCl)
Chromatofocusing

- Some similarity to ion exchange, but based on the balance of charges (isoelectric point), not the absolute amount
- The protein is bound to the positively charged phase as its negatively charged ion
- Bound proteins are eluted with a linear pH gradient
Chromatofocusing example

Variants of hemoglobin are first bound at pH 8.1 to a mono P column. The proteins are eluted by passage of polybuffer 96 with methane sulfonic acid, pH 6.65.

Summary of chromatofocusing

- Quite high capacity
- Best for anionic proteins
- Choice of the polybuffer is crucial
  - pH ranges 4-7 (polybuffer 74), 6-9 (polybuffer 96), 8-11 (polybuffer 118)
- Getting rid of the polybuffer!
  - An affinity step next would be very helpful
Hydrophobic interaction chromatography

- Phenyl-, butyl- and octyl-Sepharose
- Protein is mixed with (NH₄)₂SO₄ at concentrations that are below the precipitation point
- The protein binds via its hydrophobic regions to escape the strong electrolyte environment
- As the (NH₄)₂SO₄ concentration is lowered, the hydrophilic parts of the protein dominate and the protein dissociates from the stationary phase

Binding and salt concentration

Binding of proteins to a Phenyl-Sepharose column

[Graph showing protein capacity vs. initial salt concentration (M (NH₄)₂SO₄)]

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Hydrophobic interaction and reverse-phase columns

- HIC is a high capacity method
  - Has low loading of hydrophobic groups
  - Usually short alkyl groups - C₄, C₈
  - Eluted without organic solvent
- Reverse-phase columns are low capacity, but high resolution
  - Longer alkyl groups - C₄, C₈, C₁₈
  - High loading
  - Elution requires organic solvent

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Hydroxyapatite chromatography

- A mixture of affinity and ion exchange
- Hydroxyapatite is a calcium phosphate insoluble matrix, often embedded in an agarose matrix
- Very sensitive to pH (pH 6.5-12)
- Elution occurs with a gradient of phosphate buffer (200-500 mM)
  - Use potassium salts - greater solubility in cold

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Affinity Chromatography

Very selective absorption of the protein based on local structure

The affinity group is often put on the end of a hook created by adding a spacer arm

Affinity ligands

- The ligands can be small molecules (often inhibitors of an enzyme)
- Or antibodies
  - The affinities for the binding must >10^{-4} M, but not too high
  - Monoclonal antibodies are better than polyclonal antibodies
- Elution can occur with alterations of pH or ionic strength, or with specific elutants
The ideal result

Non-bound protein
eluted protein

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Gel filtration

- Based on hydrodynamic radii of protein
  - See video at
    [http://www1.amershambiosciences.com/aptrix/upp00919.nsf/4a0f132842ea4d354a25685d0011fa04/50c849d0d5b16ba0c1256e92003e865b/$FILE/GE_Gel%20Filtration.swf](http://www1.amershambiosciences.com/aptrix/upp00919.nsf/4a0f132842ea4d354a25685d0011fa04/50c849d0d5b16ba0c1256e92003e865b/$FILE/GE_Gel%20Filtration.swf)
- It always dilutes the sample whether conventional or HPLC forms are used
- The smaller molecules are retained by the phase - they tarry inside the spaces in the phase
- The large molecules are excluded from the phase and elute fast

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A purification table: important part of purifying a protein

- The goal is to obtain enough protein with highest possible activity and the greatest purity

<table>
<thead>
<tr>
<th>Step</th>
<th>Total activity (nmol/min)</th>
<th>Total protein (mg)</th>
<th>Specific activity (nmol/min/mg)</th>
<th>Fold purification</th>
</tr>
</thead>
<tbody>
<tr>
<td>Homogenate</td>
<td>100</td>
<td>1000</td>
<td>0.1</td>
<td>1.0</td>
</tr>
<tr>
<td>Cytosol</td>
<td>90</td>
<td>600</td>
<td>0.15</td>
<td>1.5</td>
</tr>
<tr>
<td>DEAE column</td>
<td>80</td>
<td>80</td>
<td>1.0</td>
<td>10</td>
</tr>
<tr>
<td>Affinity column</td>
<td>75</td>
<td>2.0</td>
<td>37.5</td>
<td>375</td>
</tr>
</tbody>
</table>

Examples from Barnes’ lab

- Rat liver bile acid PAPS sulfotransferase
- Human bile acid CoA:amino acid N-acyltransferase (hBAT)
- Rat liver bile acid CoA ligase
- Recombinant hBAT expressed in *E. coli*
Bile acid PAPS sulfotransferase

Rat liver cytosol → Dialysis with 5 mM Na-Pi buffer → DEAE-Trisacryl 30 x 2 cm Eluted with 0-100 mM NaPi

PAP-Sepharose 6 x 1 cm Eluted with 5'AMP, 3'AMP and 3',5'-PAP → Active fractions diluted with water to 2 mhos → Unbound proteins

Purifying BAST

Anion exchange

PAP Affinity

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BAST purification data

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Protein (mg)</th>
<th>Activity (nmol/min)</th>
<th>Spec Act (nmol/min/mg)</th>
<th>Fold purification</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cytosol</td>
<td>1206</td>
<td>144</td>
<td>0.119</td>
<td>1.00</td>
</tr>
<tr>
<td>DEAE peak</td>
<td>50</td>
<td>48</td>
<td>0.96</td>
<td>8.1</td>
</tr>
<tr>
<td>PAP-purified</td>
<td>0.385</td>
<td>7.2</td>
<td>18.7</td>
<td>157</td>
</tr>
</tbody>
</table>

Purified BAST

N-terminal sequence:

- pdytwfeglipfpaflsketlqdv
- pdytwfeglipfpaflsketlqnv

Determined by cDNA cloning

Fractions from PAP-Sepharose affinity absorption and elution

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Human bile acid CoA:amino acid N-acyltransferase (hBAT)

Liver cytosol → dialysis → DEAE-cellulose 5 x 30 cm Eluted with 0-200 mM NaCl → Chromatofocusing 1.6 x 70 cm Eluted with polybuffer 74, pH 5

→ TSK-250 gel filtration 2.5 x 50 cm

Glycocholate-AH-Sepharose 1 x 10 cm Eluted with 5 mM glycocholate

Purification of hBAT

Martin Johnson
### hBAT purification table

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Protein (mg)</th>
<th>Activity (nmol/min)</th>
<th>Spec Act (nmol/min/mg)</th>
<th>Recovery (%)</th>
<th>Fold</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cytosol</td>
<td>18,000</td>
<td>1,200</td>
<td>0.067</td>
<td>100</td>
<td>1.0</td>
</tr>
<tr>
<td>DEAE-cellulose</td>
<td>1,764</td>
<td>987</td>
<td>0.56</td>
<td>82</td>
<td>8.4</td>
</tr>
<tr>
<td>Chromatofocusing</td>
<td>52</td>
<td>271</td>
<td>5.22</td>
<td>22</td>
<td>78.0</td>
</tr>
<tr>
<td>GC-Sepharose</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gel filtration</td>
<td>7.7</td>
<td>246.7</td>
<td>31.87</td>
<td>20</td>
<td>475.7</td>
</tr>
</tbody>
</table>

### Purifying rat liver bile acid CoA ligase

- **Brij-58**
- **Q-Sepharose** 13 x 2.5 cm (flow through)
- **CM-Sepharose** 3 x 1.0 cm Eluted with 80 mM NaCl
- **Hydroxyapatite** 5 x 1.0 cm (bound then eluted with 80 mM NaCl)

Wheeler, Shaw Barnes
J Lip Res 1997

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James Wheeler
Bile acid CoA ligase

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Protein (mg)</th>
<th>Activity (nmol/min)</th>
<th>Sp. Act. (nmol/min/mg)</th>
<th>Yield (%)</th>
<th>Fold purified</th>
</tr>
</thead>
<tbody>
<tr>
<td>microsomes</td>
<td>176</td>
<td>341.4</td>
<td>1.94</td>
<td>100</td>
<td>1.00</td>
</tr>
<tr>
<td>Sol microsomes</td>
<td>176</td>
<td>1010.2</td>
<td>5.74</td>
<td>295</td>
<td>2.96</td>
</tr>
<tr>
<td>Q-Sepharose pool</td>
<td>44</td>
<td>545.6</td>
<td>12.4</td>
<td>150</td>
<td>6.39</td>
</tr>
<tr>
<td>Hydroxyapatite</td>
<td>0.74</td>
<td>72.67</td>
<td>98.2</td>
<td>21</td>
<td>50.6</td>
</tr>
<tr>
<td>CM-Sepharose</td>
<td>0.055</td>
<td>21.18</td>
<td>385.0</td>
<td>6.2</td>
<td>198.4</td>
</tr>
</tbody>
</table>

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Affinity purification of rBAL

FIG. 1. SDS-PAGE analysis of reduced, denatured proteins in fractions from the purification procedure. Equal amounts of rBAL activity were loaded in lanes A–D. (A) Solubilized microsomes, (B) Q-Sepharose chromatography pool, (C) Hydroxyapatite pool, (D) CM-Sepharose pool, (E) CM-Sepharose pool overloaded (rBAL) calculated as being 33% of total protein loaded.

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James Wheeler
**Recombinant hBAT**

6xHis-tag BAT was expressed in *E. coli* but when the cytosol was passed over a Ni-affinity column, the imidazole eluate gave rise to 23 kDa and 56 kDa bands in addition to the 50 kDa hBAT band. p23 was shown to be peptidyl prolyl cis-trans isomerase, a protein with 14 His residues in a 30 residue C-terminal region. p56 is a bacterial GRoEL chaperone.

Mindan Sfakianos

Stephen Barnes 1-30-07

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**hBAT-avi**

The recombinant *E. coli* contains both hBAT-avi and a biotin ligase. Once hBAT-avi is overexpressed, biotin (5 mM) is added to label hBAT. The biotinylated hBAT-avi is recovered by use of soft avidin resin affinity phase. The bound hBAT-avi is eluted with biotin. This generates two proteins, p50 and p56. Once again, the p56 protein is a GRoEL chaperone. We removed it by pre-incubating the cytosol with an ATP generating system.

Mindan Sfakianos

Erin Shonsey

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Pure hBAT-avi

(1) is the bacterial cytosol, (2) the DEAE column flow through, (3) is the avidin column flow through, (4) is the avidin column wash, and (5) is the biotin elution fraction from the avidin column. We optimized the recovery to over 80% in just two steps.

Erin Shonsey

But is this what we want?

A 172-180 GLLEFRASL
B 208-219 LEYFEAAANNFLL
C 237-247 GVQIGLSMAIY
D 292-298 LLELYRR
E 337-346 AEQAIGQLKR
F 392-408 AAAQEHWKEIQRFLRK

1 101-108 FQVQVKLY
2 149-153 RGALF
3 163-166 VIDL
4 ?
5 229-234 VGVVS
6 252-257 TATVL
7 320-324 FLFIV
8 353-357 TLLSY

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Thanks

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- Mindan Sfakianos
- GE Healthcare