Chromatofocusing

High resolution and high capacity

The pH-gradient is created by an equilibration buffer (high pH) which is later replaced by a chromatofocusing buffer (anion-exchange),

The stationary phase has to include charged groups that are not affected in the used pH-interval.

Separation based on isoelectric point (pI)
Precipitation- a problem

- Most proteins have their lowest solubility close to their pI value, where the electrostatic repulsion between the molecules is at a minimum.

- Isoelectric precipitation is counteracted by the fact that proteins need to have a slight net-charge at when they are adsorbed to the matrix.

- Higher ionic strength helps protein solubility but requires higher charge for efficient adsorption.

- Urea or detergents can be included in the buffer.

Covalent chromatography

Can be used when the target protein has a free cystein.

1. S-S \overset{\text{SH}}{\rightarrow} P-SH
   - Activation of the stationary phase by 2,3-diprydyl disulphide

2. S-S \overset{\text{P}}{\rightarrow} R-SH
   - Reduce the target protein
   - Bind to the gel, batchwise
   - Wash with 0.1-0.3 M NaCl

3. S-S \overset{\text{P}}{\rightarrow} R-SH
   - Elute with a reducing agent, usually 10-25mM DTT
   - Thiopyrtyl is released more easily than the protein, gradient or step-elution

4. S\overset{\text{SH}}{\rightarrow} P\overset{\text{SH}}{\rightarrow} R-S-S-R
   - Gelfiltration
Affinity chromatography

- Concentration and purification in the same unit operation
- Often very selective
- The binding has to be reversible
- Good first step in a purification process

**Pros:**
- High specificity
- Fewer purification steps
- Counterligand with a low concentration can be purified

**Cons:**
- Expensive stationary phases
- Few available ligands
- Protein ligands have low stability
- Harsh elution conditions
Examples of ligands

<table>
<thead>
<tr>
<th>Ligand</th>
<th>Counterligand</th>
</tr>
</thead>
<tbody>
<tr>
<td>Antibody</td>
<td>Antigen, Cell, Virus</td>
</tr>
<tr>
<td>Enzyme inhibitors</td>
<td>Enzymes</td>
</tr>
<tr>
<td>Lectin</td>
<td>Polysaccarides, Glycoproteins</td>
</tr>
<tr>
<td>Nucleic acids</td>
<td>Proteins binding to nucleic acids</td>
</tr>
<tr>
<td>Hormones, Vitamins</td>
<td>Receptors, binding proteins</td>
</tr>
<tr>
<td>Sugars</td>
<td>Lectin, Sugar-binding proteins</td>
</tr>
</tbody>
</table>

Properties of the ligand

- Ability to form a reversible complex with the target protein
- Specificity should be high
- Affinity has to be sufficiently high for the target protein \((K_a \approx 10^5-10^6)\)
- It should be possible to break the complex without damage to the target protein.
- It should be possible to bind the ligand to a matrix.
The matrix

• Has to contain active groups that the ligand can be coupled to.

• Maintain stability during the whole chromatographic cycle.

• Should not interact with the molecules in the starting material.

• Should tolerate high flow-rates.

• Macroporous to allow large molecules to diffuse into the pores.

Coupling chemistry

• NHS-activated matrix:
  • -NH₂

• CNBr-activated matrix:
  • -NH₂

• Aktivated CH matrix:
  • -NH₂

• Epoxiactivated matrix:
  • -OH
  • -SH (tioeter)
  • -NH₂

• Thiolpropylmatrix:
  • -SH (disulfid)

• EAH-activated matrix:
  • -COOH

If the ligand is small, a “spacer-arm” may be required to get it out from the matrix surface.
Group-specific affinity ligands

**Lectines:** used to purify glycosulated proteins. There are different lectines which are specific for different types of sugar (glucose, mannose, galactose…).

**Protein A & protein G:** used for purification of antibodies. Different affinity for antibodies from different species and classes.

**Heparin:** Used for purification of coagulation proteins and also DNA-binding enzymes.

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Purification

**Pre-fractionation:**
- Remove solid particles and cell-debris (centrifugation/filtration)
- Set a pH value, ionic strengh (gelfiltration/dialysis)

**Binding – washing - elution:**
- Normally a short and wide column $\rightarrow$ quick separation
  - If the affinity is low, the target protein is only slowed. A longer column is required.
- Batchwise binding is sometimes used (elution)
- If binding kinetics is slow a low flow-rate is required (stopped flow).
- Washing with high ionic strength and including detergent to minimize non-specific binding.
- Low elution flow-rate gives a higher resolution and a more concentrated protein.
- Gradient elution can be used if there are several binders.
- Elution can be achieved by changing the pH up or down, to ionize residues responsible for binding.
- Sometimes a high salt solution is used for elution.
- Competitive elution, low flow-rate, otherwise broad peaks, mild elution procedure.
Regeneration

- In the lab, the usual procedure is to re-equilibrate the column with starting buffer.

- In industrial procedures a solution containing 0.1-1 M NaOH is usually used to clean the column. This is usually a problem since many protein ligands cannot withstand the high pH-value.

Purification of target proteins

Gene-fusiones

- General method for many target proteins
- Fusion systems are available commercially
- The purification tag can affect the target protein

Native target protein

- Purification of the native protein
- Affinity ligand may not be available
Examples of commonly used purification tags

<table>
<thead>
<tr>
<th>Tag</th>
<th>Molecular Weight</th>
<th>Functionality</th>
<th>Elution Method</th>
</tr>
</thead>
<tbody>
<tr>
<td>ProteinA</td>
<td>31 kDa</td>
<td>binds IgG</td>
<td>elution by low pH</td>
</tr>
<tr>
<td>Z</td>
<td>7 kDa</td>
<td>binds IgG</td>
<td>elution by low pH</td>
</tr>
<tr>
<td>ABP</td>
<td>5-25 kDa</td>
<td>binds HSA</td>
<td>elution by low pH</td>
</tr>
<tr>
<td>GST</td>
<td>25 kDa</td>
<td>binds glutathione</td>
<td>elution by low pH</td>
</tr>
<tr>
<td>His6</td>
<td>6 aa</td>
<td>binds metal ions</td>
<td>elution by low pH or imidazole</td>
</tr>
<tr>
<td>Biotin</td>
<td>13 kDa</td>
<td>binds avidine</td>
<td>elution by biotin</td>
</tr>
<tr>
<td>Flag-peptide</td>
<td>8 aa</td>
<td>binds a monoclonal antibody</td>
<td>elution by low pH or EDTA</td>
</tr>
</tbody>
</table>

Immobilized Metal Ion Affinity Chromatography, IMAC

- Metallbinding amino acids (His, Cys, Trp) has to be exposed on the surface.
- The distribution of the amino acids is important.
- Ion-ion-interactions can be inhibited by the right choice of buffer (high salt).
- pH-dependent (adsorption at high pH, desorption at low pH)
- Several ways to elute has been reported (pH, competitive elution, organic solvents, chelating substances)
Choice of metal-ion when the amino acid content on the surface of the target protein is known

<table>
<thead>
<tr>
<th>Content of His/Trp on the surface</th>
<th>Metal ions able to Adsorb</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 His</td>
<td>Cu²⁺</td>
</tr>
<tr>
<td>&gt;1 His</td>
<td>Cu²⁺, Ni²⁺</td>
</tr>
<tr>
<td>Cluster of His</td>
<td>Cu²⁺, Ni²⁺, Zn²⁺, Co²⁺</td>
</tr>
<tr>
<td>Several Trp</td>
<td>Cu²⁺</td>
</tr>
</tbody>
</table>

Purification tag, His₆

• Genetic fusion between the target protein and a His₆-tag
• Very common when producing recombinant proteins
• Simple, rather specific purification with is not dependent on the target protein.
• Possibility to purify under denaturing conditions. This is advantageous when the protein is produced as inclusion bodies in the host or when it has a poor solubility.
• Elution by low pH or imidazole (His)
Expanded bed adsorption (EBA)

- Minimize the number of unit operations in a separation process.
- The material is applied directly from the cultivation to the column. Centrifugation/filtration can be avoided.

Possibilities

- Cultivation
  - Centrifugation
    - Cultivation
      - Column chromatography
      - Chromatography Fluidized bed
      - Chromatography EBA
Expanded bed adsorption (EBA)

**Operation**

- The bed is expanded by pumping buffer from the bottom of the column.
- The top adaptor is adjusted slightly over the top of the bed.
- The cell suspension with the target protein is applied. The bed is usually expanded some more.
- Washing with buffer until the absorption of the washing solution emerging from the top of the column is decreased to 0.
- The flow is reversed and the top adaptor is lowered.
- Elution from a packed bed (usually)
- CIP, Cleaning In Place, often NaOH and NaCl over night.

Cultivation

First purification step

EBA
Expansion

- The flow has to be even, the adaptors clean and free of air-bubbles, most important in the bottom.
- Column has to be vertical
- Cell debris should pass through the column but the stationary phase should remain.
- Different size of the particles in the stationary phase (50-400 µm) and density (1.1-1.3 g/cm³) for an even distribution in the column.

Stationary phase

High density is necessary to keep the stationary phase in the column
High flow-rate → not too short contact time for the target protein.

- Agarose with a quartz core to increase the density is the most common matrix material
Feed-stock/Raw material

- DNA-content gives high viscosity $\Rightarrow$ unstable bed.

- An-ion exchange $\Rightarrow$ positive charges on the stationary phase and negatively charged DNA can give rise to canals in the column.

Stationary phase, things to consider

- Lots of proteases in the feed-stock applied to the column.

- CIP neccessary

- Group selective ligands with high stability is often used. EBA is usually (should be) an early purification step and high selectivity is therefore not as important.
Column cleaning-Sanitation

- Important when loading whole cells or parts of cells on the column.
- 0.5M NaOH for at least 1 h.
- CIP, Cleaning In Place
- Ion-exchangers and HIC stationary phases can withstand this treatment, but it may cause problems for affinity stationary phases with protein ligands.

(Example)
Electrostatic potentials of basic variants

- Red-negative potential
- Blue-positive potential
(Example)
Fusion proteins for purification by ion-exchange chromatography

<table>
<thead>
<tr>
<th>Protein</th>
<th>Molecular Weight</th>
<th>Calculated pI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Klenow</td>
<td>68215.9</td>
<td>5.65</td>
</tr>
<tr>
<td>Z_{basic2}-Klenow</td>
<td>77050.9</td>
<td>6.19</td>
</tr>
</tbody>
</table>

(Example)
Cation-exchange chromatography of Z_{basic2}-Klenow polymerase