Biological Chemistry Laboratory
Biology 3515/Chemistry 3515
Spring 2021

Lecture 24:

Quantifying Results from the RNAse A Digestion Experiment

and

Introduction to Chromatography

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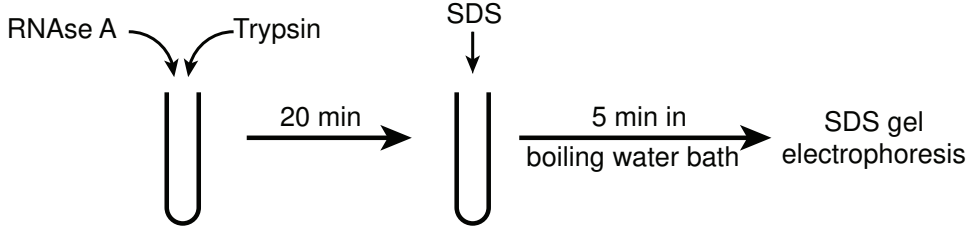
Trypsin Digestion Experiment

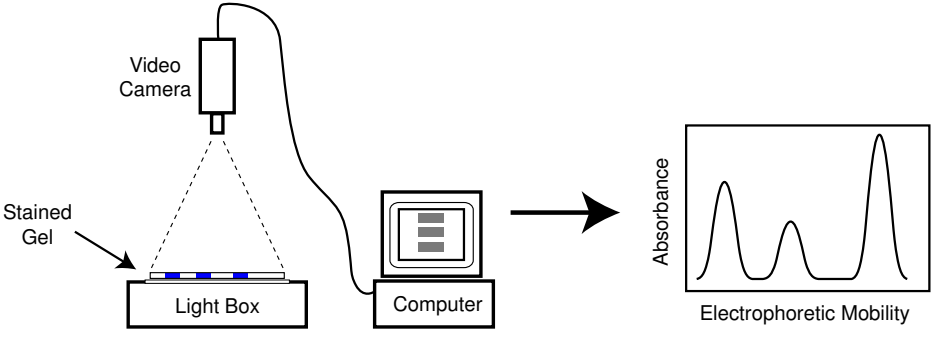
- The questions:
  - Are different forms of RNAse A substrates for trypsin?
  - Are some forms better substrates than others?
  - Can we compare RNAse A forms to chromogenic peptide as substrates for trypsin?
- The strategy:
  - SDS gel electrophoresis separates polypeptides on the basis of molecular weight. (number of amino acid residues)
  - Follow the disappearance of intact RNAse A to monitor hydrolysis.
- Possible experimental variables:
  - Time of reaction.
  - Enzyme concentration.
  - Substrate concentration.
  - Solution conditions: Temperature, pH, other solutes.
- We will only vary enzyme concentration.

Trypsin Digestion Experiment



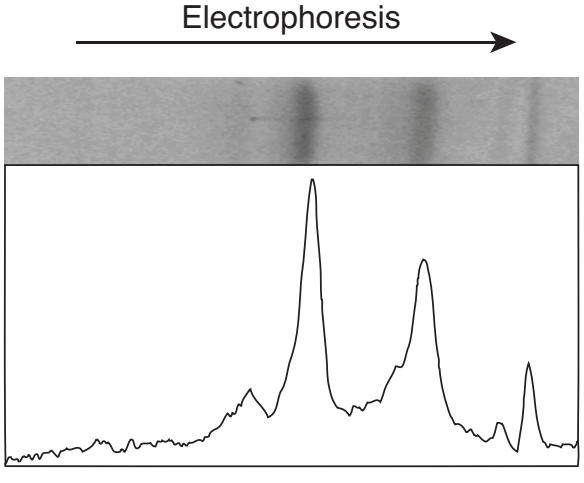
- For each RNAse A form (N, RCM, RCAM):
  - Reaction with no trypsin.
  - Two reactions with different trypsin concentrations.
- Reactions stopped after 20 min by adding SDS and heating (95°C).
- RNAse A analyzed by SDS gel electrophoresis.
- Relative concentrations estimated by quantitative analysis of gel image.

Video Densitometry of Gels



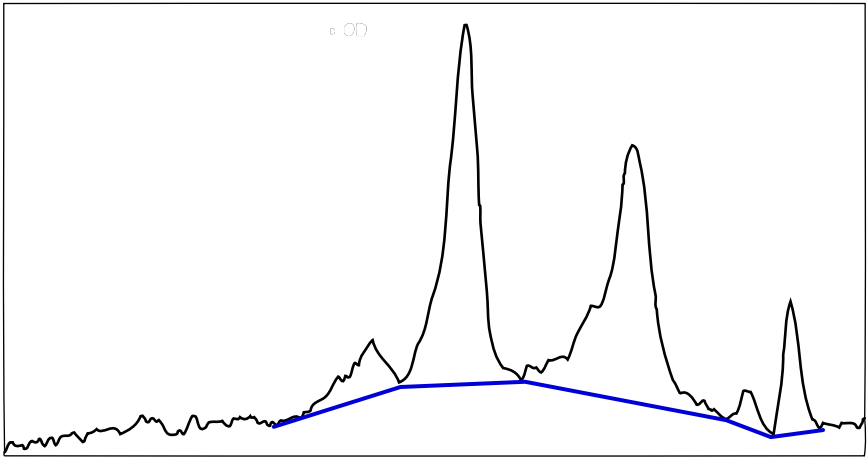
- Lightbox and camera function as a position-specific spectrophotometer.

Scans of SDS Gel Lanes



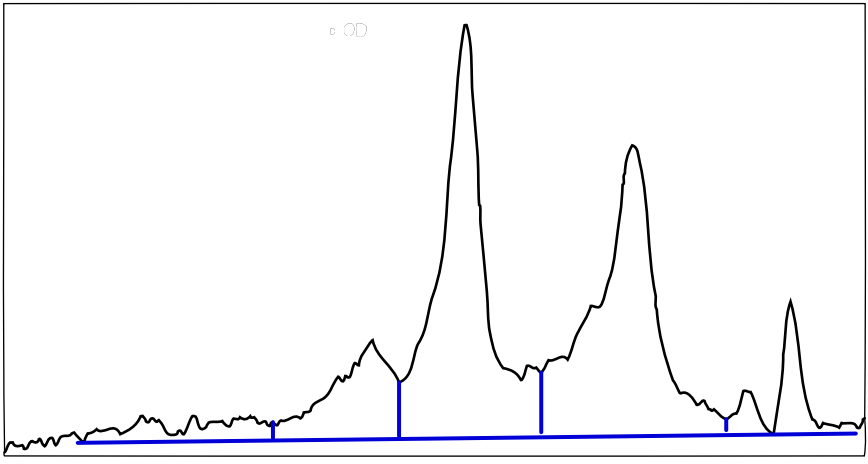
- Integrals of peaks are proportional to protein concentrations.
- Have to define limits of individual peaks and baseline.

One Way to Draw Baselines



- Implies that there is a broad distribution of stain below the peaks that is unrelated to protein concentration.

Another (Correct) Way to Draw Baselines



Kinetic Analysis of the RNAse A Digestion Experiment

- Michaelis-Menten Equation: $V = \dfrac{[\mathrm{E}]_\mathrm{T} k_\mathrm{cat} [\mathrm{S}]}{K_\mathrm{m} + [\mathrm{S}]}$

- If $[\mathrm{S}] \ll K_\mathrm{m}$:
  (We will assume this as a simplification, without much justification.)

$$V = \frac{[\mathrm{E}]_\mathrm{T} k_\mathrm{cat} [\mathrm{S}]}{K_\mathrm{m}}$$

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

  where:

$$k_\mathrm{app} = \frac{[\mathrm{E}]_\mathrm{T} k_\mathrm{cat}}{K_\mathrm{m}}$$

The Time Course for Digestion

- First-order rate expression:

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

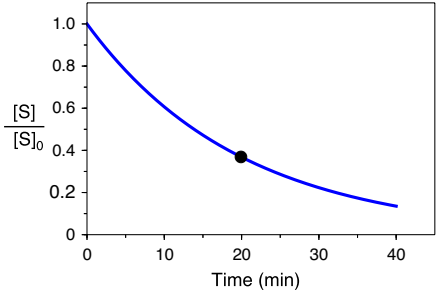
- After integration:

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

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

- Take logarithms and solve for k_{app}:

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



Clicker Question #1

If 70% of the protein is cleaved in 20 min,
what is the apparent first-order rate constant, k_{app}?

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

A) $3\times10^{-4}\,s^{-1}$

B) $1\times10^{-3}\,s^{-1}$

C) $2\times10^{-2}\,s^{-1}$

D) $6\times10^{-2}\,s^{-1}$

$$k_{app} = -\frac{\ln\left([S]/[S]_0\right)}{t} = -\frac{\ln\left(1-0.7\right)}{20\,min} = 0.06\,min^{-1} = 0.001\,s^{-1}$$

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 $[E]_T$:

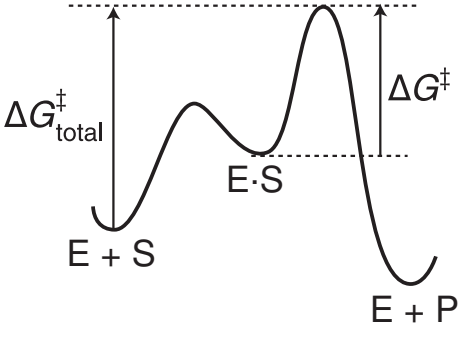
$$k_{app} = \frac{[E]_T k_{cat}}{K_m}$$

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

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?

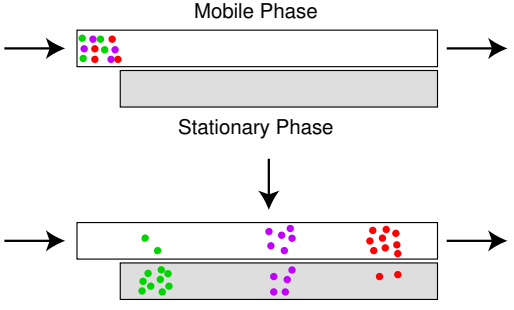
Warning!

Direction Change

Introduction to Chromatography

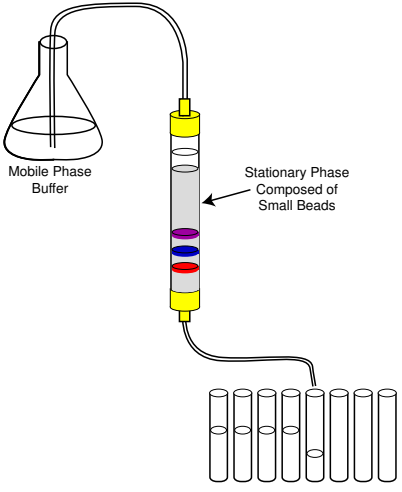
(More on electrophoresis later)

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.

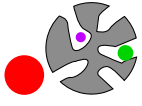
Column Chromatography:
The Most Commonly Used Format for Biochemistry



- 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.

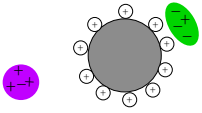
Chromatography Methods Commonly Used for Biomolecules

- Gel filtration chromatography - based on molecular size

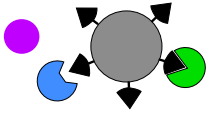


Form that we will use in lab.

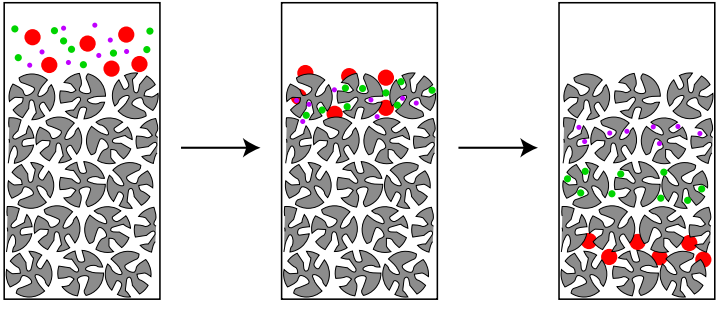
- Ion exchange chromatography - based on electric charge



- Affinity chromatography - based on specific biochemical interactions

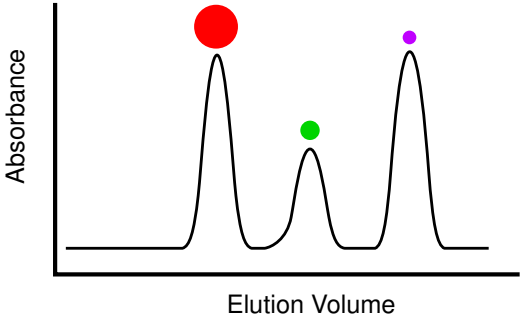


Gel Filtration Chromatography



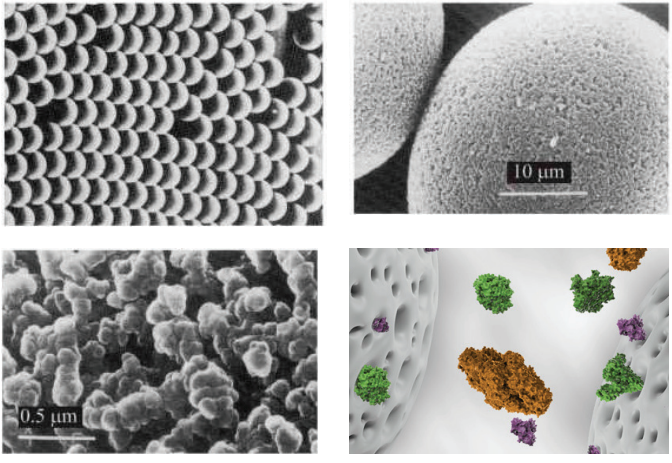
- Also called “gel permeation”, “size exclusion”, “sizing” or “molecular sieve” chromatography.
- Beads are made of a porous gel. (similar to gels used for electrophoresis)
- Separates molecules on basis of size.
- Larger proteins elute first. (opposite of gel electrophoresis!)
- Beads have a distribution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

Scanning Electron Micrographs (and a cartoon) of Gel Filtration Media



Micrographs by A. Medin, Department of Biochemistry, Uppsala University.
Found at: http://macromol.sbcs.qmul.ac.uk/oldsite/expertise/Beads2a.jpg
Cartoon by GE HealthCare:
http://www.gelifesciences.com/webapp/wcs/stores/servlet/catalog/en/GELifeSciences-us/brands/superdex/

Clicker Question #2

- Suppose that we have a protein with a molecular weight of 85 kDa, and we want to separate this protein from both smaller and larger molecules?
- What range of pore diameters should the gel filtration media have?

A) 0.4–2 nm

B) 2–10 nm

C) 10–50 nm

D) 50–250 nm

All answers count for now!

How Do We Estimate the Diameter of a Protein?

- What do we know?
  - The mass of the molecule:

$$85,000\,\mathrm{g/mol} \div 6.02 \times 10^{23}\,\mathrm{molecule/mol}$$
$$= 1.41 \times 10^{-19}\,\mathrm{g/molecule}$$

- What else do we need to know (or assume)?
  - The density of the molecule (mass/vol)
  - Is the density of a protein greater or less than 1 g/mL?
  - Proteins sediment in the ultracentrifuge!
    Density must be greater than that of water.
  - Because of protein interaction with water, defining density is a bit tricky.
    But, we will assume $\approx 1\,\mathrm{g/mL}$.
- Anything else?
  - What is the shape of the molecule?
  - Determines relationship between volume and linear dimensions.
  - We will assume a sphere.