Biological Chemistry Laboratory
Biology 3515/Chemistry 3515
Spring 2023

Lecture 24:

Variations on Gel Electrophoresis

and

Introduction to Chromatography

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Kinetic Analysis of the RNAse A Digestion Experiment

- Michaelis-Menten Equation: $V = \frac{[E]_T k_{cat} [S]}{K_m + [S]}$
- If $[S] \ll K_m$:
  (We will assume this as a simplification, without much justification.)

$$V = \frac{[E]_T k_{cat} [S]}{K_m}$$

- A pseudo first-order rate expression: $\frac{d[S]}{dt} = -V = -k_{app}[S]$

  where:

$$k_{app} = \frac{[E]_T k_{cat}}{K_m}$$

The Time Course for Digestion

- First-order rate expression:

$$\frac{d[S]}{dt} = -k_{app}[S]$$

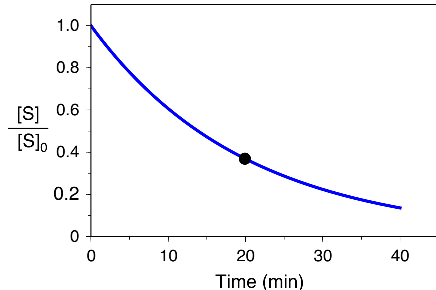
- After integration:

$$\frac{[S]}{[S]_0} = e^{-k_{app} \cdot t}$$

$$[S]_0 = \text{initial substrate concentration}$$

- Take logarithms and solve for k_{app}:

$$k_{app} = -\frac{\ln\left([S]/[S]_0\right)}{t}$$



Estimating k_{cat}/K_m from a Single Time Point

- From integrated rate expression:

$$\ln\left([S]/[S]_0\right) = -k_{app} \cdot t$$

$$k_{app} = -\frac{\ln\left([S]/[S]_0\right)}{t}$$

- Calculate k_{cat}/K_m from k_{app} and $[E]_T$:

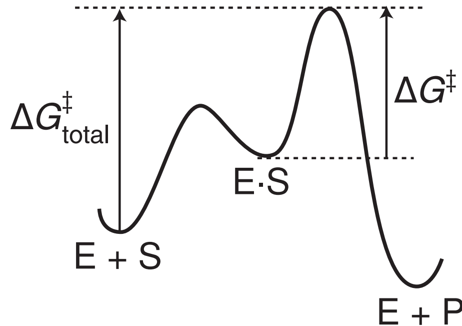
$$k_{app} = \frac{[E]_T k_{cat}}{K_m}$$

$$k_{cat}/K_m = k_{app}/[E]_T$$

The Significance of k_{cat}/K_m

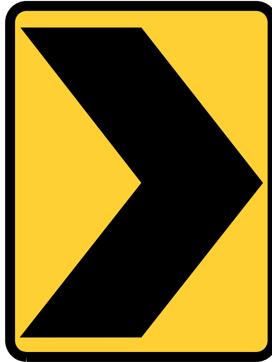
- If $K_d \approx K_m$:

$$\Delta G^{\ddagger}_{total} = C - RT \ln\left(\frac{k_{cat}}{K_m}\right)$$



- Catalytic efficiency is favored by a large value of k_{cat} and a small value of K_m.
- How does k_{cat}/K_m compare for a protein and a synthetic substrate?

Warning!



Direction Change

Other Aspects of Electrophoresis

Methods for Detecting Proteins in Gels

- To detect all proteins:
  - Staining with coomassie blue:
    Dye binds to proteins and excess is washed away
    Sensitivity: $\approx$ 0.1–1 μg/band
  - “Silver staining”:
    Based on reduction of silver ions to metallic silver in vicinity of protein.
    Much more sensitive than coomassie blue; as little as 10 ng/band
    Messy, finicky and sensitive to artifacts.
  - Staining with fluorescent dyes
    Dye fluorescence is enhanced when bound to proteins
    Much more sensitive than coomassie blue
  - Other dyes and metal ions.
- To detect specific protein classes:
  - Specific dyes for phosphoproteins
  - Specific dyes for glycoproteins

Steinberg, T. H. (2009). Protein gel staining methods: An introduction and overview. *Methods Enzymol.*, 463, 541–563. http://dx.doi.org/10.1016/S0076-6879(09)63031-7

Detection of Gel-fractionated Proteins with Antibodies: Western Blotting

- Allows selective detection of specific proteins: Requires specific antibodies.
- After electrophoresis, proteins are electrophoresed out of the gel and transferred ("blotted") onto a membrane (usually nitrocellulose or polyvinylidene difluoride)

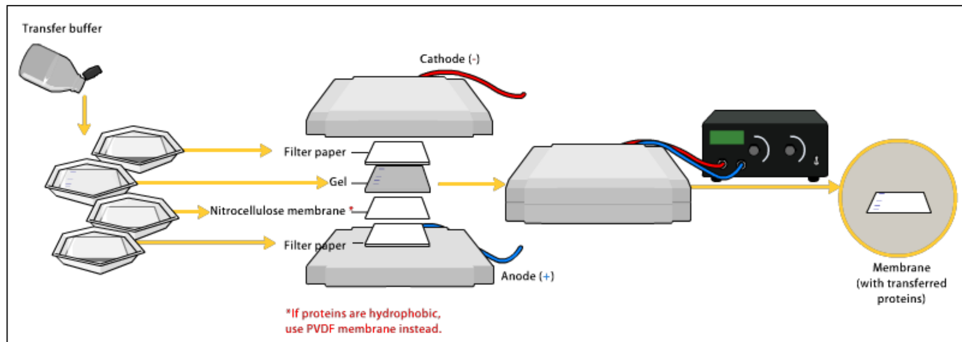
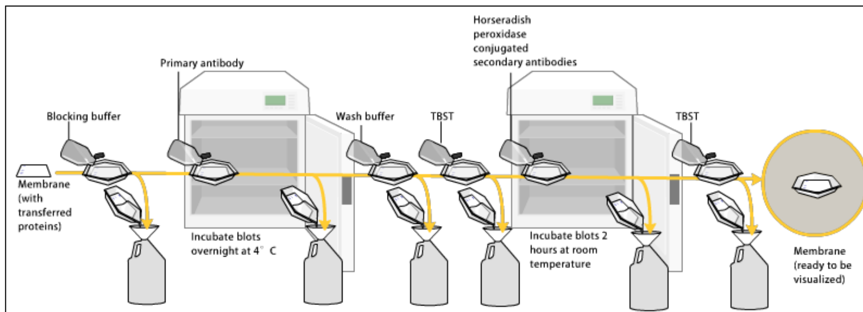


Illustration from `https://en.wikipedia.org/wiki/Western_blot`

Detection of Gel-fractionated Proteins with Antibodies: Western Blotting

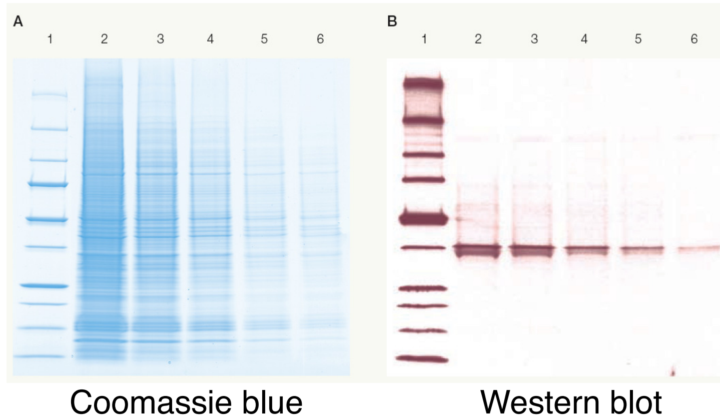
- Multistep treatment of membrane with bound proteins.



  - Generic protein solution to block all protein binding sites.
  - “Primary” antibody specific for protein of interest.
  - “Secondary” antibody, binds to primary antibody and is labeled for detection. Radioactive label or enzyme that generates colored precipitate or chemiluminescence.

Illustration from `https://en.wikipedia.org/wiki/Western_blot`

Western Blot Example



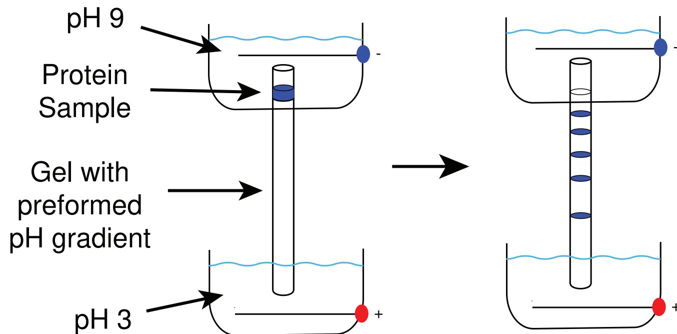
- Sample: HeLa cell lysate.
- Antibody: Specific for human CDK7 protein. (cyclin-dependent kinase)

Illustration from Bio-Rad Bulletin 2032 Rev. E,
www.bio-rad.com/webroot/web/pdf/lsr/literature/Bulletin_2032.pdf

A Bit More About Western Blots

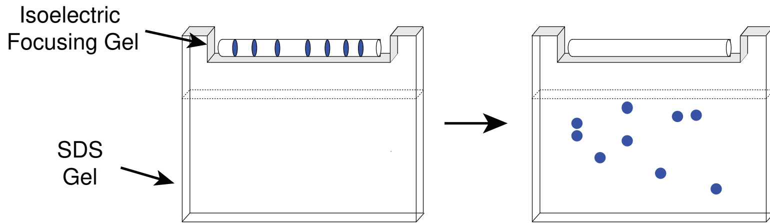
- Clinical applications: Used to detect antibodies in patients indicative of infection.
  - HIV
  - Lime disease
  - Hepatits
  - Other viral and bacterial infections
- Where does the name come from?
  - 1975: DNA blotted from gels and detected by hybridization to radiolabeled DNA. Invented by Edwin Southern. https://doi.org/10.1016/S0022-2836(75)80083-0
  - 1977: RNA blotted from gels and detected by hybridization to radiolabeled DNA. Invented by James C. Alwine, David J. Kemp, and George R. Stark and nicknamed by others “northern blotting”. https://dx.doi.org/10.1073%2Fpnas.74.12.5350
  - 1981: Proteins plotted from gels and detected by antibody binding. Invented by W. Neal Burnette, who called it “western blotting”. https://doi.org/10.1016/0003-2697(81)90281-5

Another Electrophoresis Method: Isoelectric Focusing (IEF)



- pH gradient is formed by mixtures of buffering compounds incorporated in the gel.
- Proteins migrate to point where they have no net charge, and then stop.
- Proteins are separated by isoelectric point.
- Usually performed in the absence of detergent, but often with urea present to unfold proteins and keep them soluble.

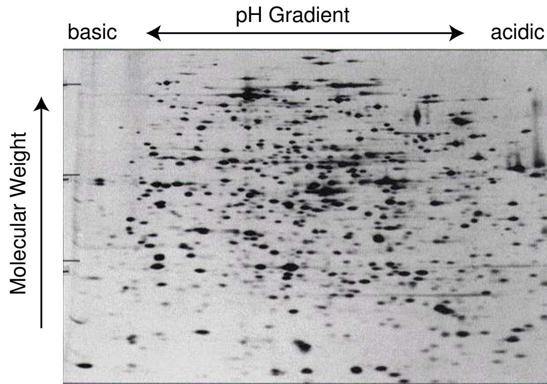
Two-dimensional Gel Electrophoresis



- Isoelectric point and chain molecular weight are largely independent properties.
- Greatly increases ability to resolve proteins in complex mixtures.
- Simultaneously provides information about molecular weight and isoelectric point.
- Other variations are possible (*e.g.,* native gel/SDS gel)

O'Farrell, P. H. (1975). High resolution two dimensional electrophoresis of proteins. *J. Biol. Chem..*, 250, 4007–4021. http://www.jbc.org/content/250/10/4007.abstract

A 2-dimensional Gel of Bacterial Proteins



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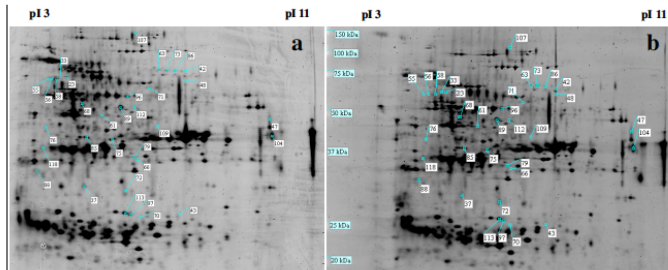
2-dimensional Gel Electrophoresis in Proteomics

- Proteomics: A (sort of) new discipline focused on analysis of protein compositions of cell, tissues and organisms under different physiological, developmental or environmental conditions. One of many new “omics”!
- An example using 2-d gel electrophoresis:

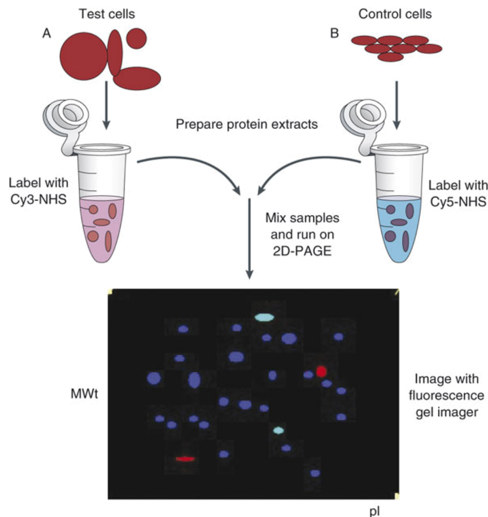


Comparison of cultured tissue (callus) of vanilla orchid under different conditions.

- Spots in gel can be excised and chemically analyzed to identify proteins: Protease digestion, mass spectrometry, sequencing

Palama, T. L., Menard, P., Fock, I., Choi, Y. H., Bourdon, E., Govinden-Soulange, J., Bahut, M., Payet, B., Verpoorte, R. & Kodja, H. (2010). *BMC Plant Biology*, 10, 82. http://dx.doi.org/10.1186/1471-2229-10-82

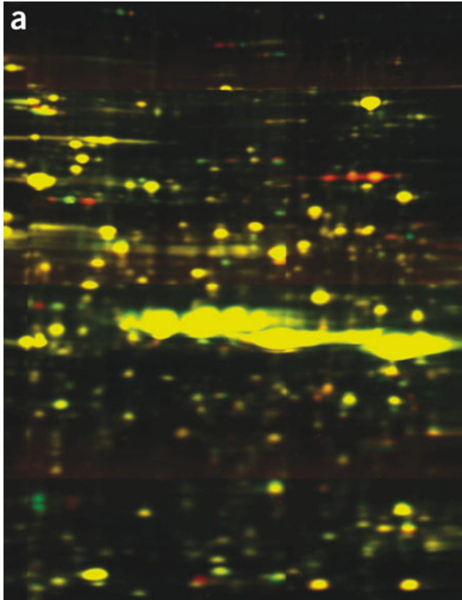
Differential Fluorescent Labeling of Proteins for 2-d Gel Analysis



- 2-d gels are difficult to reproduce precisely.
- Proteins from different samples are labeled with different fluorescent reagents.
- Reagents are matched to have same mass and electric charge.
- Samples are mixed and run on a single 2-d gel.
- Proteins from different samples are distinguished by different colors.

Viswanathan, S., Ŭniŭ, M. & Minden, J. S. (2006). Two-dimensional difference gel electrophoresis. *Nature Protocols*, 1, 1351–1358. http://dx.doi.org/10.1038/nprot.2006.234

An Example of Differential 2-d Gel Analysis



- *Drosophila melanogaster* proteins from different mutants during development, labeled with red and blue fluorescent dyes.
- Yellow indicates proteins present at same levels in both strains.
- Red or green indicates proteins present at higher levels in one or the other strain.

Viswanathan, S., Ŭniŭ, M. & Minden, J. S. (2006). Two-dimensional difference gel electrophoresis. *Nature Protocols*, 1, 1351–1358.
http://dx.doi.org/10.1038/nprot.2006.234

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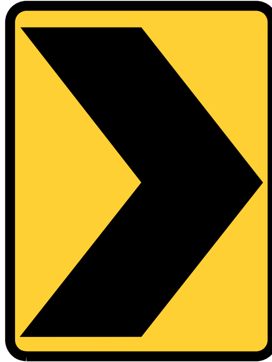
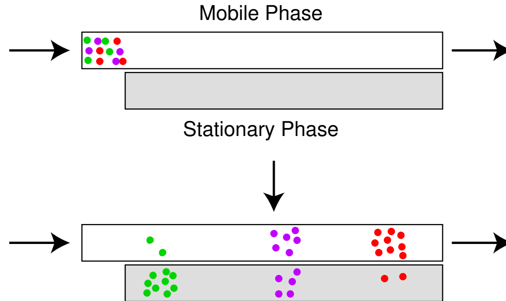


Direction Change

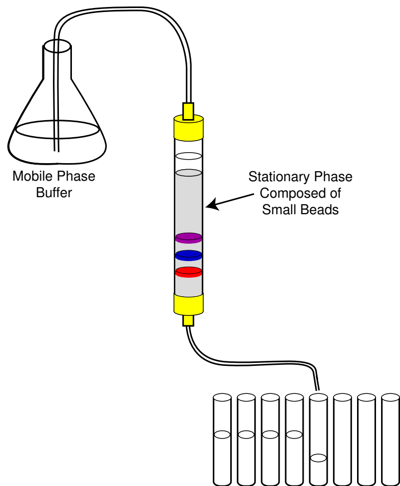
Introduction to Chromatography

General Description of Chromatography



- Stationary and mobile phases have distinct chemical or physical properties.
- Molecules partition (thermodynamic equilibrium) between two phases.
- Molecules that partition preferentially into mobile phase move more rapidly than molecules that prefer the stationary phase.

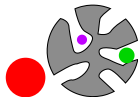
Column Chromatography:
The Most Commonly Used Format for Biochemistry



- Sample is placed at top of column.
- Buffer flows through and around beads in stationary phase and caries sample molecules.
- Molecules that interact the least with the beads elute from the column first.
- Eluent is fractionated into tubes as it leaves the column. (automatic fraction collector)
- Eluent can be analyzed as it leaves the column (*e.g.*, spectrophotometer with flow cell) or from fractions.

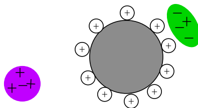
Chromatography Methods Commonly Used for Biomolecules

- Gel filtration chromatography - based on molecular size

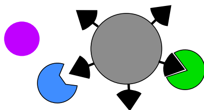


  Form that we will use in lab.

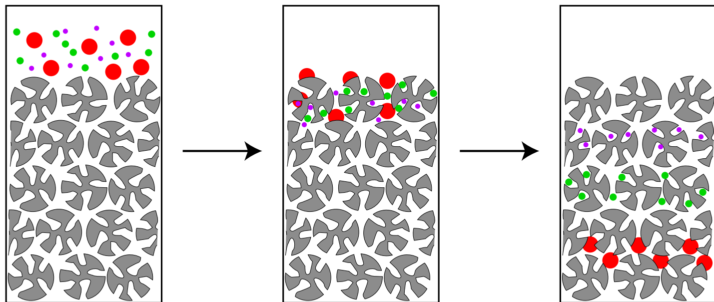
- Ion exchange chromatography - based on electric charge



- Affinity chromatography - based on specific biochemical interactions

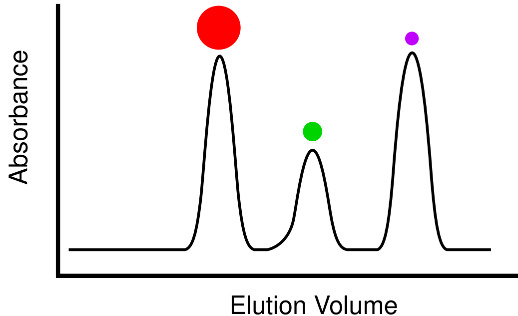


Gel Filtration Chromatography



- Also called “gel permeation”, “size exclusion”, “sizing” or “molecular sieve” chromatography.
- Beads are made of a porous gel. (similar to gels used for electrophoresis)
- Separates molecules on basis of size.
- Larger proteins elute first. (opposite of gel electrophoresis!)
- Beads have a distribution of pore sizes.

Elution Profile for a Gel Filtration Column



- Elution volume is the volume of buffer that flows through the column between when the sample is applied to the top and when a particular protein leaves the column.
- Can be calibrated with proteins of known size in order to estimate molecular weights of other proteins.
- Unless a denaturant is present, elution volume usually reflects molecular weight of native protein, with quaternary and tertiary structure intact, unlike SDS gel electrophoresis.
- Media with different distributions of pore sizes are used to separate molecules of different size ranges.