Two-dimensional gel electrophoresis for proteome analysis

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Outline

- Sample preparation
- First dimension: isoelectric focusing
  - Acid-base properties of peptides and proteins
  - Immobilized pH gradient
- Second dimension: SDS-PAGE
  - Rehydration and denaturation
  - Migration
- Staining procedures
- Image analysis
- Differential Gel Electrophoresis
Sample preparation
Sample preparation for 2D-GE

- Extract and solubilize as many proteins as possible from cells (or body fluid)

- Disrupt all interactions to recover individual proteins

- Preserve the protein charge for first dimension IEF (see below)

- Remove interfering substances

- Preserve the sample from alteration
Role of urea

Urea is almost always present in sample preparation protocols for 2D-GE

- It is neutral.

- It disrupts hydrogen bonds between proteins (inter-molecular) and between amino acids (intra-molecular).

- It can be used up to 9.8 M (solubility limit in water at room temperature).
Never heat urea-protein solutions!

Urea degrades at room temperature into cyanate, which can react with amine and cysteine groups in proteins:

\[
\begin{align*}
\text{H}_2\text{N-C-NH}_2 & \quad \leftrightarrow \quad \text{NH}_3 + \text{HNCO} & \quad \leftrightarrow \quad \text{NH}_4^+ + \text{O-C}:\equiv\text{N} \\
\text{HCNO} + \text{H}_3\text{N-Prot} & \quad \rightarrow \quad \text{H}_2\text{N-C-N-Prot} \\
\text{HCNO} + \text{HS-Prot} & \quad \rightarrow \quad \text{H}_2\text{N-C-S-Prot}
\end{align*}
\]

Occurs over 48 h at RT, equilibrium displaces with T

Optimal at pH 7 (Nter) or 8.5-9.5 (Lys)

Urea solutions should be prepared fresh, or stored in the freezer. The degradation is slow compared to sample preparation and separation procedures.
Thiourea

- Thiourea is another chaotrope that has been showed to help solubilizing hydrophobic proteins.
- It is used only with 5-7M urea, because thiourea is poorly soluble in water.
- Usually used at 0.5-2M. Above this, results in decreased resolution and problems of transfer to the second dimension.
Necessary reduction

• Disulfide bridges stabilize protein 3D structures.

• To obtain fully denatured proteins, it is necessary to reduce disulfide bridges.
Dithiothreitol, or Cleland’s reagent (DTT)

Is reactive only when thiolate -S⁻ is formed, i.e. at pH > 7

Used at 20-100 mM, but migrates during IEF below pH 7, thus leading to possible protein reoxidation of basic proteins (disulfide scrambling).
Dithioerythritol (DTE)

Is reactive only when thiolate \(-S^-\) is formed, i.e. at pH \(>7\)

Same mechanism of action than for DTT

Because of steric interactions of OH groups, the formation of the oxidized form is less favorable than for DTT, thus DTE is a weaker reducing agent.

Used at 20-100 mM, but migrates during IEF below pH 7, thus leading to possible protein reoxidation of basic proteins (disulfide scrambling).
2-mercaptoethanol

Migrates during IEF below pH 7, thus leading to possible protein reoxidation of basic proteins (disulfide scrambling).

Usually less efficient than DTT and DTE
tris(2-carboxyethyl)phosphine (TCEP)

very soluble in water and works in a broad pH range
unstable in phosphate buffers
used at 5-50 mM

TCEP is negatively charged in solution, thus migrates
during IEF and leads to possible protein reoxidation of
basic proteins (disulfide scrambling).
Tributylphosphine (TBP)

more reactive reducing agent than DTT, DTE, and 2ME
mechanism is the same as TCEP used at 2-50 mM

TBP is neutral so does not migrate in IEF
Carrier-ampholytes (CA)

- Carrier ampholytes are amphoteric species
- They inhibit the interactions between proteins and Immobilines (see below)
- They scavenge cyanate ions resulting from urea degradation
- They are often used at 2% concentration
Carrier ampholytes: Ampholynes®

Statistical mixture of synthetic compounds with acid and basic groups

- Svenson-Vesterberg (1960-1970): oligoamines reacted with unsaturated acrylic acids
Carrier Ampholytes: Servalyt®

• Grubhofer (1975): oligoamines obtained by condensation of ethylene imine and propylene diamine, derivatised with:

  • propane sulfone and Na-vinylsulfonate: introduction of sulfonic acid groups

  • Na-chloromethyl phosphonate: introduction of phosphonic acid groups

  • carboxylic acids
Carrier Ampholytes: Pharmalyte®

- Williams & Söderberg (1979): co-polymerisation of amines, glycine, glycylglycine and epichlorohydrin
Detergents

• A molecule that has a lipophilic domain (with affinity for hydrophobic domains of proteins) and a hydrophilic domain (affinity for water)

• A critical feature of detergents is the Critical Micellar Concentration, the concentration at which detergent molecules self-organize to form microvesicles or micelles.
How do detergents solubilize proteins?

- At low concentration (below CMC), detergents enter biological membranes.
- At higher concentration (above CMC), there are protein detergent-micelles, detergent micelles, and protein-detergent complexes.
## Detergent categories

There are three main categories: ionic, non-ionic, and zwiterionic detergents.

<table>
<thead>
<tr>
<th>Category</th>
<th>Example</th>
<th>Properties</th>
<th>CMC (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ionic</td>
<td>Sodium dodecyl sulfate</td>
<td>Usually useful to disrupt protein-protein interactions</td>
<td>7-10</td>
</tr>
<tr>
<td>Non-ionic</td>
<td>Triton X-100</td>
<td>Usually useful to disrupt lipid-lipid and lipid-protein interactions</td>
<td>0.2-0.9</td>
</tr>
<tr>
<td>Zwiterionic</td>
<td>CHAPS</td>
<td>Combined properties of ionic and non-ionic detergents</td>
<td>6-10</td>
</tr>
</tbody>
</table>
Rules of thumbs

• Though detrimental to IEF, the ionic SDS surfactant aids in the solubilization of proteins, if used with an excess of non-ionic or zwiterionic detergent

• Always use SDS below 0.25%, with an excess of at least 8:1 of CHAPS, Triton X-100...

• The rationale is that the uncharged detergent forms mixed micelles with SDS, that are electrophorethetically removed during electrophoresis
Sample preparation cocktails...

<table>
<thead>
<tr>
<th>Sample</th>
<th>Urea</th>
<th>Thiourea</th>
<th>Reducing agent</th>
<th>Detergent</th>
<th>Carrier ampholytes</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chinese Hamster Ovary cells (exogenously expressing membrane proteins)</td>
<td>7 M</td>
<td>2 M</td>
<td>50 mM DTT, 2 mM TCEP-HCl</td>
<td>2% C80 or 2% CHAPS or 2% ASB14</td>
<td>0.5% (3–10)</td>
<td>Henningsen et al. [27]</td>
</tr>
<tr>
<td>Mouse brain</td>
<td>5 M</td>
<td>2 M</td>
<td>60 mM DTT</td>
<td>2% CHAPS</td>
<td>2% (3–10)</td>
<td>Riederer and Shaw [3]</td>
</tr>
<tr>
<td>Bacterial outer membrane proteins (after carbonate washing)</td>
<td>7 M</td>
<td>2 M</td>
<td>30 mM DTT</td>
<td>1% ASB14 0.5% Triton X-100</td>
<td>0.5% (3–10)</td>
<td>Molloy et al. [28]</td>
</tr>
<tr>
<td>E. coli outer membrane proteins (after carbonate washing)</td>
<td>7 M</td>
<td>2 M</td>
<td>2 mM TBP</td>
<td>1% ASB14</td>
<td>0.5% (3–10)</td>
<td>Molloy et al. [29]</td>
</tr>
<tr>
<td>Human plasma</td>
<td>8 M</td>
<td>None</td>
<td>10 mM DTE</td>
<td>2% CHAPS</td>
<td>0.8% (4–8)</td>
<td>Liberatori et al. [30]</td>
</tr>
<tr>
<td>Mouse liver</td>
<td>8 M</td>
<td>None</td>
<td>60 mM DTT</td>
<td>0.5% Triton X-100</td>
<td>2% (3–10)</td>
<td>O’Connell and Stults [31]</td>
</tr>
<tr>
<td>Disaggregated human kidney tissue</td>
<td>9 M</td>
<td>None</td>
<td>65 mM DTE</td>
<td>4% CHAPS</td>
<td>4% (9–11)</td>
<td>Sarto et al. [32]</td>
</tr>
</tbody>
</table>
Getting rid of interfering substances

• Nucleic acids: add DNase and RNase to the sample preparation cocktail, or precipitate them with polyamines at high pH.

• Lipids: detergents and / or delipidation with a mixture of organic solvents.

• Salts: dialysis, gel filtration, TCA precipitation

• Proteases: use protease inhibitors.

• Polysaccharides: ultracentrifugation to remove high MW polysaccharides, TCA, ammonium sulfate, or phenol/ammonium acetate precipitation to remove low MW polysaccharides.
TCA precipitation

- Add one volume of 100% trichloroacetic acid for 4 volumes of protein sample.

- Incubate 4 min at 4°C.

- Centrifuge at 14000 RPM 5 min.

- Discard the supernatant and rinse the pellet with 200 µL of ice-cold acetone.

- Centrifuge at 14000 RMP 5 min.

- Repeat up to 2 volumes of acetone (compared to original protein sample).
First dimension: isoelectric focusing
Isoelectric focusing is a *steady-state* separation technique for *amphoteric species* based on acid-base equilibria in a *pH gradient*.
Peptides and proteins

- Peptides and proteins are made of 20 natural amino acids
<table>
<thead>
<tr>
<th>Symbol</th>
<th>Name and composition</th>
<th>Structure</th>
<th>Isotopic mass</th>
<th>Average mass</th>
<th>pKÅ</th>
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<tr>
<td>Ala A</td>
<td>Alanine C₃H₅NO</td>
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<td>71.0788</td>
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<tr>
<td>Arg R</td>
<td>Arginine C₅H₁₂N₄O</td>
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<td>156.1876</td>
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<tr>
<td>Asn N</td>
<td>Asparagine C₄H₆N₂O₂</td>
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<td>114.04293</td>
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<tr>
<td>Asp D</td>
<td>Aspartic acid C₄H₅NO₃</td>
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<td>115.02694</td>
<td>115.0886</td>
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<tr>
<td>Cys C</td>
<td>Cysteine C₃H₅NOS</td>
<td></td>
<td>103.00919</td>
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<tr>
<td>Gln Q</td>
<td>Glutamine C₅H₈N₂O₂</td>
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<td>128.05858</td>
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<tr>
<td>Glu E</td>
<td>Glutamic acid C₅H₇NO₃</td>
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<td>129.04259</td>
<td>129.1155</td>
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<td>Gly G</td>
<td>Glycine C₂H₃NO</td>
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<td>His H</td>
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<tr>
<td>Ile I</td>
<td>Isoleucine C₆H₁₁NO</td>
<td></td>
<td>113.08406</td>
<td>113.1595</td>
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<tr>
<td>Leu L</td>
<td>Leucine C₆H₁₁NO</td>
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<tr>
<td>Lys K</td>
<td>Lysine C₆H₁₂N₂O</td>
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<td>Thr T</td>
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<tr>
<td>Nter</td>
<td></td>
<td></td>
<td></td>
<td>9.7</td>
<td></td>
</tr>
<tr>
<td>Cter</td>
<td></td>
<td></td>
<td></td>
<td>2.5</td>
<td></td>
</tr>
</tbody>
</table>
Positively charged amino acids

**Histidine**

![Histidine structure](image)

\[ \text{C}_2\text{H}_4\text{N}^+ \leftrightarrow \text{C}_2\text{H}_4\text{N} + \text{H}^+ \]

\[ pK_a = 6.1 \]

**Lysine**

![Lysine structure](image)

\[ \text{C}_6\text{H}_{13}\text{N}^+ \leftrightarrow \text{C}_6\text{H}_{13}\text{N} + \text{H}^+ \]

\[ pK_a = 10.8 \]

**Arginine**

![Arginine structure](image)

\[ \text{C}_6\text{H}_{14}\text{N}_2\text{O}^+ \leftrightarrow \text{C}_6\text{H}_{14}\text{N}_2\text{O} + \text{H}^+ \]

\[ pK_a = 13.2 \]

**N-ter**

\[ \text{NH}_3^+ \leftrightarrow \text{NH}_2 + \text{H}^+ \]

\[ pK_a = 9.7 \]
Negatively charged amino acids

Cysteine \[ \overset{\text{COOH}}{\overset{\text{H}_2}{\text{C-SH}}} \rightleftharpoons \overset{\text{COO}^- + \text{H}^+}{\overset{\text{H}_2}{\text{C-S}}} \]

Tyrosine \[ \overset{\text{OH}}{\overset{\text{H}_2}{\text{C-OH}}} \rightleftharpoons \overset{\text{COO}^- + \text{H}^+}{\overset{\text{H}_2}{\text{C-O}^-}} \]

Aspartic acid \[ \overset{\text{COOH}}{\overset{\text{H}_2}{\text{C-C-OH}}} \rightleftharpoons \overset{\text{COO}^- + \text{H}^+}{\overset{\text{H}_2}{\text{C-C}^-}} \]

Glutamic acid \[ \overset{\text{COOH}}{\overset{\text{H}_2}{\text{C-C-C-OH}}} \rightleftharpoons \overset{\text{COO}^- + \text{H}^+}{\overset{\text{H}_2}{\text{C-C-C}^-}} \]

C-ter \[ \overset{\text{COOH}}{\overset{\text{H}_2}{\text{C-}}} \rightleftharpoons \overset{\text{COO}^- + \text{H}^+}{\overset{\text{H}_2}{\text{C-[}}} \]
Net charge of one amino acid residue

\[ \text{AH} \rightarrow \text{A}^- + \text{H}^+ \]

\[ K_a = \frac{[\text{A}^-][\text{H}^+]}{[\text{AH}]} \]

\[ [\text{AH}] + [\text{A}^-] = C_0 \]

\[ K_a = \frac{[\text{A}^-]10^{-pH}}{C_0 - [\text{A}^-]} \]

\[ [\text{A}^-] = \frac{C_0}{1 + \frac{10^{-pH}}{10^{-pK_a}}} \]

\[ \text{BH}^+ \rightarrow \text{B} + \text{H}^+ \]

\[ K_a = \frac{[\text{B}][\text{H}^+]}{[\text{BH}^+]} \]

\[ [\text{BH}^+] + [\text{B}] = C_0 \]

\[ K_a = \frac{(C_0 - [\text{BH}^+])10^{-pH}}{[\text{BH}^+]} \]

\[ [\text{BH}^+] = \frac{C}{1 + \frac{10^{-pH}}{10^{-pK_a}}} \]
Charge of a polypeptide

\[
\text{net charge} = \sum_{\text{positively charged}} \frac{n_i}{K_i} \frac{10^{-pH}}{10^{-pH}} + 1 - \sum_{\text{negatively charged}} \frac{n_j}{K_j} + 1
\]

Well-defined pl

Poorly-defined pl
Manufacturing of the pH gradient (IPG strip)

- Co-polymerisation of acrylamide with amino- or carboxylic acrylamide monomers

acrylamide:

\[ -\text{CH}_2=\text{C}N\text{-C}N\text{-H} \]

\[ \begin{array}{c}
\text{O} \\
\text{H}
\end{array} \]

derivatised acrylamide:

\[ -\text{CH}_2=\text{C}N\text{-C}N\text{-R} \]

\[ \begin{array}{c}
\text{O} \\
\text{H}
\end{array} \]

where R is a carrier ampholyte
How does IPG strip look like?

Narrow gel strip of given length (7-24 cm) incorporating an immobilized pH gradient (3-10 or others, linear or non linear)
Commercially available IPG strips

<table>
<thead>
<tr>
<th>pH range</th>
<th>2</th>
<th>4</th>
<th>6</th>
<th>8</th>
<th>10</th>
<th>12</th>
<th>Strip length</th>
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</thead>
<tbody>
<tr>
<td>3–5.6 NL</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>24 cm</td>
</tr>
<tr>
<td>5.3–6.5</td>
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<td></td>
<td></td>
<td></td>
<td></td>
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<td>18 cm</td>
</tr>
<tr>
<td>6.2–7.5</td>
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<td></td>
<td></td>
<td>13 cm</td>
</tr>
<tr>
<td>7–11 NL</td>
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<td></td>
<td></td>
<td></td>
<td>11 cm</td>
</tr>
<tr>
<td>3–11 NL</td>
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<td>7 cm</td>
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<td>3.5–4.5</td>
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<td>5.0–6.0</td>
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<tr>
<td>3–7 NL</td>
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<td>6–9</td>
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<td>6–11</td>
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<td>3–10 NL</td>
<td></td>
<td></td>
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<td></td>
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<td></td>
</tr>
</tbody>
</table>

From Amersham
Isoelectric focalisation

Diffusion-migration flux (linear pH gradient):

$$
\mathbf{J}_i = -c_i \tilde{u}_i \text{grad} \tilde{\mu}_i = -c_i \tilde{u}_i \text{grad} \mu_i - c_i \tilde{u}_i z_i F \text{grad} \phi
$$

Flux conservation:

$$
\frac{\partial c_i}{\partial \text{pH}} = -\text{div} \mathbf{J}_i = 0
$$

$$
- \frac{\partial}{\partial \text{pH}} \left[ - \frac{c_i \tilde{u}_i RT}{c_i} \frac{\partial c_i}{\partial \text{pH}} - c_i \tilde{u}_i z_i F \frac{\partial \phi}{\partial \text{pH}} \right] = 0
$$

$$
RT \frac{\partial c_i}{\partial \text{pH}} = c_i z_i FE
$$
Resolution

If the charge is proportional to the pH:

\[ z_i = -p_i \times pH \]

\[ RT \frac{\partial c_i}{\partial pH} = -c_i p_i FE \times pH \]

Which then gives the concentration:

\[ c_i = c_i^{\text{max}} \exp \left( -\frac{p_i FE (pH - pI)^2}{2 RT} \right) \]
Concentration profile
Concentration profile
Procedure for IEF in IPG strips

- Rehydrate the strip
- Load the sample
- Perform electrophoresis
- Wash the strip
- Re-equilibrate the strip for second dimension
IPG strip rehydration and sample loading

- IPG strips are sold dried-frozen
- The simplest procedure is to rehydrate the strip directly in the sample
- Rehydration should take place during at least 10 hours (overnight)
- Active rehydration can be used (under 50-120 V) to help large proteins to enter the gel.
Sample loading

• If sample was not applied during rehydration, there are several ways to apply sample

Direct loading  
Cup loading  
Paper bridge loading
### IEF run

<table>
<thead>
<tr>
<th>Step</th>
<th>kV</th>
<th>mA</th>
<th>W</th>
<th>kV.h</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.3</td>
<td>2</td>
<td>20</td>
<td>0.2</td>
</tr>
<tr>
<td>2</td>
<td>0.8</td>
<td>2</td>
<td>20</td>
<td>0.4</td>
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<tr>
<td>3</td>
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<td>0.6</td>
</tr>
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<td>4</td>
<td>1.8</td>
<td>2</td>
<td>20</td>
<td>0.8</td>
</tr>
<tr>
<td>5</td>
<td>2.6</td>
<td>2</td>
<td>20</td>
<td>1.3</td>
</tr>
<tr>
<td>6</td>
<td>3.6</td>
<td>2</td>
<td>20</td>
<td>1.8</td>
</tr>
<tr>
<td>7</td>
<td>5</td>
<td>2</td>
<td>20</td>
<td>100</td>
</tr>
</tbody>
</table>

Electrical limit are set to ensure 1) maximum migration 2) gel does not burn 3) run does not stop
At the end of IEF run

• Proteins are denatured, unfolded, globally neutral.

• Proteins need to be alkylated

• Proteins need to be charged to migrate in the second dimension
Why alkylate proteins?

- Block -SH reactivity for the formation of mixed disulfide
- Block -SH groups for interaction with acrylamide
- Both hold specially true at basic pH (8.5-9.5) where -SH is ionized in -S⁻
Why alkylate proteins? (2)

From Herbert et al, Electrophoresis 2001, 22, 2046–2057
Alkylation with iodoacetic acid (IAA) or iodoacetamide

- Prepare IAA solution fresh in 1M NaOH
- Because residual I⁻ can oxidize proteins, limit the reaction time as much as possible.
Risk of deamidation during alkylation

Because alkylation is performed at high pH, the peptide bond nitrogen is deprotonated, which favors the formation of the succinimide intermediate.

A

Asparagine

\[
\begin{align*}
\text{NH}_2 & \quad \text{O} = \text{C} \\
\text{H}_2 & \quad \text{C} \quad \text{NH} \quad \text{R} \quad \text{NH} - \text{CH} - \text{NH} \\
\text{NH} - \text{CH} & \quad \text{R} \quad \text{NH} \quad \text{R} \quad \text{NH} \quad \text{R} \\
\text{NH} - \text{CH} & \quad \text{C} \quad \text{NH} \\
\end{align*}
\]

Succinimide Intermediate

\[
\begin{align*}
\text{NH}_2 & \quad \text{O} = \text{C} \\
\text{H}_2 & \quad \text{C} \quad \text{NH} \quad \text{R} \quad \text{NH} \\
\text{NH} - \text{C} & \quad \text{N} \quad \text{CH} - \text{NH} \\
\end{align*}
\]

B

Aspartic Acid

\[
\begin{align*}
\text{OH} & \quad \text{O} \\
\text{H}_2 & \quad \text{C} \quad \text{R} \\
\text{H} & \quad \text{C} \quad \text{NH} \quad \text{R} \quad \text{NH} \quad \text{R} \quad \text{NH} \quad \text{R} \\
\text{NH} - \text{CH} & \quad \text{C} \quad \text{NH} \\
\text{NH} - \text{CH} & \quad \text{C} \quad \text{O} \\
\text{NH} & \quad \text{CH} - \text{C} \quad \text{OH} \\
\end{align*}
\]

Degradation of Succinimide Intermediate

\[
\begin{align*}
\text{OH} & \quad \text{O} \\
\text{H}_2 & \quad \text{C} \quad \text{R} \\
\text{H} & \quad \text{C} \quad \text{NH} \quad \text{R} \quad \text{NH} \quad \text{R} \quad \text{NH} \\
\text{NH} - \text{CH} & \quad \text{C} \quad \text{NH} \\
\text{NH} - \text{CH} & \quad \text{C} \quad \text{O} \\
\text{NH} & \quad \text{CH} - \text{C} \quad \text{OH} \\
\end{align*}
\]

IsoAspartic Acid
Role of SDS in re-equilibration

Unfolded, neutral protein after IEF

Unfolded protein linearly charged with SDS

Approximately 1.4 g SDS per gram of protein
All proteins have the same mass to charge ratio and thus the same electrophoretic mobility
Reequilibration protocol

- Incubate the strip for 12 min in DTE 2%, Tris-HCl 0.5 M, Urea 6 M, glycerol 33%, SDS 0.07 M

- Incubate the strip for 5 min in iodoacetamide 2.5%, Tris-HCl 0.5 M, Urea 6 M, glycerol 33%, SDS 0.07 M

- Apply the strip on top of an SDS-PAGE gel of choice

- Run the second dimension
Polyacrylamide gels

- Acrylamide + linker + catalyst
- Linker = N,N'-methylenebisacrylamide
- Initiator: ammonium persulfate
- Catalyst = TEMED (N,N,N',N'-tetramethylethylenediamine)

Usually around 30 mM of unpolymerized acrylamide remains in the gel
Pore size

- if %T increases, pore size diminishes
- For a given %T, pore size is minimal at %C = 5%

For T = 3%

\[
\%T = \frac{g(\text{acrylamide} + \text{bisacrylamide})}{100 \text{ ml}} \times 100
\]

\[
\%C = \frac{g(\text{bisacrylamide})}{g(\text{acrylamide} + \text{bisacrylamide})} \times 100
\]
Migration through the gel

“blob”

Hernia

Head
Size-migration distance relationship

In a gel with constant porosity, the relationship between migration distance and size is semi-log.
Background of sieving mechanism

Large proteins have more difficulties to find a way through the gel (sponge-like) than short proteins:

\[
\frac{\mu_e}{\mu_e^0} = \exp\left(-\frac{\pi}{4}\left(\frac{d + r}{R}\right)^2\right)
\]

where \(\mu_e\) is the electrophoretic mobility in the gel, \(\mu_e^0\) in solution, \(d\) the radius of gel fibers, \(R\) the pore diameters, and \(r\) the protein radius.
Use of gels with constant porosity

<table>
<thead>
<tr>
<th>Polyacrylamide gel (%T)</th>
<th>Mr range (kDa)</th>
</tr>
</thead>
<tbody>
<tr>
<td>6</td>
<td>30-200</td>
</tr>
<tr>
<td>8</td>
<td>20-175</td>
</tr>
<tr>
<td>10</td>
<td>15-150</td>
</tr>
<tr>
<td>12</td>
<td>10-100</td>
</tr>
<tr>
<td>15</td>
<td>6-50</td>
</tr>
</tbody>
</table>

- The larger %T (acrylamide + bis concentration), the smaller the pores
- The smaller the pores, the smaller the proteins that can be resolved
- The gel porosity has to be adapted to the sample
Pore gradient gel electrophoresis (PGGE)

- By varying the acrylamide or bis concentration along the gel, the pore size can be adapted
- Gels from T=5-20% can be prepared
Advantage of PGGE

• Proteins migrate till they reach a region of the gel where pores are too small for them to continue migration

• At this point, their effective mobility is close to zero

• A larger range of molecular weights can be analysed

• For example, for a PGGE gel with %T=5-20, proteins from 6000 to 250000 Da can be analysed
Gel staining
Coomassie staining

- Binds to arginine, aromatic amino acids, and histidine
- Has low sensitivity (100-500 ng per spot)
- The protocol is simple
- Staining is directly compatible with mass spectrometry
Silver staining

- Silver staining is based on the reduction of AgNO₃ to metallic silver by formaldehyde.

- Proteins provide nucleation site for metallic silver clustering and growth.

- Sensitivity is better than with Coomassie (1-10 ng per spot).

- Silver staining is less compatible with mass spectrometry.
Fluorescent staining

- Different fluorescent stains exist, either as pre-electrophoresis labeling, or post-electrophoresis stains.

- Usually better sensitivity, dynamic range, and linearity than coomasie and silver stains

- Requires specific scanners

Linearity and dynamic range of SYPRO Ruby
Comparison of stains

Rat fibroblast proteins

Lopez et al, Electrophoresis 2000, 21, 3673
Is response linearity so important?

A change in concentration from 55 to 70 results in a change of silver intensity of 30 and of fluorescence intensity of 1.4...
Use of imaging software

Control sample    Disease sample
Spot detection
Spot matching (reference gel)
Differential analysis
Identification of differentially expressed proteins

Identification with mass spectrometry techniques
DIGE principle

Protein extract 1
Label with fluor 1 → Mix labelled extracts → Protein extract 2
Label with fluor 2

Separate by 2-D PAGE

Excitation wavelength 1
Image analysis: overlay images

Image gel
Analysis of difference

Excitation wavelength 2
Image analysis: data quantitation
CyeDye labeling

Currently three colors are available, and thus three samples can be analyzed together.
Transfusion plasma treated with methylene blue for pathogen inactivation
Take-home message

• For setting up a two-dimensional gel electrophoresis experiment, first check the literature for a protocol corresponding to the sample of interest

• Perform experiments with the standard protocols

• Improve the protocol by playing around with the different parameters, keeping in mind the background information about the roles of detergents, carrier-ampholytes, chaotropes, gel properties...