An understanding of the proteome is acquired by isolating, characterizing and cataloging proteins. In some, but not all, cases, this process begins by separating a particular protein from all other biomolecules in the cell.
**PROTEIN PURIFICATION**

- Need to be able to separate the protein of interest from the rest of the cellular components after lysing the cells
- Most proteins are soluble, first separation step is centrifugation
- The crude extract is assayed to determine if protein of interest is present before further purification begins

**PNPA Assay for PAF-AH**

**PROTEIN PURIFICATION**

- Proteins are separated on the basis of differences in solubility, size, charge and specific binding affinity.

1. **Separation by Solubility**
   - Salting out – high salt concentration causes proteins to become insoluble in solution; degrades protein & not desirable
   - Dialysis – semi-permeable membrane used to adjust the concentration of salt in the protein solution.
PROTEIN PURIFICATION

1. Separation by Size
   - Gel Filtration Chromatography
     - Column filled with polymer beads that allow proteins to pass through
     - Small molecules enter and get trapped in the beads, moving slower
     - Large molecules can’t enter the packed column and follow a shorter path to the bottom emerging first

![Diagram of Gel Filtration Chromatography]

PROTEIN PURIFICATION

1. Separation by Charge
   - Ion-exchange Chromatography
     - Positively charged proteins will bind to a column that contains negatively charged beads, and vice versa
     - Proteins are then eluted from the column with a salt buffer

![Diagram of Ion-exchange Chromatography]
PROTEIN PURIFICATION

1. Separation by Specific binding affinity
   - Affinity Chromatography
     - Takes advantage of proteins that have high affinity for specific chemical groups or specific molecules
     - Protein of interest will bind and the rest flow through
     - Elute protein of interest off column

PROTEIN PURIFICATION

- High-pressure liquid chromatography (HPLC)
  - Improves resolving power of purification
  - Very fine beads used in column
  - Pressure is applied to obtain adequate flow rates
  - Gives high resolution and rapid protein separation

Gel filtration by HPLC clearly defines the individual proteins because of its greater resolving power: (1) thyroglobulin (669 kd), (2) catalase (232 kd), (3) bovine serum albumin (67 kd), (4) ovalbumin (43 kd), and (5) ribonuclease (13.4 kd).
**PROTEIN PURIFICATION**

- **Polyacrylamide Gel Electrophoresis (PAGE)**
  - A way to visualize the number of proteins present during each step of purification.
  - Gels are run from negative to positive.
  - Smaller proteins will move through the gel faster and larger proteins will be stuck at the top.

- **SDS PAGE**
  - All proteins are negatively charged, reduced and denatured.

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**SDS PAGE**

Graphic depicting the process of electrophoresis with SDS-PAGE, showing the separation of proteins based on their molecular weight.
PROTEIN PURIFICATION

- Isoelectric Focusing
  - Proteins separated by electric charge
  - Uses an acrylamide gel with a pH gradient
  - Can separate proteins that differ by 1 net charge

- Two-dimensional Electrophoresis
  - Isoelectric focusing first, then SDS PAGE
  - Horizontally separated by charge and vertically separated by mass
  - Can be used to determine differences in healthy vs disease state
**IMMUNOLOGICAL TECHNIQUES**

- Can test the presence of proteins that lack enzyme activity- ex Estrogen receptor
- Estrogen receptor is known to bind estradiol
  - Exploit this property with radiolabeling and gradient centrifugation

- **Gradient Centrifugation**
  - Quantify the rate of movement by calculating the sedimentation coefficient
    \[ s = \frac{m(1-v\rho)}{f} \]
  - \( s \) is sedimentation coefficient, \( m \) is mass of particle, \( v \) is partial specific volume, \( \rho \) is the density and \( f \) is the frictional coefficient. \((1-v\rho)\) is the buoyant force exerted by the medium

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<table>
<thead>
<tr>
<th>Protein</th>
<th>S value (Svedberg units)</th>
<th>Molecular weight</th>
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</thead>
<tbody>
<tr>
<td>Pancreatic trypsin inhibitor</td>
<td>1</td>
<td>6,520</td>
</tr>
<tr>
<td>Cytochrome c</td>
<td>1.80</td>
<td>12,330</td>
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<tr>
<td>Ribonuclease A</td>
<td>1.78</td>
<td>13,680</td>
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<tr>
<td>Myoglobin</td>
<td>1.97</td>
<td>17,600</td>
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<tr>
<td>Trypsin</td>
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<tr>
<td>Carbonic anhydrase</td>
<td>3.35</td>
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<tr>
<td>Concanavalin A</td>
<td>3.8</td>
<td>51,260</td>
</tr>
<tr>
<td>Malate dehydrogenase</td>
<td>5.76</td>
<td>74,600</td>
</tr>
<tr>
<td>Lactate dehydrogenase</td>
<td>7.54</td>
<td>146,200</td>
</tr>
</tbody>
</table>
IMMUNOLOGICAL TECHNIQUES

- Gradient Centrifugation Provides and Assay for the Estradiol-Receptor Complex

The steps are as follows: (A) form a density gradient, (B) layer the sample on top of the gradient, (C) place the tube in the rotor and centrifuge it, and (D) collect the samples.

- Collects a fraction containing the complex, but also other proteins with similar S values

IMMUNOLOGICAL TECHNIQUES

- Since the fraction from gradient centrifugation is impure, we need to consider the receptors immunological properties to isolate it.
- Need to generate an antibody for the protein
  - Antibodies have specific and high affinity for the antigens that elicited their synthesis.
  - Proteins, polysaccharides, and nucleic acids can be effective antigens.
  - An antibody recognizes a specific group or cluster of amino acids on a large molecule called an antigenic determinant, or epitope.
IMMUNOLOGICAL TECHNIQUES

- Antigen-Antibody interactions

A protein antigen, in this case lysozyme, binds to the end of an F\textsubscript{ab} domain from an antibody. The end of the antibody and the antigen have complementary shapes, allowing a large amount of surface to be buried on binding.

IMMUNOLOGICAL TECHNIQUES

- Depend on our being able to generate antibodies to a specific antigen.
- To obtain antibodies that recognize a particular protein, a biochemist injects the protein into a rabbit twice, 3 weeks apart.
- The injected protein stimulates the reproduction of cells producing antibodies that recognize the foreign substance.
- Blood is drawn from the immunized rabbit several weeks later and centrifuged to separate blood cells from the supernatant, or serum, which contains the antibodies.
**IMMUNOLOGICAL TECHNIQUES**

- Antibodies of a given specificity are not a single molecular species.
  - 2,4- dinitrophenol (DNP) has been used to generate antibodies to DNP.
  - Anti-DNP antibodies revealed a wide range of binding affinities.
  - Many bands were evident in isoelectric focusing.
- **These results indicate that cells are producing many different antibodies, each recognizing a different surface feature of the same antigen.**
  - The antibodies are heterogeneous, or *polyclonal*.
  - This heterogeneity is a barrier, which can complicate the use of these antibodies.

**IMMUNOLOGICAL TECHNIQUES**

- Just as working with impure proteins makes it difficult to interpret data and understand function, so too does working with an impure mixture of antibodies.
- The ideal would be to isolate a clone of cells that produce only a single antibody, or *monoclonal antibodies*.
- Monoclonal antibodies are all identical, produced by clones of a single antibody-producing cell. They recognize one specific epitope.
Cesar Milstein and Georges Köhler discovered that large amounts of homogeneous antibody of nearly any desired specificity could be obtained by fusing a short-lived antibody-producing cell with an immortal myeloma cell.

Now that we have antibody for the estrogen receptor, we can purify it a number of ways

1. Immunoprecipitation
IMMUNOLOGICAL TECHNIQUES

1. Immunoprecipitation

2. Enzyme-Linked Immunosorbent Assay (ELISA)
   - An enzyme, which reacts with a colorless substrate to produce a colored product, is covalently linked to a specific antibody that recognizes a target antigen.
   - If the antigen is present, the antibody-enzyme complex will bind to it, and the enzyme component of the antibody-enzyme complex will catalyze the reaction generating the colored product.
   - Thus, the presence of the colored product indicates the presence of the antigen.
   - Rapid, convenient, can detect low levels of protein and can use polyclonal or monoclonal antibodies
IMMUNOLOGICAL TECHNIQUES

1. Immunoprecipitation
2. Enzyme-Linked Immunosorbent Assay (ELISA)
3. Western Blotting
   - Very small quantities of a protein of interest in a cell or in body fluid can be detected
   - The sample is resolved via SDS PAGE
   - Blotting transfers the proteins on the gel to the surface of a membrane to make them more accessible.
   - Antibody is added to the membrane sheet and reacts with the antigen.
   - The antibody-antigen complex on the sheet then can be detected by rinsing the sheet with a second antibody specific for the first (e.g., goat antibody that recognizes mouse antibody).
   - The film is developed
   - Western blotting makes it possible to find a protein in a complex mixture, the proverbial needle in a haystack.
Once a protein has been purified, the next step is characterization. Start with determining the primary structure, or amino acid sequence:

\[
\text{Ala – Gly – Asp – Phe – Arg – Gly}
\]

Determine the amino acid composition:

1. The peptide is hydrolyzed into its constituent amino acids by heating it in 6 N HCl at 110°C for 24 hours.

Amino acids can be separated by ion-exchange chromatography.

The identity of the amino acid is revealed by its elution volume and quantified by reaction with ninhydrin. Amino acids treated with ninhydrin give an intense blue color, except for proline, which gives a yellow color because it contains a secondary amino group.

The concentration of an amino acid in a solution, after heating with ninhydrin, is proportional to the optical absorbance of the solution.

A comparison of the chromatographic patterns of our sample with that of a standard mixture of amino acids would show that the amino acid composition of the peptide is
**PROTEIN STRUCTURE AND FUNCTION**

- Determine the amino acid composition

![Graph showing elution profile of peptide hydrolysate and standard amino acids](image)

(Asp – Gly₂ – Ala – Phe – Arg)

**PROTEIN STRUCTURE AND FUNCTION**

- Determine the amino acid sequence with Edmond degradation
  - Sequentially removes one residue at a time from the N-term of the peptide
  - Phenyl isothiocyanate reacts with the N-term amino acid, which then cyclizes and breaks off the peptide, making the peptide one amino acid shorter
  - The cyclic compound produced is a PHT-amino acid that can be identified with chromatographic procedures
  - The Edman procedure is repeated sequentially to determine the sequence
PROTEIN STRUCTURE AND FUNCTION

- Determine the amino acid sequence with Edmond degradation

Edmond degradation is only useful for peptides up to 50 amino acids – proteins are much larger.

- Use specific cleavage to “divide and conquer”

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Cleavage site</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chemical cleavage</td>
<td></td>
</tr>
<tr>
<td>Cyanogen bromide</td>
<td>Carboxyl side of methionine residues</td>
</tr>
<tr>
<td>O-benzoxyphenylacetate</td>
<td>Carboxyl side of tryptophan residues</td>
</tr>
<tr>
<td>Hydrazinolysis</td>
<td>Ammonolysis of glycine bonds</td>
</tr>
<tr>
<td>2,4-Dinitrofluorobenzene</td>
<td>Amino side of cysteine residues</td>
</tr>
<tr>
<td>Enzymatic cleavage</td>
<td></td>
</tr>
<tr>
<td>Trypsin</td>
<td>Carboxyl side of lysine and arginine residues</td>
</tr>
<tr>
<td>Chortopain</td>
<td>Carboxyl side of arginine residues</td>
</tr>
<tr>
<td>Staphylococcal proteinase</td>
<td>Carboxyl side of serine and glutamine residues (glutamine only under certain conditions)</td>
</tr>
<tr>
<td>Thermolysin</td>
<td>Carboxyl side of arginine</td>
</tr>
<tr>
<td>Chymotrypsin</td>
<td>Carboxyl side of tyrosine, tryptoph, 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>

Tryptic peptide: Ala — Ala — Trp — Gly — Lys
Chymotryptic peptide: Val — Lys — Ala — Ala — Trp

Thrombin: Thr — Phe — Val — Lys — Ala — Ala — Trp — Gly — Lys

Chymotryptic overlap peptide
PROTEIN STRUCTURE AND FUNCTION

Insight from Amino Acid Sequences

1. The sequence of a protein of interest can be compared with all other known sequences to determine if similarities exist. Does the protein belong to an established family?

2. Comparison of sequences of the same protein in different species yields a wealth of information about evolutionary pathways.

3. Amino acid sequences can be searched for the presence of internal repeats.

4. Many proteins contain amino acid sequences that serve as signals designating their destinations or controlling their processing.

5. Sequence data allows for a molecular understanding of diseases.

6. Proteins sequences served as a guide to nucleic acid information.
SUMMARY

- The proteome is a functional representation of the genome
- The purification of proteins is the first step in understanding their function
- Immunological techniques are used to purify and characterize proteins
- Determination of primary structure facilitates an understanding of protein function

DON’T HAVE THE SECOND EDITION?

1. Protein Purification

2. Immunological Techniques

3. Protein Structure & Function