Done By: -

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Mariam Ababneh
Protein Purification & Characterizing Techniques

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.

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.

3. What Is Electrophoresis?

In electrophoresis, differences in charge and in size are the criteria for separation. The sieving action of gel slabs is used in conjunction with the charge on proteins to achieve separation. The electrophoretic mobilities of proteins can be used to estimate their molecular weights.

- Column in different sizes
- mobile phase, stationary phase
- charge, polarity and size of molecule
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.

**PROTEINS & PEPTIDES MUST BE PURIFIED PRIOR TO ANALYSIS**

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.

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**Table 5.1**

Example of a Protein Purification Scheme: Purification of the Enzyme Xanthine Dehydrogenase from a Fungus

<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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**هذا الجدول مهم**

* percent recovery = 100 × percent recovery

* buffer

* crushing

* كاملاً
Protein purification

2. The volume of the clear solution decreased.

***

Specific activity = total activity / total protein

Benzidines are highly purified if they contain the smallest amount of protein and highest specific activity.

Highly purified enzymes have the highest amount of specific activity.

Dialysis.

Protein molecules (red) are retained within the dialysis bag, whereas small molecules (blue) diffuse.

Dialysis bags are semipermeable membranes that separate protein according to their size. Small molecules will pass through the membrane, whereas large molecules will be trapped inside.

Centrifugation uses different speeds, sizes, and types of centrifuges, such as ultracentrifugation (one million gravity) and small and refrigerated. Separation of cells is achieved where precipitation will take place.
Protamine purification

Nuclei and broken cell centrifugation at \( 600 \text{G} \) for 10 min, obtaining supernatant 1. Use electrophoresis and always follow centrifugation and settle mitochondria, lysosomes, and microbodies. Supernatant 2 is obtained by centrifugation at \( 100,000 \text{G} \) for 24 hours, sedimenting ribosome, Golgi apparatus, ER, plasma membrane, and others. We can perform centrifugation for 22 hours to sediment the smallest particles of the cell. supernatant and precipitation are examined by the electron microscope and their components are observed.
Protein purification

2 phases:
- 1 stationary phase -powder we will put it in column
- 2Mobile phase-sample

We will put the sample in a column. The stationary phase will be a powder that we put in the column. The mobile phase will be the sample.

We will mix the sample with ionized water to create a solution. We will then add buffer to the solution to create a homogenate. We will then add the homogenate to the column.

We will then add buffer continuously to the column. We will then collect the fractions. We will then take the collection and continue the process.

Agarose - sugar

Agarose is a type of substance that we can use to add to the column. It helps to separate the substances.

Gel filtration-size exclusion chromatography.

The small molecules enter the pores in the beads. The large molecules go around the beads. The large molecules are separated from the small ones.
Protein purification

Small molecule (beads) will enter into the beads of column, large molecule will not enter into beads.

Affinity Chromatography

Column with substance S (supporting material)

The column has substance and already we put in it another substance

p1, p2, and p3
**Protein purification**

**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.
column charged positively or negatively

positive beads (a) Cation-Exchange Media

(b) Add mixture of Asp, Ser, Lys
(c) Add Na⁺ (NaCl)
(d) Serine is eluted next
(e) Lysine, the most positively charged amino acid, is eluted last

<table>
<thead>
<tr>
<th>(a)</th>
<th>Structure</th>
</tr>
</thead>
<tbody>
<tr>
<td>Strongly acidic: polystyrene resin (Dowex-50)</td>
<td><img src="image" alt="Strongly acidic structure" /></td>
</tr>
<tr>
<td>Weakly acidic: carboxymethyl (CM) cellulose</td>
<td><img src="image" alt="Weakly acidic structure" /></td>
</tr>
<tr>
<td>Weakly acidic, chelating: polystyrene resin (Chelex-100)</td>
<td><img src="image" alt="Weakly acidic, chelating structure" /></td>
</tr>
</tbody>
</table>
Protein purification

(b) Anion-Exchange Media

<table>
<thead>
<tr>
<th>Structure</th>
<th>CH₃</th>
</tr>
</thead>
<tbody>
<tr>
<td>CH₂N⁺CH₃</td>
<td></td>
</tr>
<tr>
<td>CH₃</td>
<td></td>
</tr>
</tbody>
</table>

| Strongly basic: polystyrene resin (Dowex-1) |

| Weakly basic: diethylaminoethyl (DEAE) cellulose |

CH₂CH₂N⁺H |
| CH₃      |

Prot ein purification

لائم يمر بكل الخطوات مرة و مرتين وثلاث لحتى نحصل على protein purification نحصل على ال protein with charge نعمل لحتى نحصل على الانزيمات الغالبة جدا التي نستخدمها في ال purification لنتعرف على اشياء محددة for tumor slides for histology and pathology

Page 10
Chromatographic Separations

- 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)

Electrophoresis

- Is 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 (positive and negative)

- 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

https://www.youtube.com/watch?v=yosecfE980k
Protein purification

This is the Polyacrylamide Gel Electrophoresis. This is also the ventricle Electrophoresis. This is the buffer. This is the mixture of macromolecules. This is the direction of movement from the top to the bottom in different levels.

https://www.youtube.com/watch?v=vtxb6Tr8Y3s

https://www.youtube.com/watch?v=eaETFKXtNRA
Prot  

**Electrophoretic Analysis of a Protein Purification**

![Graph showing electrophoretic mobility vs. log molecular weight]

With the completion of the gel, the separation is achieved. The gel is then dried and the solution is stained, which allows the visualization of the separated proteins. The unknown standard is compared to the homogenate and the remaining standard is compared to the un-separated proteins. The analyses are performed under different conditions, and initially, the work is done on the proteins that are known. Afterward, the more subtle differentiations are carried out, leading to the use of affinity chromatography. The mobility of the proteins is lower, implying a higher molecular weight.
2 Dimensional gel electrophoresis

By adding a buffer and separating the proteins, gel electrophoresis is a technique used to separate proteins based on their size. Another separation technique is SDS-Polyacrylamide slab gel electrophoresis, which is used to determine the 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</td>
<td>1</td>
<td>6,520</td>
</tr>
<tr>
<td>inhibitor</td>
<td></td>
<td></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>Concanavalin 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?**

A simple protein needs electrophoresis to work the unknown molecular weight to the unknown. This gives us a clue about the protein's structure and then allows us to determine the general characteristics. How do we determine the primary structure of a protein?

- **Polypeptide linkage** of proteins is by an enzyme called both trypsin and chymotrypsin. Each of these enzymes acts on a specific amino acid either from the L- or N-terminal of the protein. Certain amino acid either from L or N terminal.
Protein purification

(a) Trypsin produces fragments of the original protein. Each fragment is purified.

(b) Chymotrypsin digestion of the original protein yields products.

Original protein:

H₃N⁺ — Met — Tyr — COO⁻
N-terminal

H₃N⁺ — Met — Tyr — COO⁻
C-terminal

H₃N⁺ — Leu — Trp — COO⁻
N-terminal

H₃N⁺ — Leu — Trp — COO⁻
C-terminal

H₃N⁺ — Glu — Phe — COO⁻
N-terminal

H₃N⁺ — Glu — Phe — COO⁻
C-terminal

H₃N⁺ — Ser — COO⁻
N-terminal

H₃N⁺ — Ser — COO⁻
C-terminal
Chemical Cleavage

Cyanogen bromide

OVERALL REACTION:

\[
\begin{align*}
\text{CH}_3 & \\
\text{S} & \\
\text{CH}_2 & \\
\text{CH}_2 && \text{BrCN} \\
\text{O} & \\
\text{N} & \\
\text{C} & \\
\text{C} & \\
\text{N} & \\
\text{H} & \\
\text{H} & \\
\text{H} & \\
\end{align*}
\]

\(70\%\)

\[
\text{HCOOH}
\]

\[
\begin{align*}
\text{CH}_3 & \\
\text{CH}_2 & \\
\text{CH}_3 && \text{C} \\
\text{O} & \\
\text{N} & \\
\text{C} & \\
\text{C} & \\
\text{N} & \\
\text{N} & \\
\text{H} & \\
\text{H} & \\
\text{H} & \\
\end{align*}
\]

C-terminal peptide

The C-terminal peptide is obtained through chemical cleavage with 70% HCOOH.

Edman reagent is
phenylisothiocyanate

Labeling to amino acid polypeptide

The Edman reagent is used to label polypeptide, and each time a different amino acid is identified, such as alanine or glycine, a circle is added. The Edman procedure is used to sequence polypeptides step by step.

الفرع الطبي الأكاديمي

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### Specific cleavage of polypeptides

<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>Enzymatic cleavage</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>

**Chemical & enzymatic reagent**

The doctor read the entire table and told not to worry as he would return the system in detail! 😊.. If you know if you've memorized it or not 😐!!

---

**Diagram:**

A chromatogram showing the relative fluorescence over time (minutes) for various amino acids, including Asp, Glu, Asn, Ser, Gln, Arg, Thr, Val, Ile, Met, and Lys. The graph ranges from 0 to 100% solvent B. The peaks are labeled with their respective amino acids, and the time scale is marked from 0 to 35 minutes.
Protein purification

compound

result

overall

composition structure to the protein

molecule of the DNA or RNA

amino acid sequence

DNA

base sequence to the DNA or RNA

amino acid sequence

overall sequence

Chymotrypsin

H_{3}N-Lys-Leu-Asn-Asp-Phe

Cyanogen bromide

H_{3}N-Leu-Asn-Asp-Phe-His-Met

Chymotrypsin

His-Met-Thr-Met-Ala-Trp

Cyanogen bromide

Thr-Met

Cyanogen bromide

Ala-Trp-Val-Lys-COO^- base sequence to the DNA or RNA

Cyanogen bromide

Val-Lys-COO^- base sequence to the DNA or RNA

Chymotrypsin

H_{3}N-Lys-Leu-Asn-Asp-Phe-His-Met-Thr-Met-Ala-Trp-Val-Lys-COO^- base sequence to the DNA or RNA

整体分析
Protein purification

Essential amino acids:
- Is 10 in children & 8 in adult, there is histidine and arginine essential in children and not essential in adult
- The body cannot synthesize it
- The body needs them, they must be taken by food
- They are of animal origin
- Their deficiency will lead to disease
- Example for protein contain all essential amino acids:
  1. Milk protein
  2. Animal meat
  3. Eggs

Non-Essential amino acids:
- The body can synthesize them
- They are not needed by the body
- They are of plant origin
- Their deficiency will not lead to disease
- Example:
  1. Plant protein in Legumes

“SUCCESS MEANS DOING THE BEST WE CAN WITH WHAT WE HAVE. SUCCESS IS THE DOING, NOT THE GETTING; IN THE TRYING, NOT THE TRIUMPH. SUCCESS IS A PERSONAL STANDARD, REACHING FOR THE HIGHEST THAT IS IN US, BECOMING ALL THAT WE CAN BE.”