Protein Purification III: Electrophoresis and Enzyme Assays

After you've expressed a protein and purified it, you need to know

1) How pure is it?
2) What is the concentration?
3) What is the activity (for an enzyme)?

Electrophoresis

Protein purity determination by SDS-PAGE

SDS: sodium dodecyl sulfate
PAGE: polyacrylamide gel electrophoresis

Principles

- (-) charged molecules are attracted to a (+) electrode when a charge (potential) is applied.
- If molecules have evenly spaced charge, they migrate according to size.
- The migration depends on the medium(gel) used.

Electrophoresis – general principle

$$F = q.E / d \qquad (1)$$

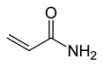
E/d is often referred to as the field strength

$$F = 6 \pi \eta r v \qquad (2)$$

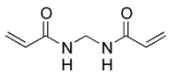
Drag force

$$v = E.q / (6 \pi \eta r d) \qquad (3)$$

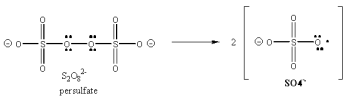
Polyacrylamide Gels

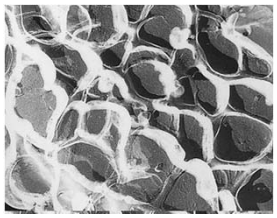


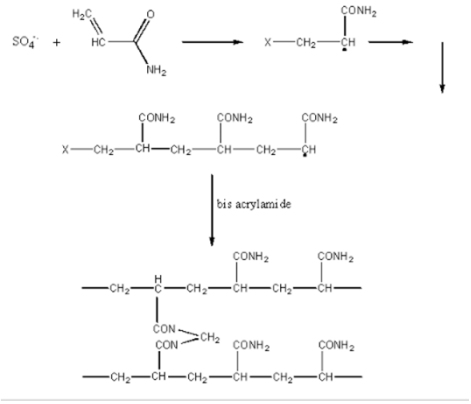
Acrylamide



N,N'-methylenebisacrylamide

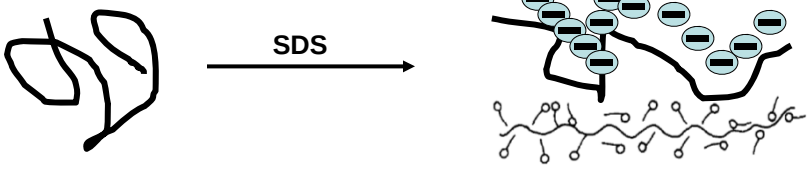






SDS PAGE = SDS polyacrylamide gel electrophoresis

- sodium dodecyl (or lauryl) sulfate, SDS : CH_3-$(CH2)_{11}$- SO_4^{--}
- CH_3-CH_2-CH_2-CH_2-CH_2-CH_2-CH_2-CH_2-CH_2-CH_2-CH_2-CH_2-SO_4^{--}



-All the polypeptides are denatured and behave as random coils
-All the polypeptides have the same charge per unit length
-All are subject to the same electromotive force in the electric field
-Separation based on the sieving effect of the polyacrylamide gel
-Separation is by molecular weight only
-SDS does not break covalent bonds. Boiling after adding SDS also help complete denaturation.

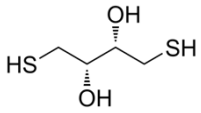
Disulfides between 2 cysteines can be cleaved in the laboratory by reduction, i.e., adding 2 Hs (with their electrons) back across the disulfide bond.

One adds a reducing agent:
2-mercaptoethanol (HO-CH2-CH2-SH).
In the presence of this reagent, one gets exchange among the disulfides and the sulfhydryls:

Protein-CH_2-S-S-CH_2-Protein + 2 HO-CH_2CH_2-SH --->

Protein-CH_2-SH + HS-CH_2-Protein + HO-CH_2CH_2-S-S-CH_2CH_2-OH

The protein's disulfide gets reduced (and the S-S bond cleaved), while the 2-mercaptoethanol gets oxidized, losing electrons and protons and itself forming a disulfide bond.



Dithiothreitol (DTT) is another common reducing agent.

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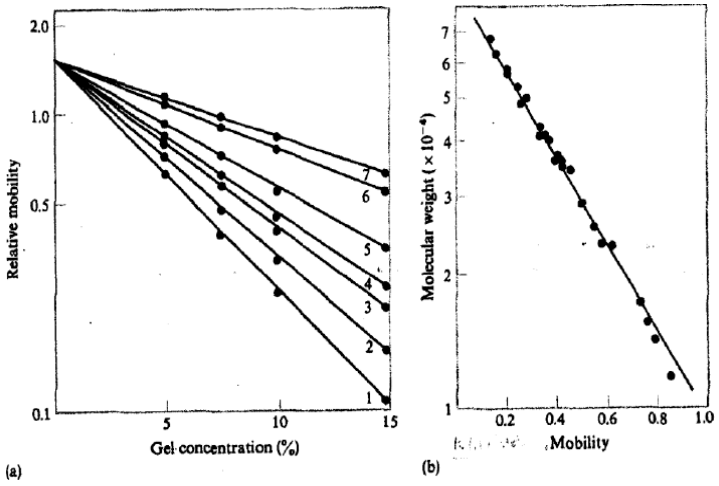
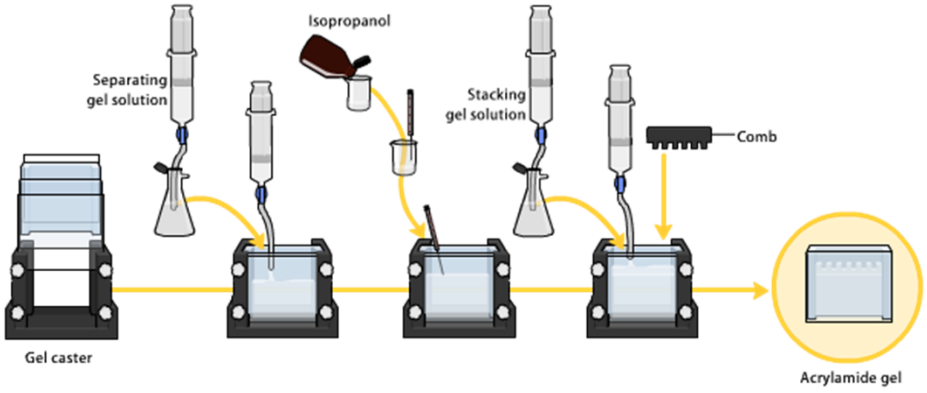


Figure 12-18

SDS polyacrylamide-gel electrophoresis of proteins. **(a)** Relative mobility of seven proteins as a function of the concentration of the acrylamide gel. The molecular weights of the proteins range from 14,000 d for the coat protein of phage R17 (curve 7) to 60,000 d for catalase (curve 1). **(b)** Relative mobility of various proteins in 10% acrylamide gels as a function of molecular weight. [After K. Weber and M. Osborn, in *The Proteins*, 3d ed., vol. 1 (New York: Academic Press, 1975), p. 179.]

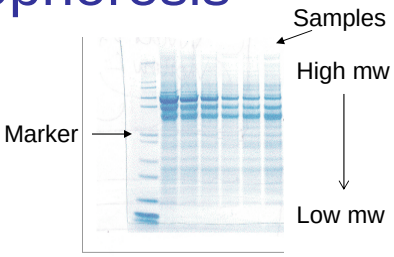
Step 1: Make Gels

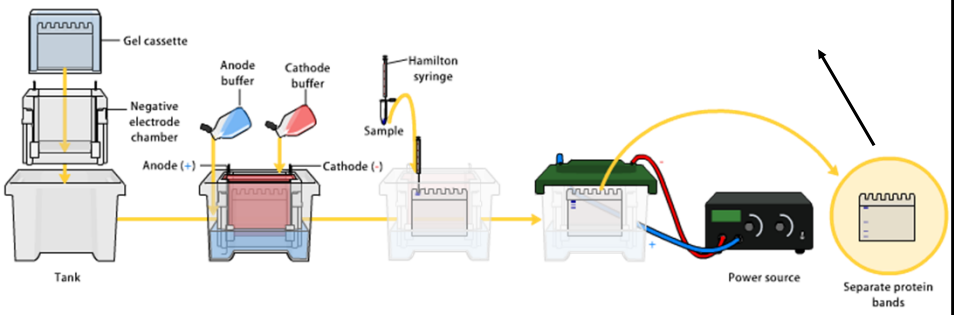


? Cathode (-) /Anode (+)

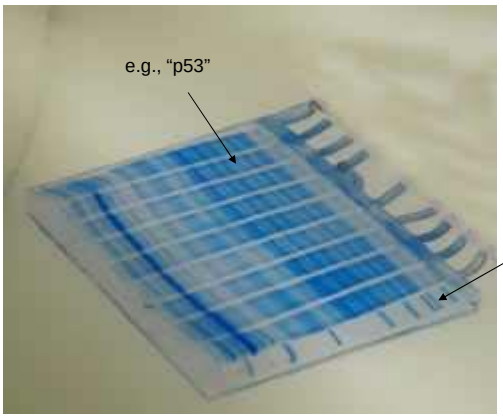
Step 2: Electrophoresis

- How do you tell the MW of the protein bands?

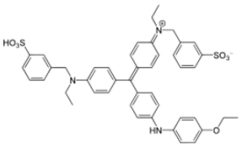




P.A.G.E.



Molecular weight markers (proteins of known molecular weight)



Coomassie Brilliant Blue

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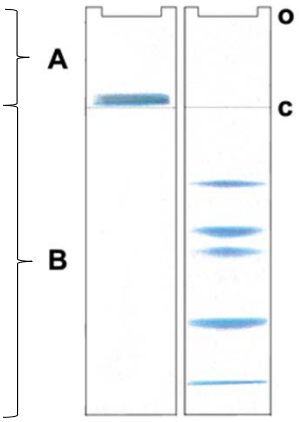
How does stacking gel work in SDS-PAGE?

http://www.biochem.arizona.edu/classes/bioc463a/Info/lecture_notes/PAGE.pdf

Stacking Gel
- pH 6.8, 4% acrylamide
- Proteins can migrate regardless of its size and stack at the boundary of two layers b/c glycine moves behind.

Glycine (pH 6.8, small charge)
SDS-coated Protein
Cl-

Resolving Gel
- pH 8.8, 12-18% acrylamide
- At pH 8.8, Glycine (completely (-)) and Cl- both run out quickly.
- Proteins now can separate according to their sizes.



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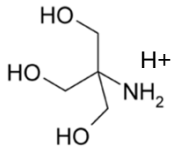
Stacking Gel Interactions:

• When an electrical current is applied to gel, ions carry the current to the anode (+).
• Cl- ions, having the highest charge/mass ratio migrate faster, being depleted at cathode end and concentrated at anode end.
• Glycine from electrophoresis buffer enters gel at pH 6.8 and becomes primarily zwitterionic moving slowly. (pKa1=2.5, pKa2=9.6 and pI=6.0)
• Protein, coated with SDS has a higher charge/mass ratio than glycine so moves fast, but slower than Cl-.
• When protein encounters resolving gel it slows down due to increased frictional resistance (smaller pore size), allowing following protein to “catch up” or stack.
• As protein is depleted from cathode end, glycine must carry current so begins to migrate behind protein, in essence concentrating the proteins further at stacking gel/resolving gel interface.

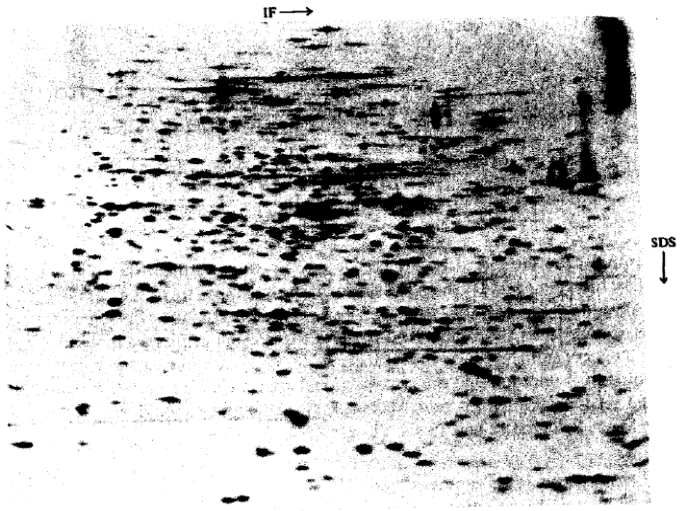
Resolving Gel Interactions:

• When glycine reaches resolving gel it becomes anionic and migrates much faster than protein due to higher charge/mass ratio.
• Now proteins are sole carrier of current and separate according to their molecular mass due to sieving effect of pores in gel.

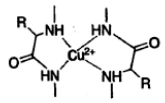
Why Tris not counted in SDS-PAGE?



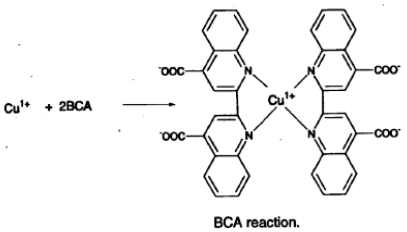
2-D electrophoresis: IEF & SDS-PAGE



Protein Concentration – BCA assay



Putative cupric complex with peptide bond.



BCA reaction.

bicinchoninic acid

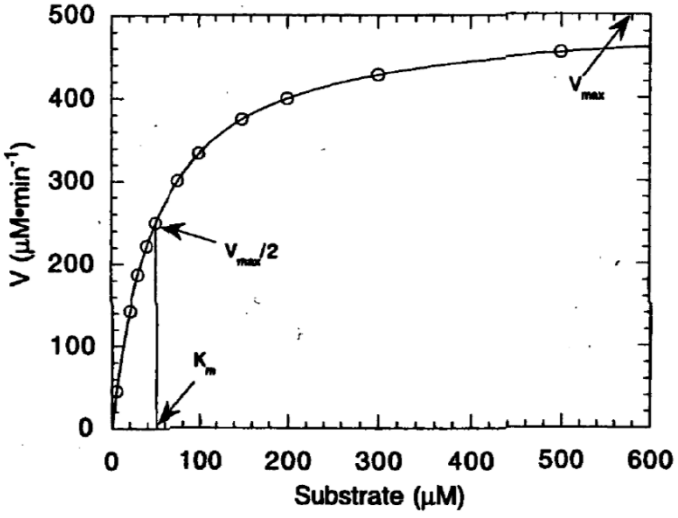
Purple

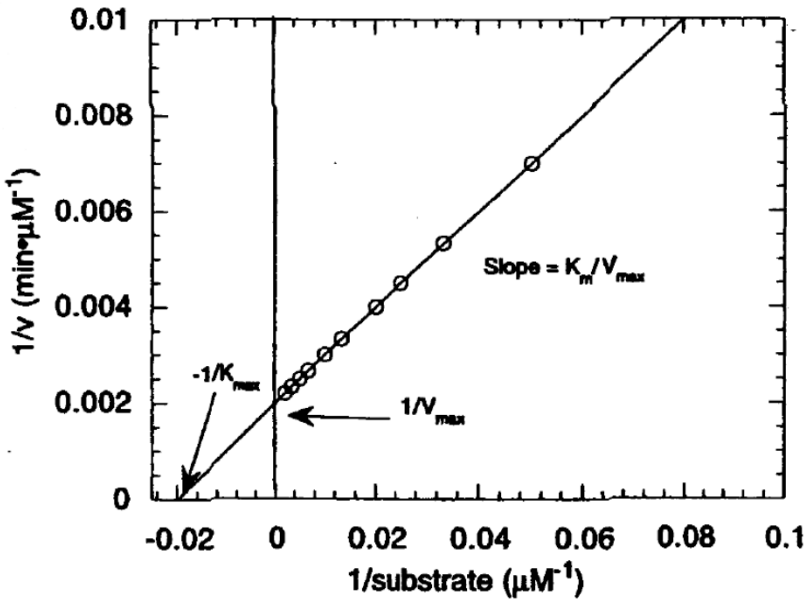
* Bradford assay
- Uses Coomassie blue.
- Abs max. change
465 nm (brown) → 595 nm (blue)

* UV absorbance at 280 nm
- Used to measure more accurate concentration.

Enzyme activity assays

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_{cat}} E + P \qquad V = \frac{k_{cat}[E_T][S]}{[S] + K_M}; K_M = \frac{k_{-1} + k_{cat}}{k_1}$$





Step	Protein (mg)	Total activity (milliunits)	Specific activity (milliunits/mg)	Yield (%)	Purification (fold)
Crude extract	1070	890	0.8	-	-
$(NH_4)_2SO_4$ precipitation	400	580	1.5	65	1.9
Gel Filtration Chromatography (Sephadex G50)	38	278	7.3	31	9.0
Ion Exchange Chromatography (Q-Sepharose)	2	96	58.0 48.0	11	73.0

One unit (U) of enzyme activity is the amount of enzyme that hydrolyzes 1 μmole of substrate per minute at 37°C