Selection and Combination of Purification Techniques

Minimise sample handling
Minimise number of steps
Use different techniques at each step

Goal: Fastest route to a product of required purity.

Every technique offers a balance between resolution, capacity, speed and recovery.
Table 3. Protein properties used during purification.

<table>
<thead>
<tr>
<th>Protein property</th>
<th>Technique</th>
</tr>
</thead>
<tbody>
<tr>
<td>Charge</td>
<td>Ion exchange (IEX)</td>
</tr>
<tr>
<td>Size</td>
<td>Gel filtration (GF)</td>
</tr>
<tr>
<td>Hydrophobicity</td>
<td>Hydrophobic interaction (HIC), reversed phase (RPC)</td>
</tr>
<tr>
<td>Biorecognition (ligand specificity)</td>
<td>Affinity (AC)</td>
</tr>
<tr>
<td>Charge, ligand specificity or hydrophobicity</td>
<td>Expanded bed adsorption (EBA) follows the principles of AC, IEX or HIC</td>
</tr>
</tbody>
</table>
The charged groups, hydrophobic regions, size, and solvation affect the biophysical properties of the protein and largely determine its purification behavior.
Chromatography

Sample containing proteins or peptides

Liquid flow

Separation according to:
- molecular weight/ size
- charge
- hydrophobicity
- affinity

Time 1 2 3 4 5

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A guide to the suitability of each purification technique for the stages in the Three Phase Purification Strategy is shown in Table 4.

<table>
<thead>
<tr>
<th>Technique</th>
<th>Main features</th>
<th>Capture</th>
<th>Intermediate</th>
<th>Polish</th>
<th>Sample Start condition</th>
<th>Sample End condition</th>
</tr>
</thead>
<tbody>
<tr>
<td>IEX</td>
<td>high resolution, high capacity, high speed</td>
<td>⭐⭐⭐</td>
<td>⭐⭐⭐</td>
<td>⭐⭐⭐</td>
<td>low ionic strength sample volume not limiting</td>
<td>High ionic strength or pH change concentrated sample</td>
</tr>
<tr>
<td>HIC</td>
<td>good resolution, good capacity, high speed</td>
<td>⭐⭐</td>
<td>⭐⭐⭐</td>
<td>⭐</td>
<td>high ionic strength sample volume not limiting</td>
<td>Low ionic strength concentrated sample</td>
</tr>
<tr>
<td>AC</td>
<td>high resolution, high capacity, high speed</td>
<td>⭐⭐⭐</td>
<td>⭐⭐⭐</td>
<td>⭐⭐</td>
<td>specific binding conditions sample volume not limiting</td>
<td>specific elution conditions concentrated sample buffer exchanged (if required) diluted sample</td>
</tr>
<tr>
<td>GF</td>
<td>High resolution using Superdex™ media</td>
<td>⭐</td>
<td>⭐⭐⭐</td>
<td>⭐⭐⭐</td>
<td>limited sample volume (&lt;5% total column volume) and flow rate range</td>
<td>in organic solvent, risk loss of biological activity</td>
</tr>
<tr>
<td>RPC</td>
<td>high resolution</td>
<td>⭐</td>
<td>⭐⭐</td>
<td>⭐⭐⭐</td>
<td>requires organic solvents</td>
<td></td>
</tr>
</tbody>
</table>

Table 4. Suitability of purification techniques for the Three Phase Purification Strategy.
Three Phase Strategy: An aid in developing the purification scheme

Capture

Intermediate purification

Remove bulk impurities

Polishing

Achieve final purity. Remove trace impurities, structural variants, aggregates, viruses, etc.

Isolate product, concentrate, stabilize

Purity

Step
Sample Preparation

General considerations:

• Select extraction procedure according to source and location of protein
• Use gentle procedures to minimize acidification and release of proteolytic enzymes
• Work quickly at sub-ambient temperatures
• Use buffer to maintain pH, ionic strength

Goal: To stabilize sample
Always Limit the Number of Steps
Maximize the Yield at Each Step

Yield (%)

Number of steps

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Gel Filtration
Gel Filtration (GF) Chromatography

GF separates proteins with differences in molecular size. The technique is ideal for the final polishing steps in a purification when sample volumes have been reduced (sample volume significantly influences speed and resolution in gel filtration). Samples are eluted isocratically (single buffer, no gradient Figure 41). Buffer conditions are varied to suit the sample type or the requirements for further purification, analysis or storage step, since buffer composition does not directly affect resolution. Proteins are collected in purified form in the chosen buffer.

Fig. 41. Typical GF elution.
The principle of gel filtration -- excluded volume

[Note: gel filtration chromatography is also sometimes called “size exclusion chromatography”]

\[ V_o = \text{“void volume”} \]
\[ V_t = \text{“bed volume”} \]
\[ V_e = \text{“elution volume”} \]
\[ V_i = V_t - V_o \]
Principles of gel chromatography (con’d)

Fig. 4. Relationship between several expressions used for normalizing elution behaviour.
Gel Filtration Elution Volumes as a Function of Molecular Weight

Ion Exchange Chromatography
**Ion Exchange (IEX) Chromatography**

![Diagram of ion exchange chromatography]

**Fig. 31.** Typical IEX gradient elution.

The net surface charge of proteins varies according to the surrounding pH. When above its isoelectric point (pI) a protein will bind to an anion exchanger, when below its pI a protein will bind to a cation exchanger. Typically IEX is used to bind the target molecule, but it can also be used to bind impurities if required. IEX can be repeated at different pH values to separate several proteins which have distinctly different charge properties, as shown in Figure 32. This can be used to advantage during a multi-step purification, as shown in the example on page 24.
Ion Exchange Chromatography (con’d)

Fig 32. Effect of pH on protein elution patterns.
Some other popular chromatographic methods:

- Hydrophobic interaction chromatography
- Affinity chromatography
- Reverse phase chromatography
Hydrophobic Interaction Chromatography (HIC)

Fig. 36. Typical HIC gradient elution
Affinity Chromatography

The target protein(s) is specifically and reversibly bound by a complementary binding substance (ligand). The sample is applied under conditions that favour specific binding to the ligand. Unbound material is washed away, and the bound target protein is recovered by changing conditions to those favouring desorption. Desorption is performed specifically, using a competitive ligand, or non specifically, by changing the pH, ionic strength or polarity. Samples are concentrated during binding and protein is collected in purified, concentrated form. The key stages in a separation are shown in Figure 40. Affinity chromatography is also used to remove specific contaminants, for example Benzamidine Sepharose 6B removes serine proteases.

Fig. 40. Typical affinity separation.
“Reversed Phase” Chromatography (RPC) (elution with organic solvents)

Fig. 43. Typical RPC gradient elution.

RPC is often used in the final polishing of oligonucleotides and peptides and is ideal for analytical separations, such as peptide mapping.

RPC is not recommended for protein purification if recovery of activity and return to a correct tertiary structure are required, since many proteins are denatured in the presence of organic solvents.

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<td>IEX</td>
<td>high resolution, high capacity, high speed</td>
<td>★★★</td>
<td>★★★</td>
<td>★★★</td>
<td>low ionic strength, sample volume not limiting</td>
<td>High ionic strength or pH change, concentrated sample</td>
</tr>
<tr>
<td>HIC</td>
<td>good resolution, good capacity, high speed</td>
<td>★★★</td>
<td>★★★</td>
<td>★</td>
<td>high ionic strength, sample volume not limiting</td>
<td>Low ionic strength, concentrated sample</td>
</tr>
<tr>
<td>AC</td>
<td>high resolution, high capacity, high speed</td>
<td>★★★</td>
<td>★★★</td>
<td>★★★</td>
<td>specific binding conditions, sample volume not limiting</td>
<td>Concentrated sample buffer, exchanged (if required), diluted sample</td>
</tr>
<tr>
<td>GF</td>
<td>High resolution using Superdex™ media</td>
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<td>★★★</td>
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<td>limited sample volume (&lt;5% total column volume) and flow rate range</td>
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<tr>
<td>RPC</td>
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<td>★★★</td>
<td>★★★</td>
<td>★★★</td>
<td></td>
<td></td>
</tr>
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Table 4. Suitability of purification techniques for the Three Phase Purification Strategy
Linking Chromatography Techniques

It is good to design your purification to have the start conditions of each step match the end conditions of the previous step in order to avoid intervening buffer exchange steps, which add to your losses.

Start conditions | Technique | End conditions
---|---|---
Small sample volume | GF | Diluted sample Buffer change (if required)
Low ionic strength | IEX | High ionic strength or pH change
High ionic strength | HIC | Low ionic strength
Specific binding conditions | AC | Specific elution conditions

Note: after IEX, HIC, or AC, sample is concentrated, too.
In addition, there are non-chromatographic protein purification techniques, e. g.:

- Ammonium sulfate precipitation
- Sedimentation (rare)
- Recombinant gene product over-expression
- Refractile body prep (see previous lecture)
- Detergent extraction
- Heat treatment (especially for recombinant thermophile proteins expressed in \textit{E. coli})
- Etc.