Protein Purification
Learning Objectives

1. How Do We Extract Pure Proteins from Cells?

2. What Is Column Chromatography?

3. What Is Electrophoresis?

4. How Do We Determine the Primary Structure of a Protein?
1. How Do We Extract Pure Proteins from Cells?

Disruption of cells is the first step in protein purification. The various parts of cells can be separated by centrifugation. This is a useful step because proteins tend to occur in given organelles. High salt concentrations will precipitate groups of proteins, which are then further separated by chromatography and electrophoresis.
Tissue-sucrose homogenate (minced tissue + 0.25 M sucrose buffer)

Centrifuge homogenate at 600 g \times 10 \text{ min}

Supernatant 1

Nuclei and any unbroken cells

Centrifuge supernatant 1 at 15,000 g \times 5 \text{ min}

Supernatant 2

Mitochondria, lysosomes, and microbodies

Centrifuge supernatant 2 at 100,000 g \times 60 \text{ min}

Supernatant 3: Soluble fraction of cytoplasm (cytosol)

Ribosomes and microsomes, consisting of endoplasmic reticulum, Golgi, and plasma-membrane fragments
Dialysis.

Protein molecules (red) are retained within the dialysis bag, whereas small molecules (blue) diffuse.
2. What Is Column Chromatography?

Two of the most important methods for separating amino acids, peptides, and proteins are chromatography and electrophoresis. The various forms of chromatography rely on differences in charge, polarity, or size of the molecules to be separated, depending on the application.
Partition molecules between two phases, one **mobile** and the other **stationary**. For separation of amino acids or sugars, the stationary phase, or matrix, may be a sheet of filter paper (paper chromatography) or a thin layer of cellulose, silica, or alumina (thin-layer chromatography);
Gel-Filtration Chromatography

Reservoir containing the eluent (the mobile phase)

Sample

Column packed with stationary phase in contact with eluent throughout its length

Time
As the eluent flows through the column, compounds of the sample migrate at different rates

Time
Three zones are being separated
Elution continues

Effluent is collected manually or automatically and analyzed for the presence (and sometimes the amount) of solute

The fastest moving substance eluted from column

Fig. 5-2, p.116
Agarose

3,6-anhydro bridge
Fig. 5-5a, p.118

(a)

Small molecule

Large molecule

Porous gel beads

The small molecules enter the pores in the beads. Large molecules go around the beads.

The large molecules are separated from the small ones.
Elution profile of a large macromolecule

A smaller macromolecule

Protein concentration

Volume (mL)
Affinity Chromatography

Column with substance S covalently bonded to supporting material

Sample containing mixture of proteins

Substance S

P₁ molecules (▲) bind to S

Rest of proteins (P₂, P₃) (●) eluted

Add high concentration of S to eluent

P₁ is eluted from column

Fig. 5-6, p.118
Affinity Chromatography

Affinity chromatography of concanavalin A (shown in yellow) on a solid support containing covalently attached glucose residues (G).

The plant protein concanavalin A can be purified by passing a crude extract through a column of beads containing covalently attached glucose residues. Concanavalin A binds to such a column because it has affinity for glucose, whereas most other proteins do not. The bound concanavalin A can then be released from the column by adding a concentrated solution of glucose.
Ion-Exchange Chromatography.

This technique separates proteins mainly according to their net charge.
(a) Cation-Exchange Media

**Strongly acidic: polystyrene resin (Dowex-50)**

![Chemical structure of Dowex-50](structure1)

**Weakly acidic: carboxymethyl (CM) cellulose**

![Chemical structure of CM cellulose](structure2)

**Weakly acidic, chelating: polystyrene resin (Chelex-100)**

![Chemical structure of Chelex-100](structure3)
(b) Anion-Exchange Media

<table>
<thead>
<tr>
<th>Strongly basic: polystyrene resin (Dowex-1)</th>
<th>![Chemical Structure]</th>
</tr>
</thead>
</table>

<table>
<thead>
<tr>
<th>Weakly basic: diethylaminoethyl (DEAE) cellulose</th>
<th>![Chemical Structure]</th>
</tr>
</thead>
</table>

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3. What Is Electrophoresis?

- **Electrophoresis**: the process of separating compounds on the basis of their electric charge & size
  - electrophoresis of amino acids can be carried out using paper, starch, agar, certain plastics, and cellulose acetate as solid supports
  - in paper electrophoresis, a paper strip saturated with an aqueous buffer of predetermined pH serves as a bridge between two electrode vessels.
- A sample of amino acids is applied as a spot (the origin) on the solid support strip.
- An electric potential is applied to the electrode vessels and amino acids migrate toward the electrode with charge opposite their own.
- Molecules with a high charge density move faster than those with a low charge density.
- Molecules at their isoelectric point remain at the origin.
- After separation is complete, the strip is dried and developed to make the separated amino acids visible.
Polyacrylamide Gel Electrophoresis.
Electrophoretic Analysis of a Protein Purification.
Two-Dimensional Gel Electrophoresis.
4. How Do We Determine the Primary Structure of a Protein?

Determination of the N-terminal and C-terminal amino acids of proteins depends on the use of these separation methods after the ends of the molecule have been chemically labeled. Selective cleavage of the protein into peptides by enzymatic or chemical hydrolysis produces fragments of manageable size for sequencing.
Highly purified protein is essential for determination of its amino acid sequence. Cells contain thousands of different proteins, each in widely varying amounts. The isolation of a specific protein in quantities sufficient for analysis presents a challenge that may require multiple successive purification techniques.
<table>
<thead>
<tr>
<th>Fraction</th>
<th>Volume (mL)</th>
<th>Total Protein (mg)</th>
<th>Total Activity</th>
<th>Specific Activity</th>
<th>Percent Recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Crude extract</td>
<td>3,800</td>
<td>22,800</td>
<td>2,460</td>
<td>0.108</td>
<td>100</td>
</tr>
<tr>
<td>2. Salt precipitate</td>
<td>165</td>
<td>2,800</td>
<td>1,190</td>
<td>0.425</td>
<td>48</td>
</tr>
<tr>
<td>3. Ion-exchange chromatography</td>
<td>65</td>
<td>100</td>
<td>720</td>
<td>7.2</td>
<td>29</td>
</tr>
<tr>
<td>4. Molecular-sieve chromatography</td>
<td>40</td>
<td>14.5</td>
<td>555</td>
<td>38.3</td>
<td>23</td>
</tr>
<tr>
<td>5. Immunoaffinity chromatography</td>
<td>6</td>
<td>1.8</td>
<td>275</td>
<td>152.108</td>
<td>11</td>
</tr>
</tbody>
</table>

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Table 5-1, p.114
molecular weights of sample proteins

<table>
<thead>
<tr>
<th>Protein</th>
<th>S value (Svedberg units)</th>
<th>Molecular weight</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pancreatic trypsin inhibitor</td>
<td>1</td>
<td>6,520</td>
</tr>
<tr>
<td>Cytochrome c</td>
<td>1.83</td>
<td>12,310</td>
</tr>
<tr>
<td>Ribonuclease A</td>
<td>1.78</td>
<td>13,690</td>
</tr>
<tr>
<td>Myoglobin</td>
<td>1.97</td>
<td>17,800</td>
</tr>
<tr>
<td>Trypsin</td>
<td>2.5</td>
<td>23,200</td>
</tr>
<tr>
<td>Carbonic anhydrase</td>
<td>3.23</td>
<td>28,800</td>
</tr>
<tr>
<td>Concanavlin A</td>
<td>3.8</td>
<td>51,260</td>
</tr>
<tr>
<td>Malate dehydrogenase</td>
<td>5.76</td>
<td>74,900</td>
</tr>
<tr>
<td>Lactate dehydrogenase</td>
<td>7.54</td>
<td>146,200</td>
</tr>
</tbody>
</table>
How Do We Determine the Primary Structure of a Protein?
**Figure 5-14, p.123**

**Step 1**
Sample 1

Hydrolyze to constituent amino acids

Separate and identify individual amino acids

**Step 2**
Sample 2

Specific reagents

Identify N-terminal and C-terminal amino acids

**Step 3**
Sample 3

Cleave protein at specific sites

Determine sequence of smaller peptides

**Step 4**
Sample 4

Cleave protein at specific sites other than those in sample 3

Determine sequence of smaller peptides

Combine information from overlapping peptides to get complete sequence
Chemical Cleavage
Cyanogen bromide

OVERALL REACTION:

Polypeptide $\xrightarrow{\text{BrCN}}$ Peptide with C-terminal homoserine lactone

$\xrightarrow{\text{HCOOH}}$ 70% $\xrightarrow{\text{H}_3^+N}$ Peptide (C-terminal peptide)
Edman reagent is phenylisothiocyanate

EDMAN DEGRADATION

 Ala — Gly — Phe — Asp — Asn
      ↓                      ↓ labeling amino-teminal residue
 Ala — Gly — Phe — Asp — Asn
      ↓                      ↓ removing the 1st residue
  Ala — Gly — Phe — Asp — Asn
      ↓                      ↓ lableing the 2nd residue
  Gly — Phe — Asp — Asn
      ↓                      ↓ removing the 2nd residue
   Gly — Phe — Asp — Asn
## Specific cleavage of polypeptides

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Cleavage site</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Chemical cleavage</strong></td>
<td></td>
</tr>
<tr>
<td>Cyanogen bromide</td>
<td>Carboxyl side of methionine residues</td>
</tr>
<tr>
<td><strong>Enzymatic cleavage</strong></td>
<td></td>
</tr>
<tr>
<td>Trypsin</td>
<td>Carboxyl side of lysine and arginine residues</td>
</tr>
<tr>
<td>Thrombin</td>
<td>Carboxyl side of arginine</td>
</tr>
<tr>
<td>Chymotrypsin</td>
<td>Carboxyl side of tyrosine, tryptophan, phenylalanine, leucine, and methionine</td>
</tr>
<tr>
<td>Carboxypeptidase A</td>
<td>Amino side of C-terminal amino acid (not arginine, lysine, or proline)</td>
</tr>
</tbody>
</table>
Chapter 5