Biological Chemistry Laboratory
Biology 3515/Chemistry 3515
Spring 2023

Lecture 26:

More on Gel Filtration Chromatography

and

Experiment 6

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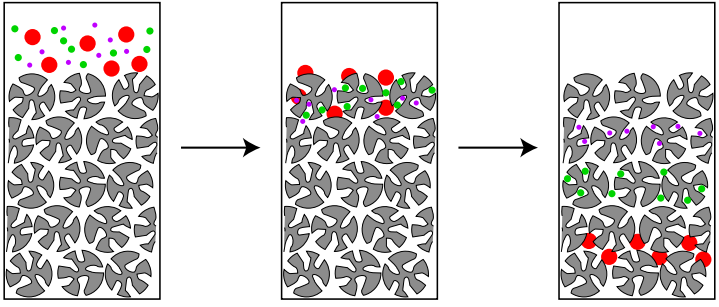
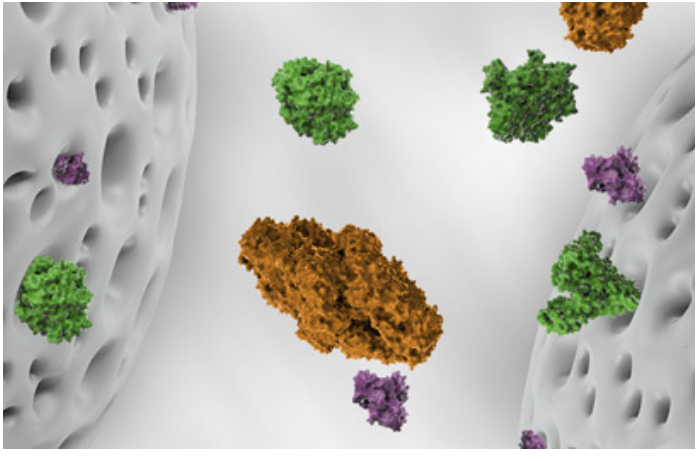
Gel Filtration Chromatography



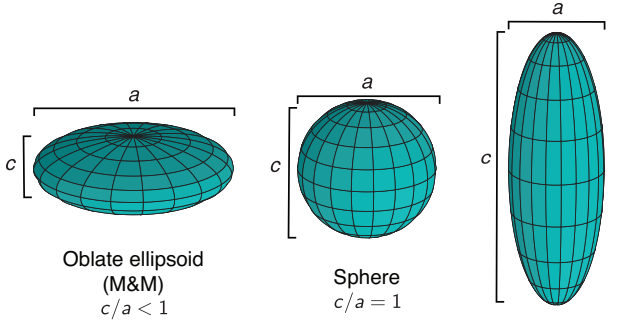
- Partition between stationary and mobile phases depends on ability of molecules to enter pores of the beads.
- K_{av} is the fraction of the bead volume that a molecule can enter.

How Does Shape Affect K_{ave} (or Elution Volume)?



Cartoon by GE HealthCare:
http://www.gelifesciences.com/webapp/wcs/stores/servlet/catalog/en/GELifeSciences-us/brands/superdex/

Useful Approximations for Non-spherical Proteins



Oblate ellipsoid
(M&M)
$c/a < 1$

Sphere
$c/a = 1$

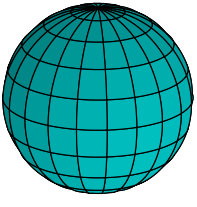
Prolate ellipsoid
(cigar)
$c/a > 1$

- Drawings scaled to represent equal volumes.
- Axial ratio, c/a, defines asymmetry.
- For a given volume ($\propto$ MW) which is more likely to enter a pore?

Clicker Question #1

If the volumes are the same, which will elute first from a gel filtration column?

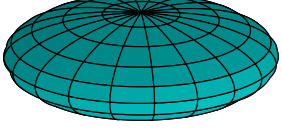
A) The sphere.



Sphere

B) The oblate ellipsoid.

C) They will elute together.



Oblate ellipsoid

All answers count for now.

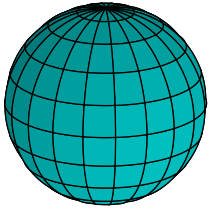
Clicker Question #2

If the volumes are the same, which will elute first from a gel filtration column?

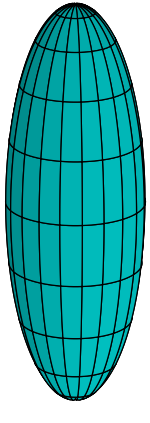
A) The sphere.

B) The prolate ellipsoid.

C) They will elute together.



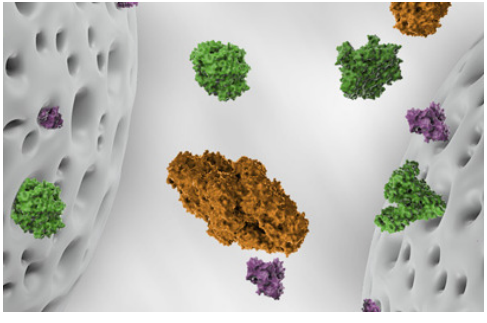
Sphere



Prolate ellipsoid

All answers count for now.

Why Does a Prolate Ellipsoid Elute First?



- The prolate ellipsoid should be able to enter smaller pores than a sphere of equal volume.
- But, ellipsoid has to be in the right orientation to enter the smaller pore.
- Most orientations make it *harder* to enter a smaller pore.
- In thermodynamic terms, the ellipsoid has to lose entropy to enter the stationary phase.
- Elution volume reflects equilibrium between stationary and mobile phase.

Effects of Size and Shape on K_{ave} (or Elution Volume)

- For molecules with the same overall shape, K_{ave} and elution volume decrease with increased molecular weight (over suitable range for a given media).
- For molecules with different shapes, elution volumes may not reflect molecular weights.
  - When compared to $\approx$ spherical proteins, more asymmetric molecules will appear to have larger molecular weights than they really do.
  - For molecules with a range of shapes, K_{ave} is best correlated with their frictional coefficients and diffusion rates in solution.
  - “Stokes radius” or ”hydrodynamic radius”, radius of a smooth sphere with the same frictional coefficient.
    Can be combined with other information (such as sedimentation velocity) to better determine molecular weight and shape.

Outline of Chromatography Experiment

Day 1:

1. Prepare column
   - Pour hydrated beads into glass column.
   - Flow several column-volumes of buffer through column.
2. Calibrate column
   - Apply a mixture of a large and a small molecule, blue dextran (MW $\approx$ 2,000,000) and phenol red (MW 354).
   - Elute column and collect fractions.
   - Measure absorbance of fractions.
3. Record image of SDS gel.

Day 2:

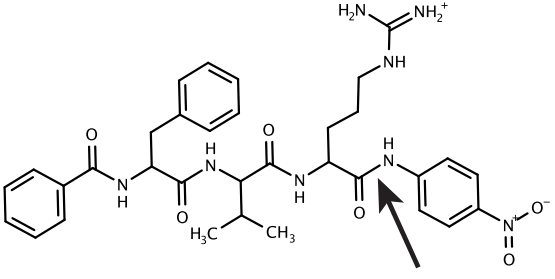
1. Separate trypsin and benzamidine
   - Mix trypsin and benzamidine - inhibit trypsin activity.
   - Apply mixture to column.
   - Elute with buffer.
   - Measure A_{280} of fractions.
   - Measure trypsin activity of peak fraction. Is activity restored?

The Last Experiment: Some Considerations

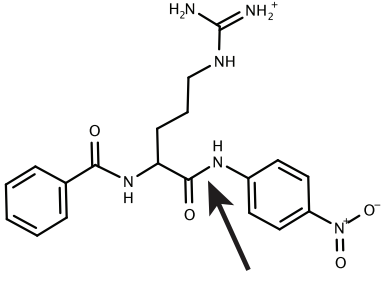
- Compare column sample with inhibited and uninhibited control samples.
- To make a meaningful comparison, the trypsin concentrations must be the same in all of the assays.
- To be detected in the column fractions, the trypsin concentration must be relatively high. ≈ 0.1 mg/mL.
- By comparison, the trypsin samples used in our previous rate measurements had concentrations of ≈ 0.001 mg/mL.
- If we dilute the trypsin samples in this experiment to make the rate slow enough to measure, we will also dilute any inhibitor present.
- The solution: Use a substrate that is cleaved more slowly than Bz-Phe-Val-Arg-*p*-NA.

Two Chromogenic Substrates for Trypsin

Bz-Phe-Val-Arg-*p*-nitroanilide

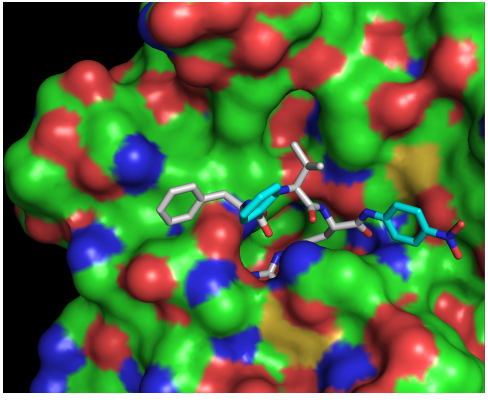


Bz-Arg-*p*-nitroanilide

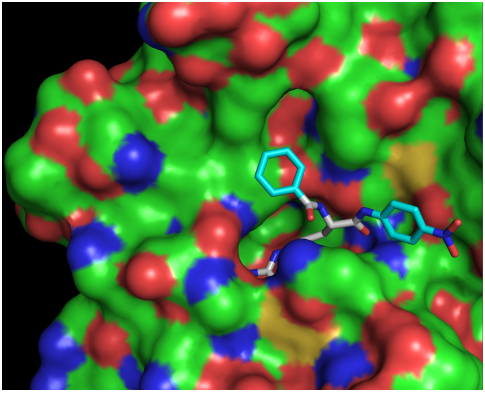


Models of the Two Substrates Bound to Trypsin

Bz-Phe-Val-Arg-*p*-nitroanilide



Bz-Arg-*p*-nitroanilide



- How will K_m and k_{cat} for the two substrates differ?

Clicker Question #3

For Bz-Arg-*p*-nitroanilide, compared to Bz-Phe-Val-Arg-*p*-nitroanilide,

A) K_m will be greater, and k_{cat} will be the same.

B) k_{cat} will be smaller, and K_m will be the same.

C) K_m will be greater, and k_{cat} will be smaller.

D) K_m will be greater, and k_{cat} will be greater.

E) K_m will be smaller, and k_{cat} will be smaller.

All answers count for now.

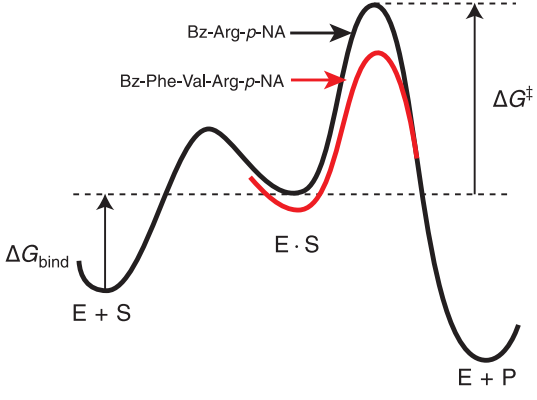
Kinetic Parameters for the Two Substrates

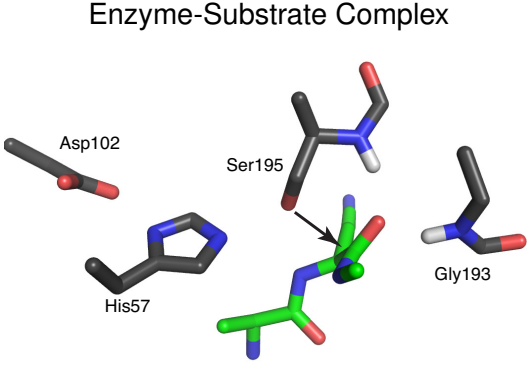
	K_m	k_{cat}	k_{cat}/K_m
Bz-Arg-*p*-nitroanilide	$600\,\mu$M	$1\,s^{-1}$	$1.7\times10^{3}\,s^{-1}M^{-1}$
Bz-Phe-Val-Arg-*p*-nitroanilide	$60\,\mu$M	$10\,s^{-1}$	$1.7\times10^{5}\,s^{-1}M^{-1}$

Adding Phe and Val to Bz-Arg-*p*-nitroanilide

- Decreases K_m by $\approx$ 10- fold.
- Increases k_{cat} by $\approx$ 10-fold.
- Increases k_{cat}/K_m by $\approx$ 100-fold.

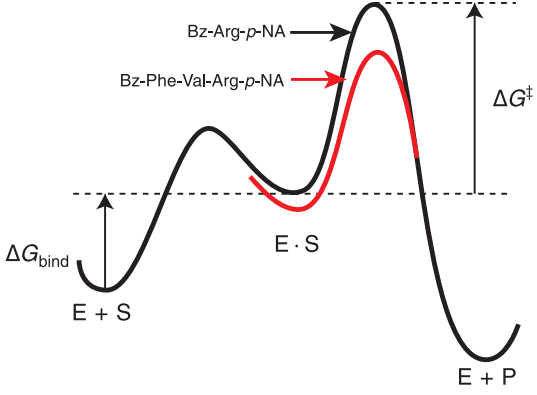
Why Does Making the Substrate Bigger Increase k_{cat}?

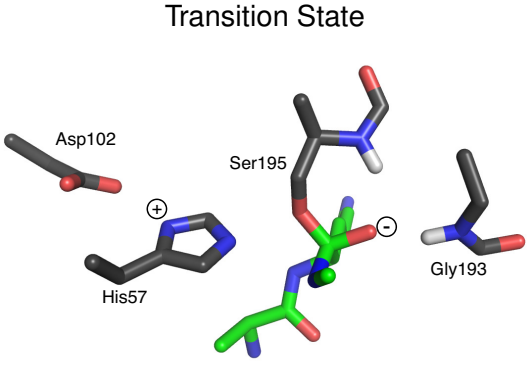




- Extra atoms of the larger substrate stabilize the E · S complex, relative to the free enzyme and substrate.
- But, the extra atoms stabilize the transition state *more*.

Why Does Making the Substrate Bigger Increase k_{cat}?





- Extra atoms of the larger substrate stabilize the E · S complex, relative to the free enzyme and substrate.
- But, the extra atoms stabilize the transition state *more*.

- A hypothesis: The extra interactions with Phe and Val in Bz-Phe-Val-Arg-*p*-NA push the carbonyl group towards the tetrahedral conformation of the transition state.