

Lecture 3

Protein function and techniques

April 5, 2016
Pyle

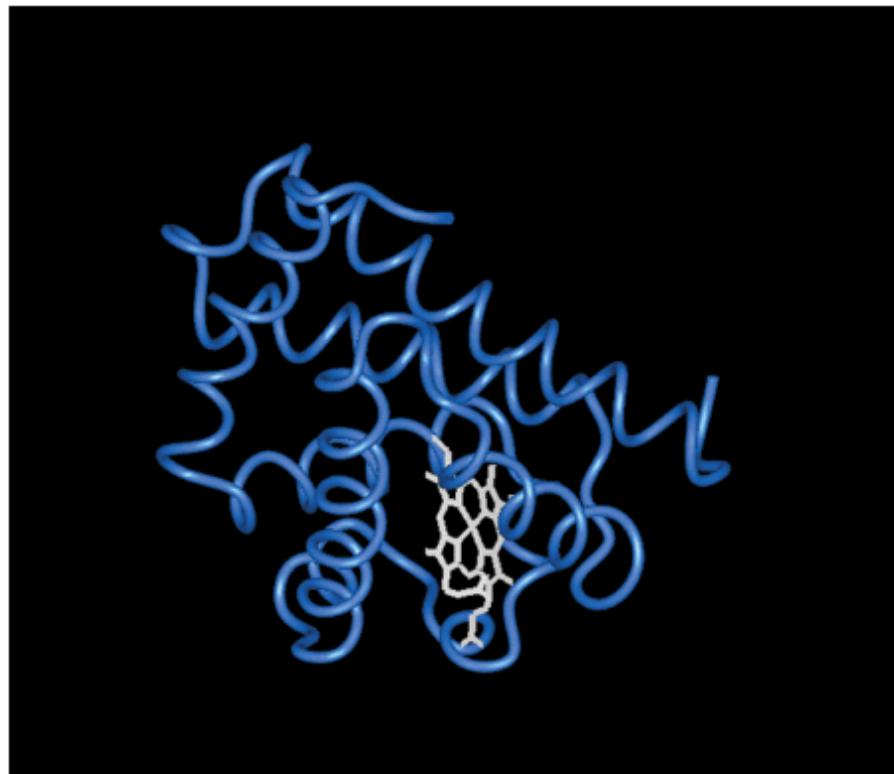
How do proteins work to regulate cell functions?

- Proteins work by binding to other molecules, called ligands or substrates, including small ones such as ions or sugars, and large ones such as proteins, DNA, and RNA.
- A protein's structure determines its function
 - structural proteins,
 - enzymes,
 - transcription factors
 - signal transduction proteins
- Proteins with the similar function can be grouped together called protein families, members of the same protein family usually have similar structures

Homology between the amino acid sequences of myoglobin and α -globin

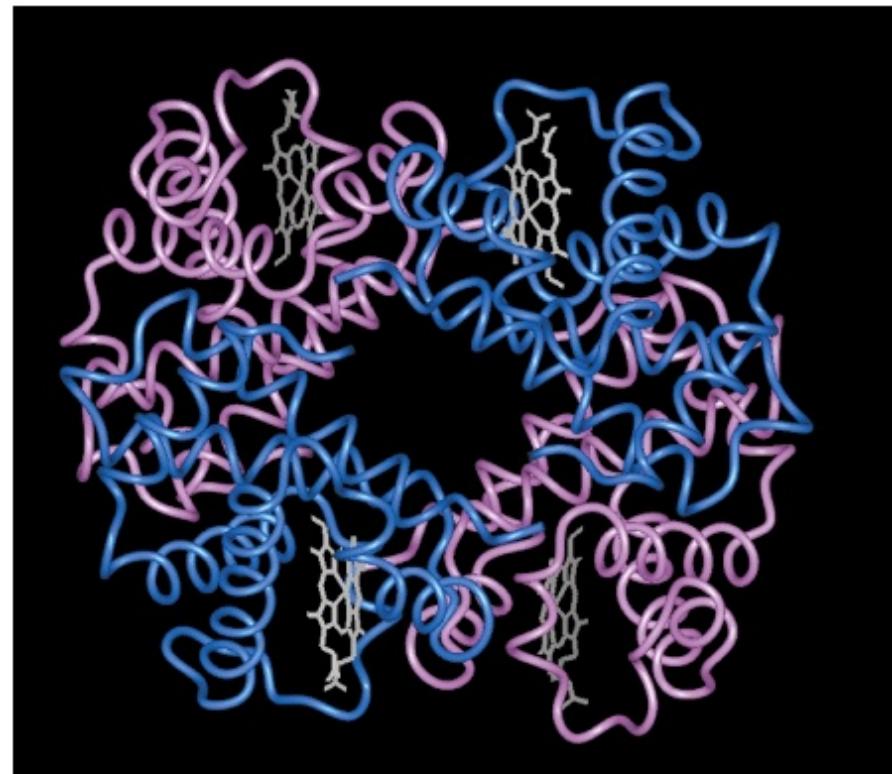
	1	Leu	Ser	Glu	5	Gly	Glu	Trp	Gln	Leu	10	Val	Leu	His	Val	15	Ala	Lys
(b)	Val	Val	Leu	Pro	Gly	Ala	Asp	Lys	Thr	Asn	Val	Val	Lys	Ala	Ala	Trp	Gly	Lys
Myoglobin	Val	Val	Glu	Ala	Asp	His	Val	Ala	Gly	Gly	His	Gly	Gly	Gln	Asp	Ile	Leu	Leu
Hb α chain	Val	Val	Gly	Ala	His	Phe	Gly	Glu	Tyr	Ala	Glu	Ala	Glu	Ala	Glu	Ile	Arg	Met
	Phe	Phe	Lys	Ser	Ser	His	Phe	Pro	Glu	Thr	Thr	Leu	Glu	Lys	Phe	Asp	Arg	Leu
	Phe	Phe	Leu	Ser	Ser	Phe	Pro	Pro	Thr	Thr	Thr	Lys	Thr	Tyr	Phe	Pro	His	Asp
	Leu	Leu	Lys	Thr	Glu	Ala	Glu	Met	Lys	Ala	Gly	Ser	Glu	Asp	Leu	Lys	Lys	His
	Gly	Gly	Val	Val	Thr	Val	Leu	Asp	Ala	Ala	Leu	Gly	Ala	Gln	Val	Lys	Gly	Asp
	His	Asp	His	Met	Glu	Ala	Asn	Glu	Leu	Leu	Lys	Ser	Ala	Asn	Ile	Leu	Asp	Lys
	His	Arg	Lys	Leu	Ile	Pro	Asp	Ile	Pro	Val	Tyr	Asn	Leu	Asp	Glu	Ile	Cys	Ile
	His	Val	Val	Thr	Leu	His	Ser	Arg	His	Leu	Pro	Gly	Asp	Phe	Gly	Ala	Asp	Gln
	Gly	Ala	Ala	Ser	Leu	Met	Asn	Lys	Lys	Phe	Pro	Ala	Glu	Phe	Thr	Pro	Ala	His
	Lys	Lys	Tyr	Tyr	Lys	Arg-141	Glu	Leu	Gly	Tyr	Gln	Gly-153						

Members of a protein family have similar structure and function



Myoglobin

16 kD
Single peptide protein
Muscle O₂ carrier



Hemoglobin

64 kD
4-subunit protein
blood O₂ carrier

Enzymes are Key Cell Regulators

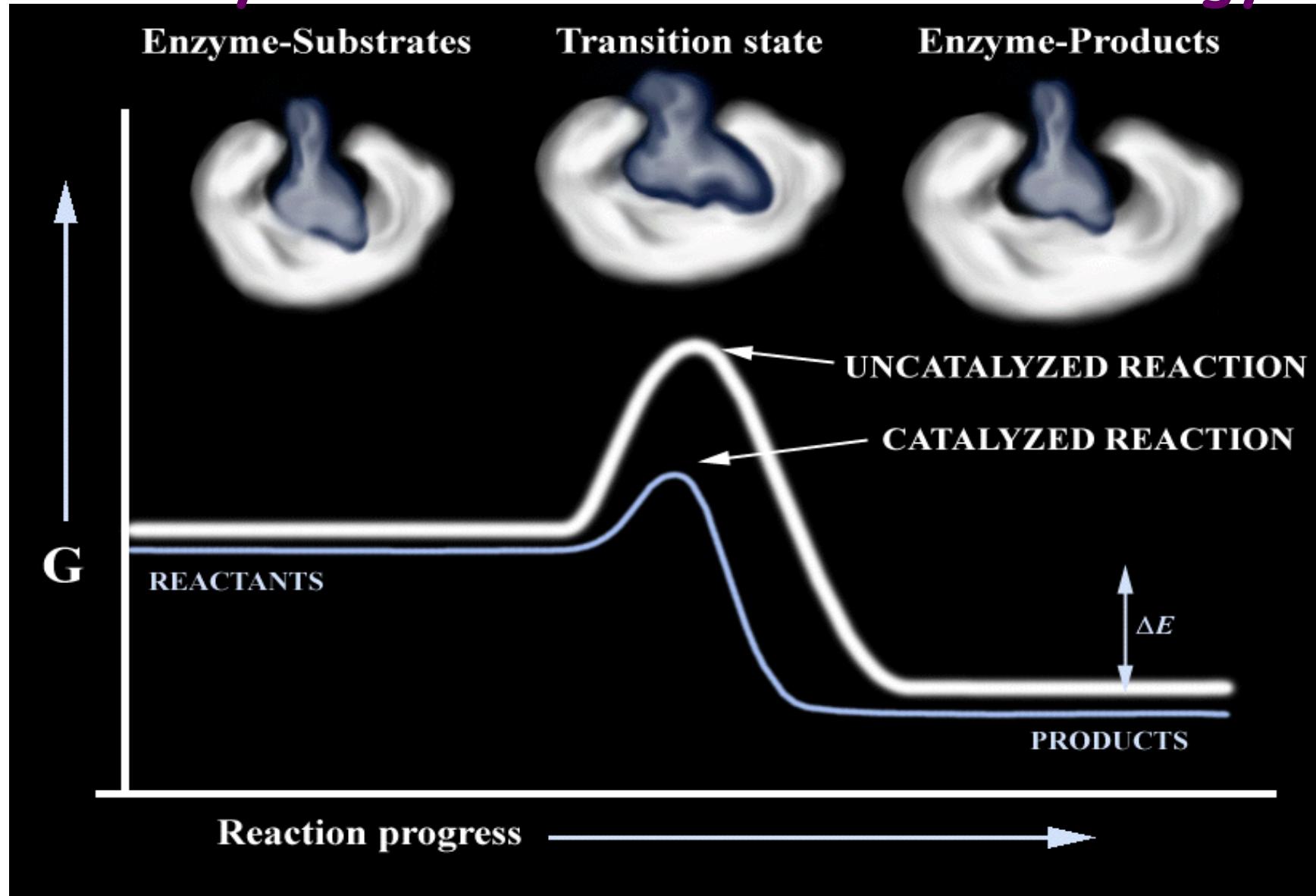
- The suffix “-ase” is usually appended to the name:
 - Protease (degrade protein)
 - Kinase (add Pi)
 - Phosphatase (remove Pi)
 - Ribonuclease (RNase:degrade RNA)

Enzymes are proteins that catalyze cellular reactions



- enzyme converts a substrate to a product without changing itself
- Enzymes speed up reactions by factors of 10^6 or more at relatively low temperature
- Enzymes are highly specific and function by lowering the activation energy of the reaction

Enzymes lower the activation energy



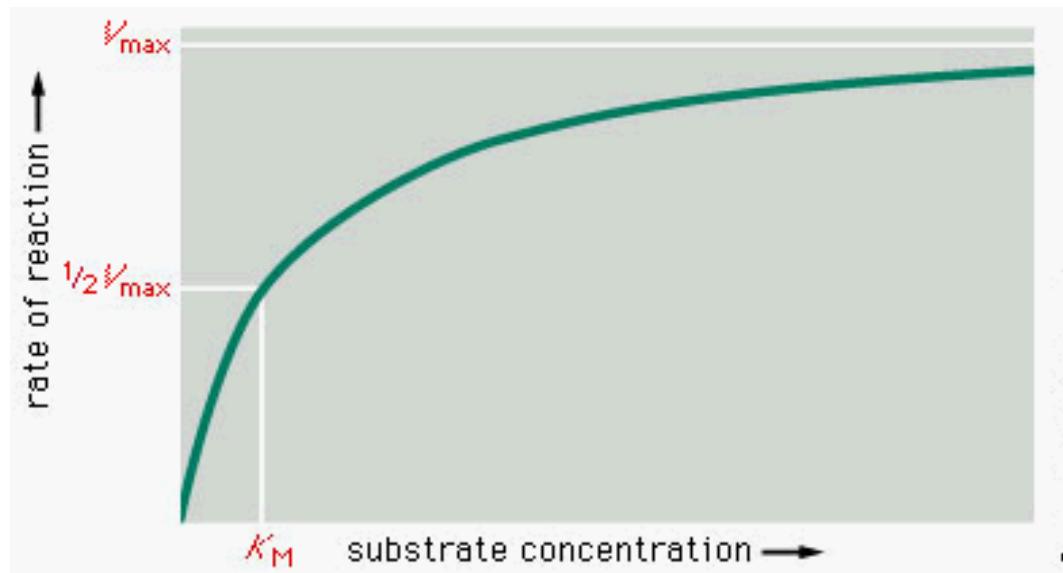
How is the enzymatic activity measured?

V_{max} - maximum rate of the reaction

the bigger the V_{max} , the more active the enzyme

K_m - substrate concentration at $1/2 V_{max}$.

the smaller the K_m , the higher the enzyme-substrate affinity (or the “better” the enzyme)

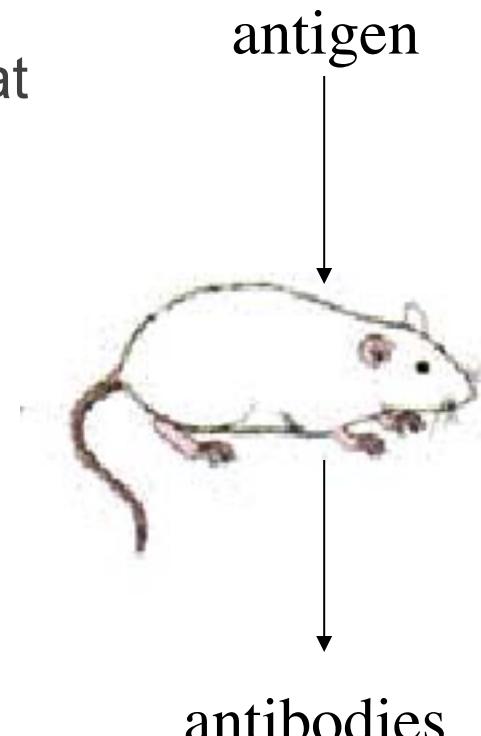
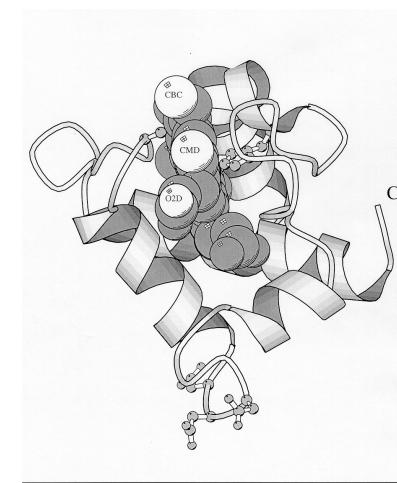


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Knowing enzyme dynamics allows for development of enzyme inhibitors too!

Antibodies as Molecular Tools

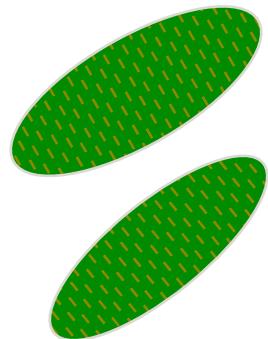
- Animals produce antibodies in response to the injection of foreign materials (Antigens).
- Antibodies bind tightly to specific antigens with $> 10^{-9}$ M affinity.
- Antibodies can distinguish among proteins that differ by only a single amino acid.
- Antibodies can be used to determine if a particular protein is present in a complex mixture of proteins.
- Antibodies can also be used to purify a particular protein away from the other components of a complex mixture.



Antibodies can be used to Neutralize Pathogens

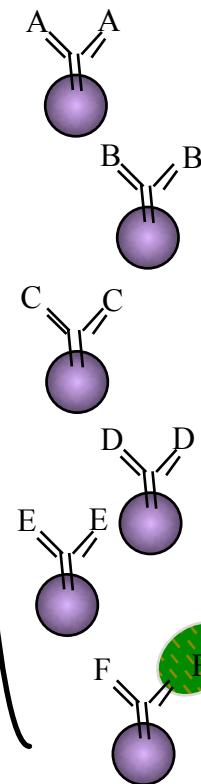
Pathogens

Bacteria
Viruses
Fungi

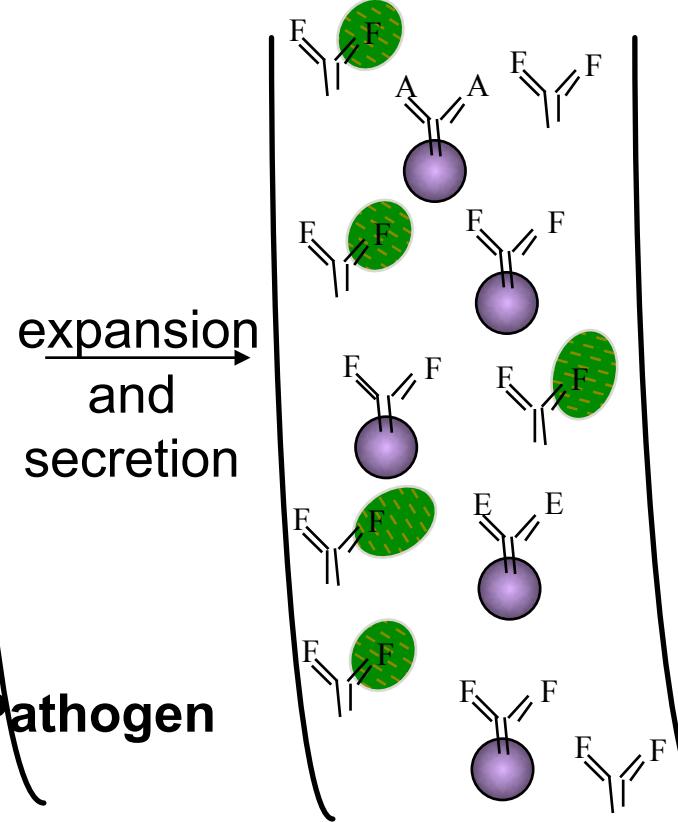


infection

Bloodstream



Bloodstream



Pathogen

Approximately 1 million B cells, each with a different antibody on the cell surface. A small fraction will be capable of binding pathogen (through appropriate non-covalent bonds).

Antibodies secreted into the bloodstream bind and neutralize pathogens.

Randy Wall discovered many of the important functions of AB's in biology!

How do we study proteins in living systems?

I. Separation

How to separate different cellular components?
and different proteins?

(centrifugation, chromatography, SDS-PAGE, etc)

II. Detection (immunological techniques)

How to detect a single protein from a mixture of
proteins

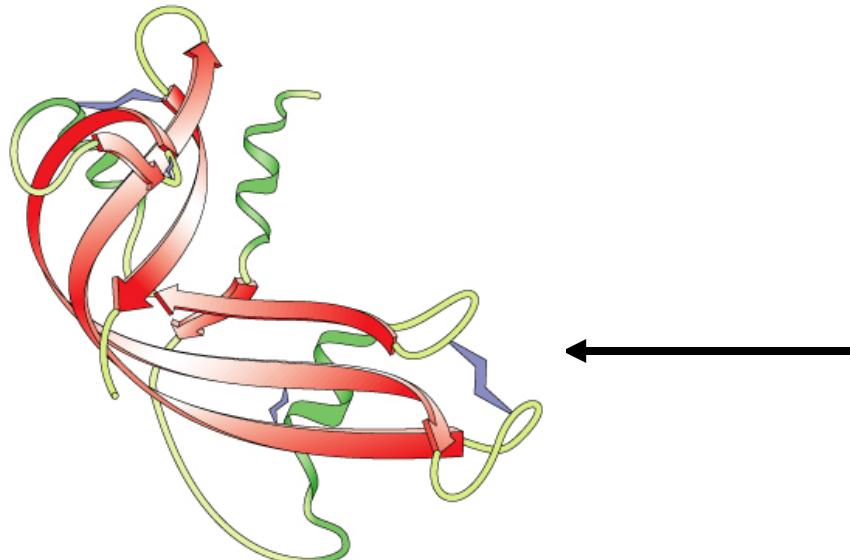
(ELISA, Immunoblot, Immunoprecipitation, etc)

III. Sequence and Structural analysis

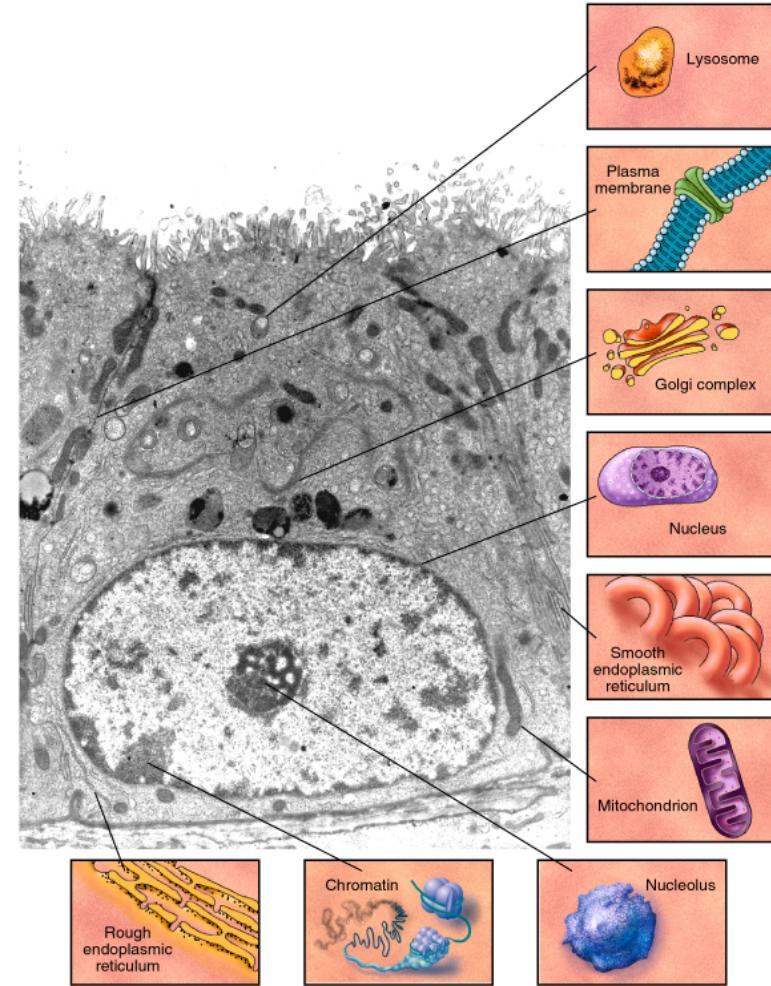
Mass spectrometry, X-ray crystallography, etc

Protein Purification

To study the structure and properties of a protein of interest, it often must be isolated in a pure form.



- A typical mammalian cell contains 3000-5000 different proteins.
- Some proteins are present at only a few molecules per cell.
- Other are present at 10^5 or more molecules per cell.



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I. Protein separation/purification

The first step in the study of a protein is to separate it from other cellular components, or to isolate it in a pure form.

(1) break-up of cells

Physical disruption, Detergent

(2) Centrifugations

Differential centrifugation
Rate-zonal centrifugation

(3) Chromatography

ion exchange, gel filtration, affinity column, etc

(4) Gel Electrophoresis

SDS-PAGE, 2-D gel

Breaking up Cells and Tissues

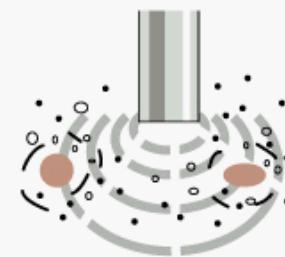
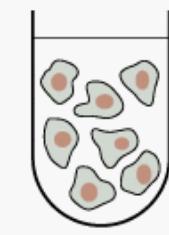
BREAKING CELLS AND TISSUES

The first step in the purification of most proteins is to disrupt tissues and cells in a controlled fashion.

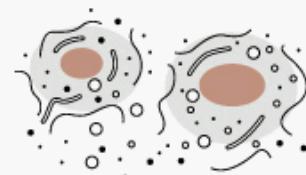
Using gentle mechanical procedures, called homogenization, the plasma membranes of cells can be ruptured so that the cell contents are released. Four commonly used procedures are shown here.

The resulting thick soup (called a homogenate or an extract) contains large and small molecules from the cytosol, such as enzymes, ribosomes, and metabolites, as well as all the membrane-bound organelles.

ultrasound

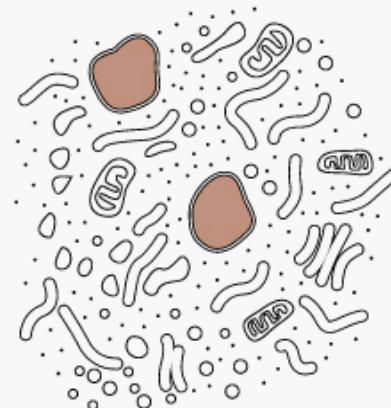


- ① break cells with high frequency sound

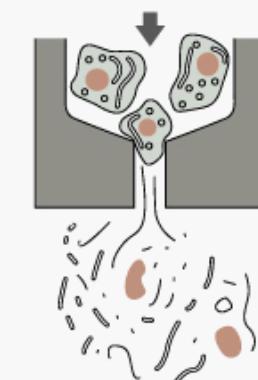


- ② use a mild detergent to make holes in the plasma membrane

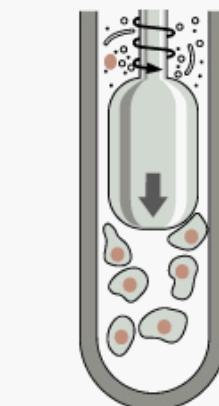
detergent



French press



- ③ force cells through a small hole using high pressure



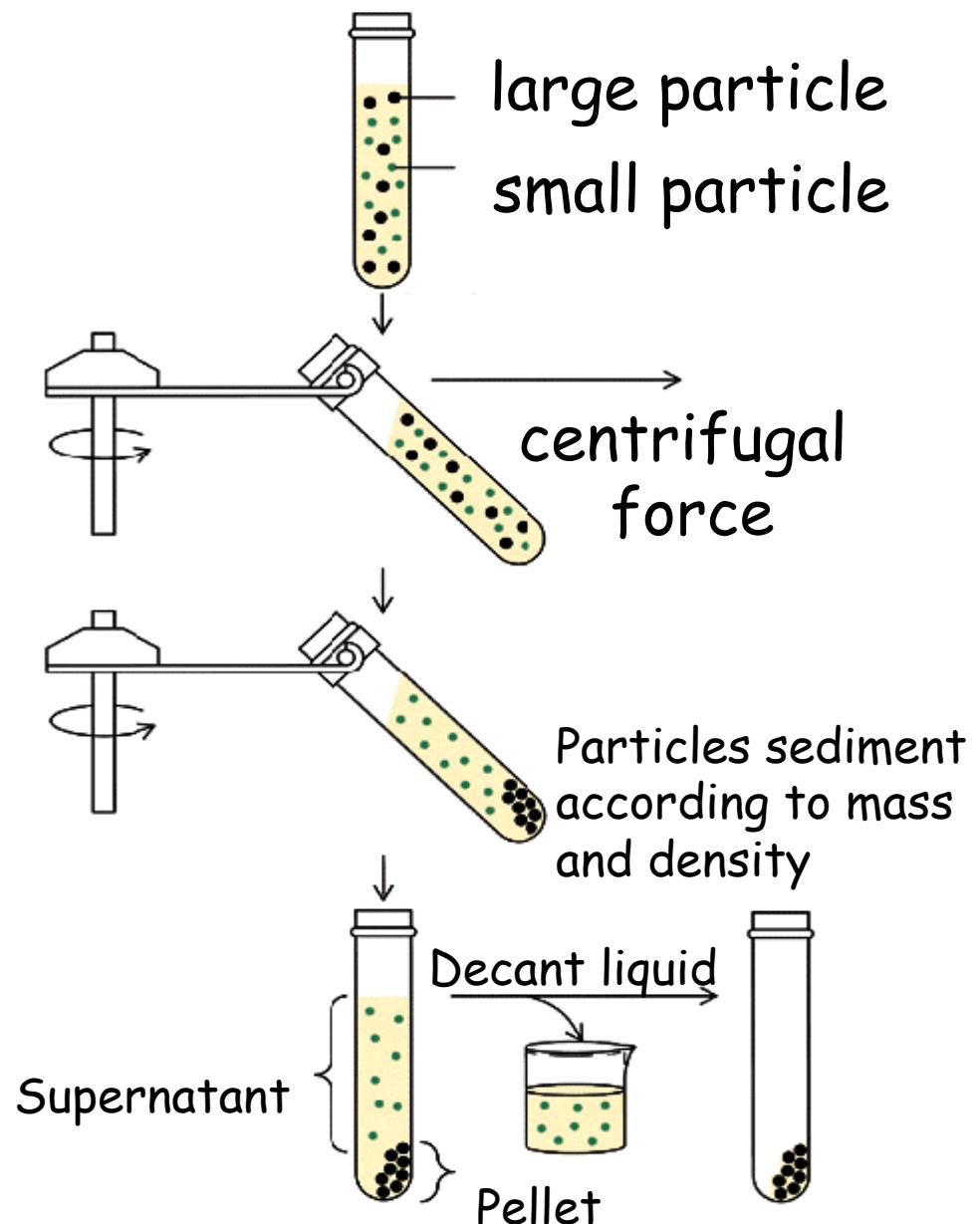
- ④ shear cells between a close-fitting rotating plunger and the thick walls of a glass vessel

homogenize

When carefully applied, homogenization leaves most of the membrane-bound organelles intact.

Differential Centrifugation

Particles (or molecules) that sediment rapidly are separated from slower sedimenting materials that remain in the supernatant. The rapidly sedimenting material is collected in the pellet.



Separates a mixture of particles (macromolecules, cell organelles) that differ greatly in size.

Rate-zonal or velocity centrifugation

A sucrose gradient (eg. 10-30%) is used to provide density stability during centrifugation.

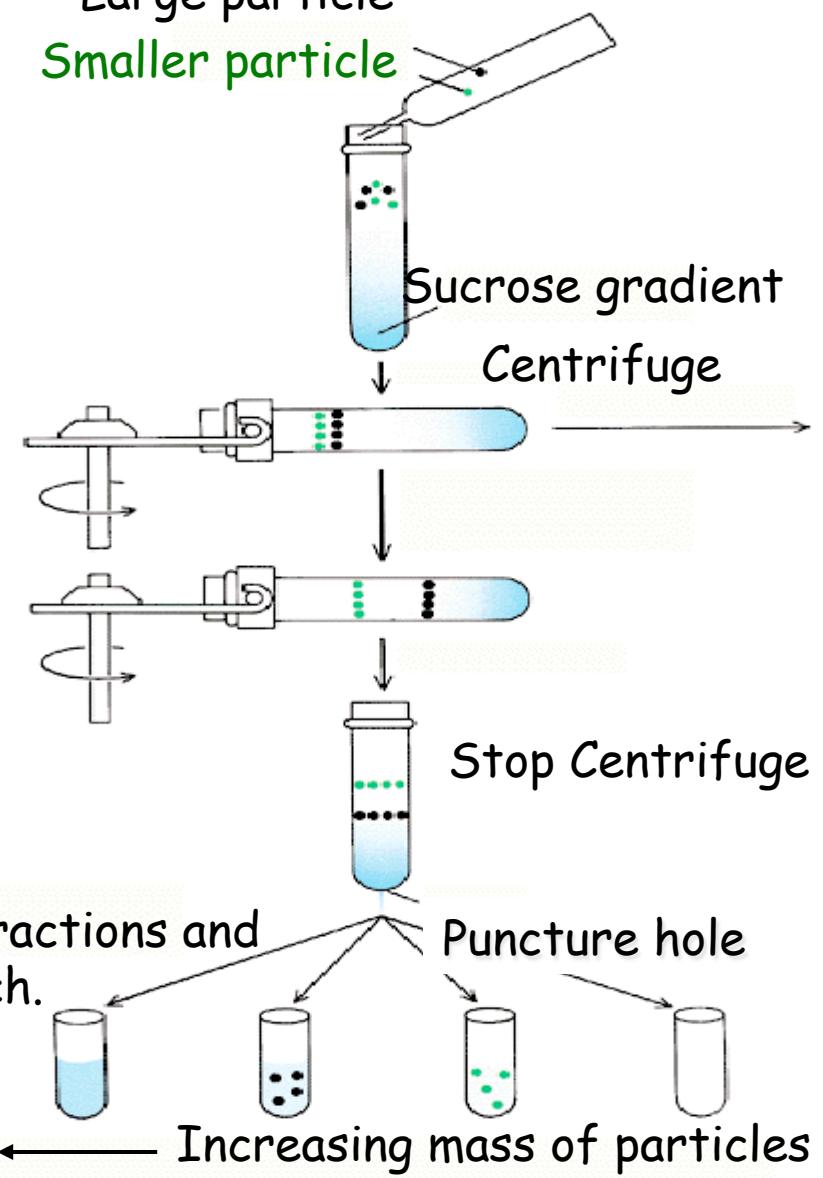
Particles of similar density sediment based primarily on mass and shape

A finer degree of separation with sucrose density gradient

Sample is layered on top of gradient

Large particle

Smaller particle



Column Chromatography

- cylindrical columns filled with water permeable solid matrix (also called bead, resin or gel) of different properties.
- Different proteins behave different in different columns, which allows separation.

(1) Ion exchange chromatography:

- Separate according to charge

(2) Gel filtration chromatography (or sizing column):

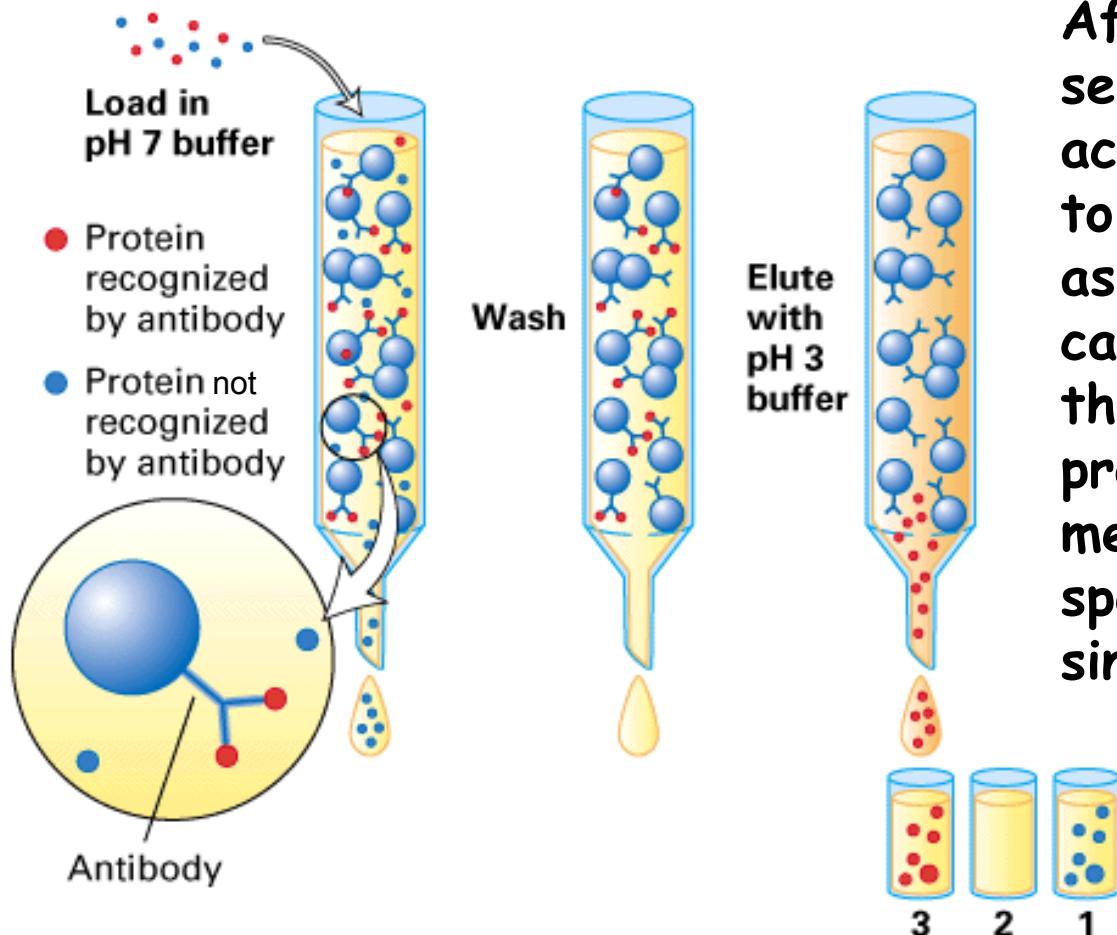
- Separate according to size

(3) Affinity chromatography:

- Separate according to ability to bind to ligands

Antibody Affinity (Immunoaffinity) Chromatography

(c) Antibody-affinity chromatography

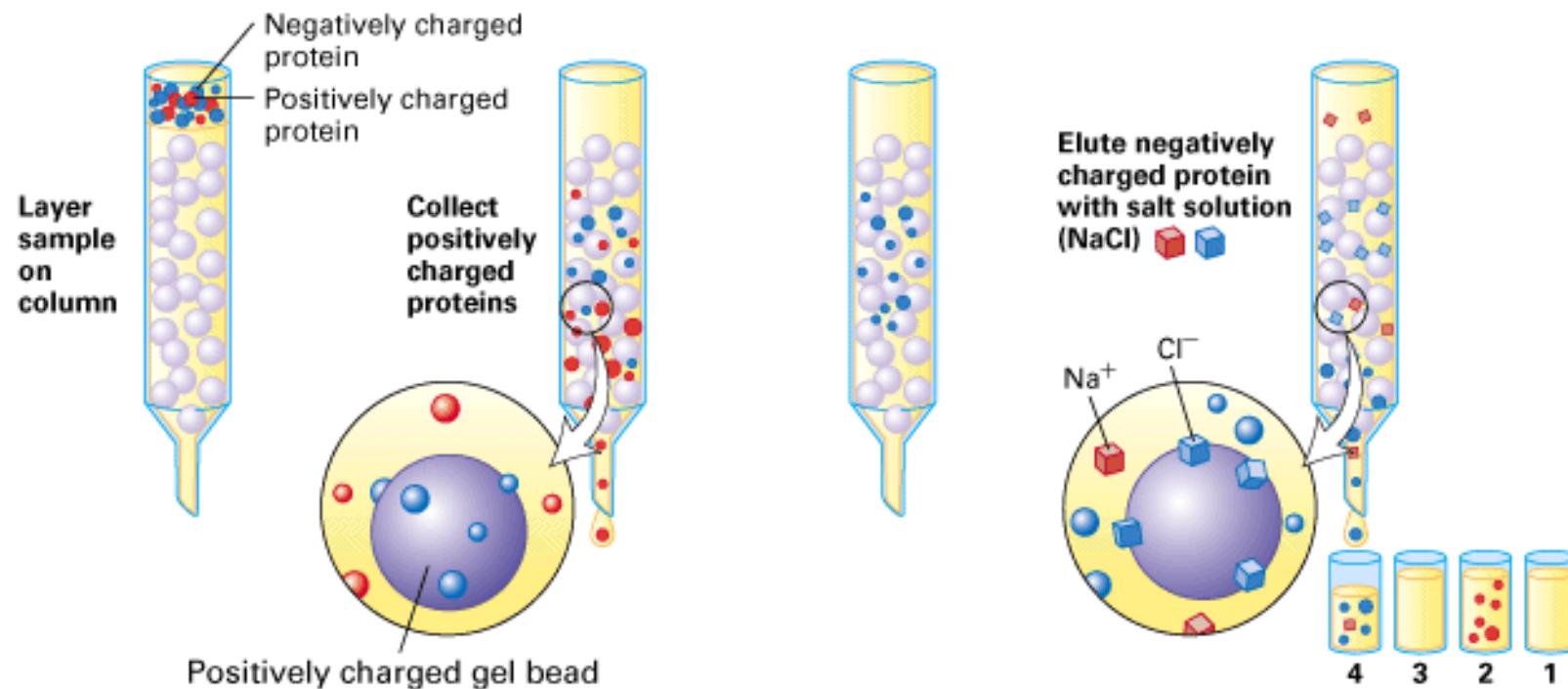


Affinity chromatography separates molecules according to their affinity to a specific ligand, such as an antibody. Beads can be covalently linked to the ligand that the target protein will bind to. This method allows highly specific purification of a single protein

- Non-specific proteins come through the column first.
- Antibody-binding proteins can be eluted by lowering pH.

Ion-Exchange Chromatography

(b) Ion-exchange chromatography



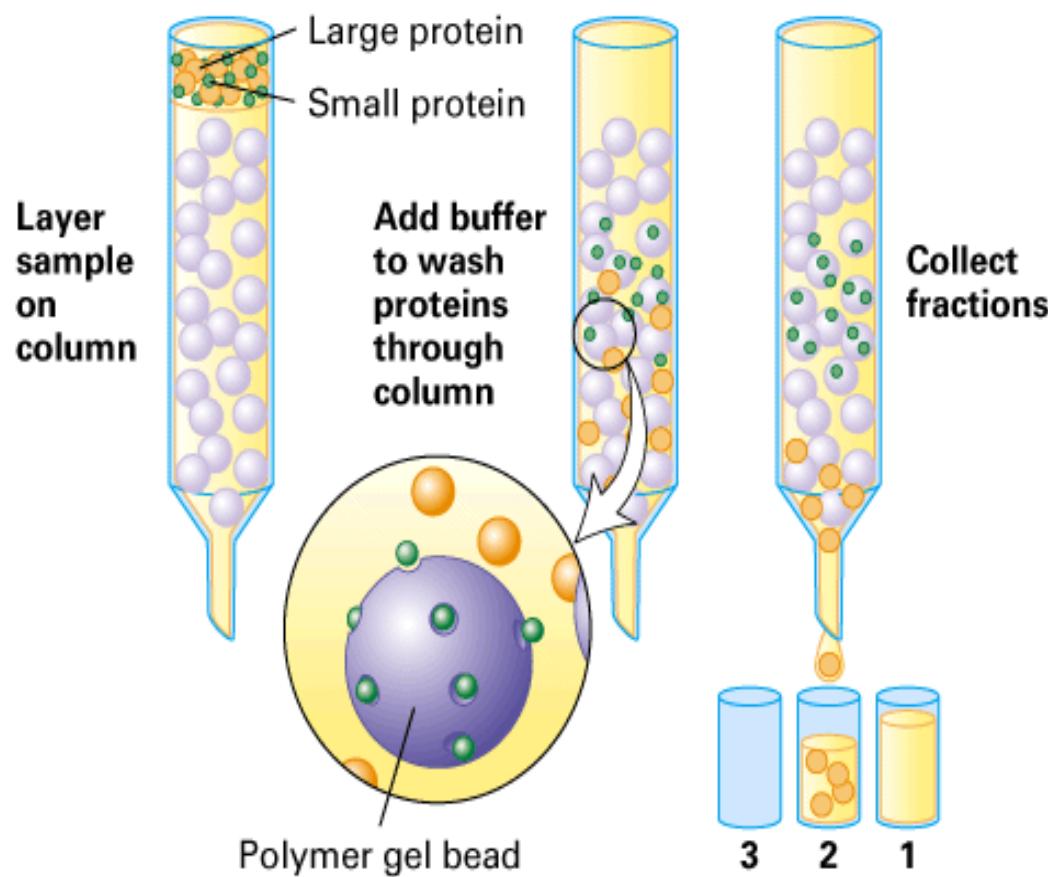
Ion exchange chromatography separate molecules according to their charge

For positively charged beads: Positively charged particles will come through the column first. Negatively charged particles bind the beads, can be eluted later.

For negatively charged beads: Opposite

Gel Filtration Chromatography

(a) Gel filtration chromatography

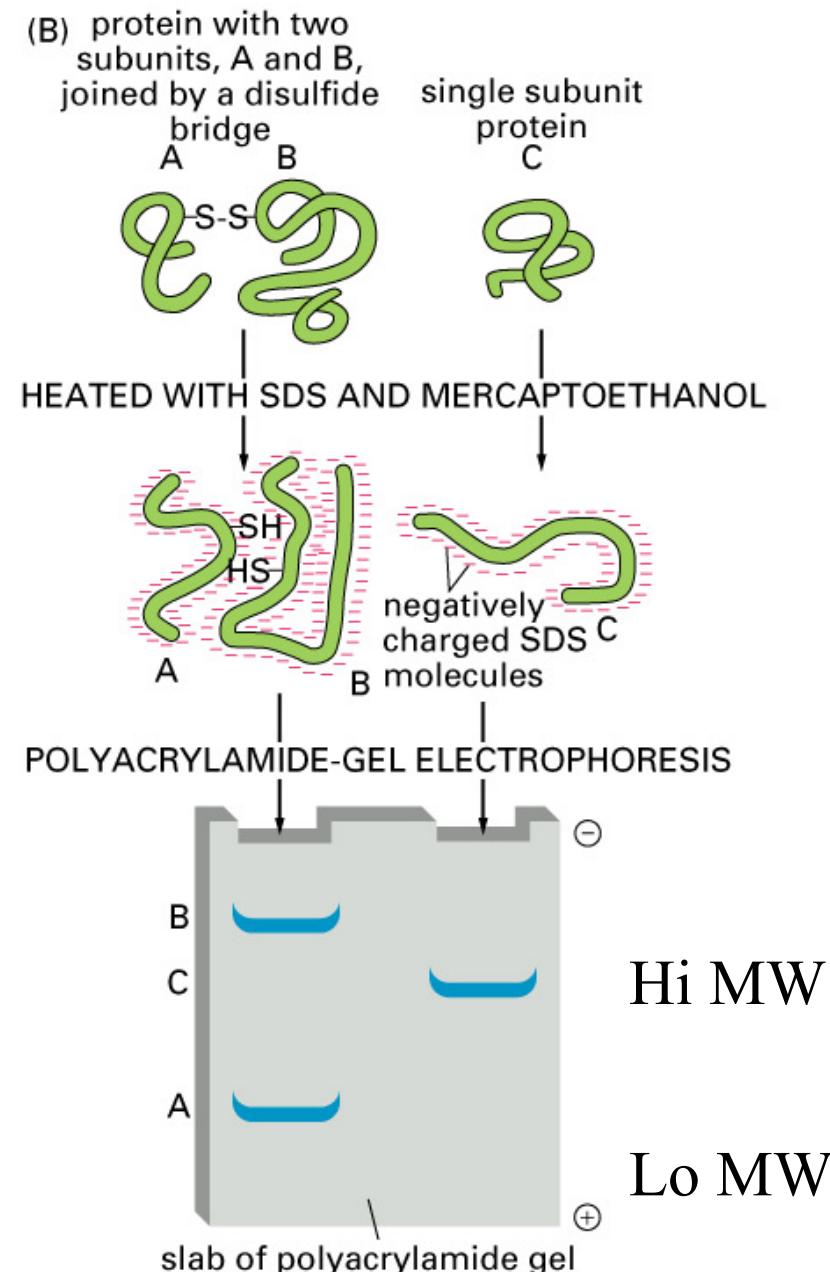


Gel filtration separates molecules according to their size. The larger molecules migrate faster in the gel filtration column. The smaller molecules migrate slower because they take longer to travel through pores of beads

Larger particles come out of the column before smaller particles

How do we detect proteins after chromatography?

- different fractions eluted from column can be collected into a series of tubes, presence of the target protein in each tube can be tested using different methods
- measure absorption at 280nm (A_{280}), protein solution absorbs 280 nm light due to tryptophan and tyrosine.
- detect target protein according to **special properties** (e.g. enzymatic activity, ligand-binding activity, special calorimetric reaction, etc.)
- detect target protein using **immunological** method such as Western blot, ELISA, etc
- run target protein using **gel electrophoresis**



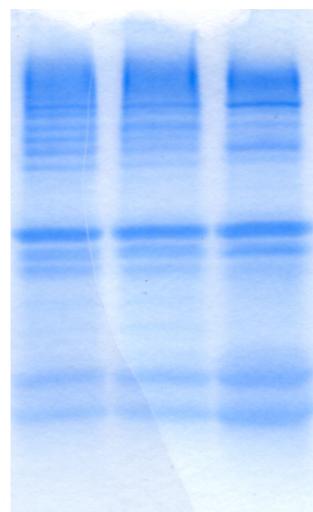
SDS-PAGE: Sodium Dodecyl Sulfate polyacrylamide gel electrophoresis

Denatured proteins are separated according to their Molecular Weight on polyacrylamide gels

Immunoblot (Western blot)

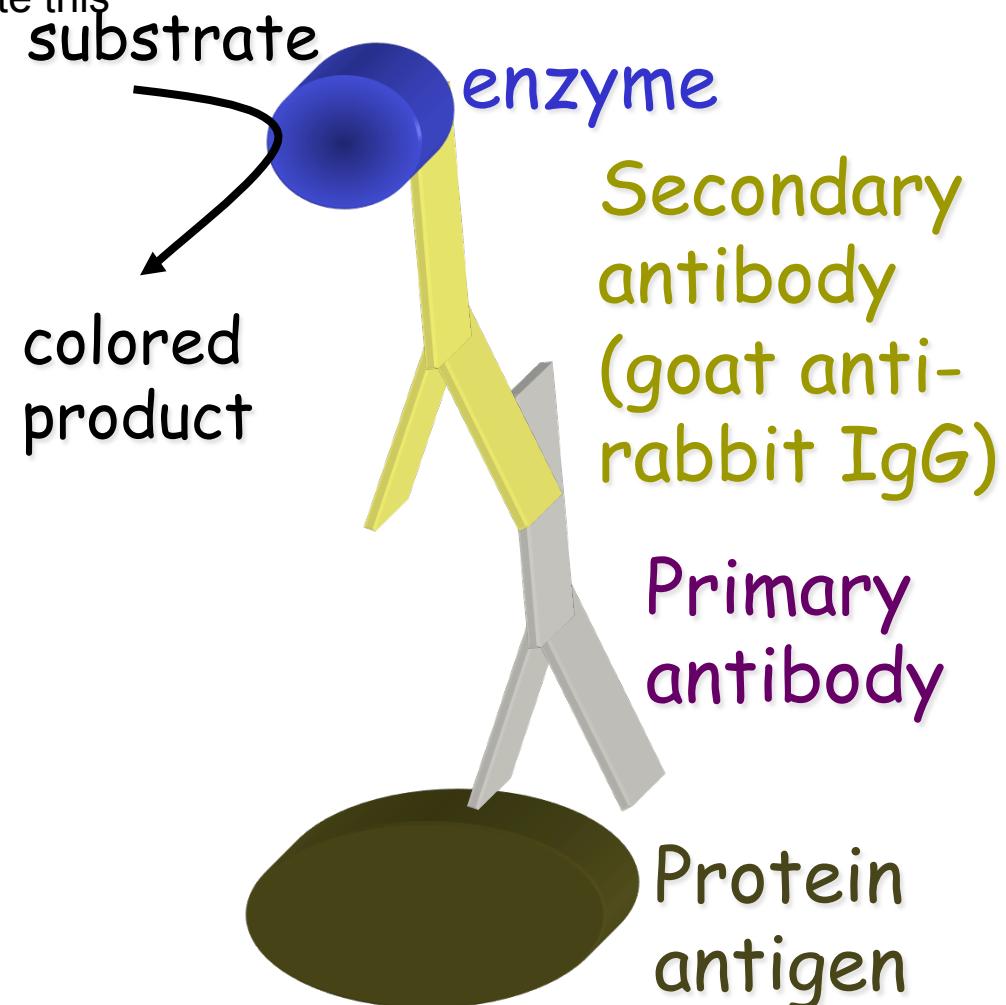
An immunoblot can detect a specific protein within a crude extract based on the ability of an antibody to selectively bind the protein of interest.

-When exposed to an appropriate substrate this enzyme drives a colourimetric reaction and produces a color

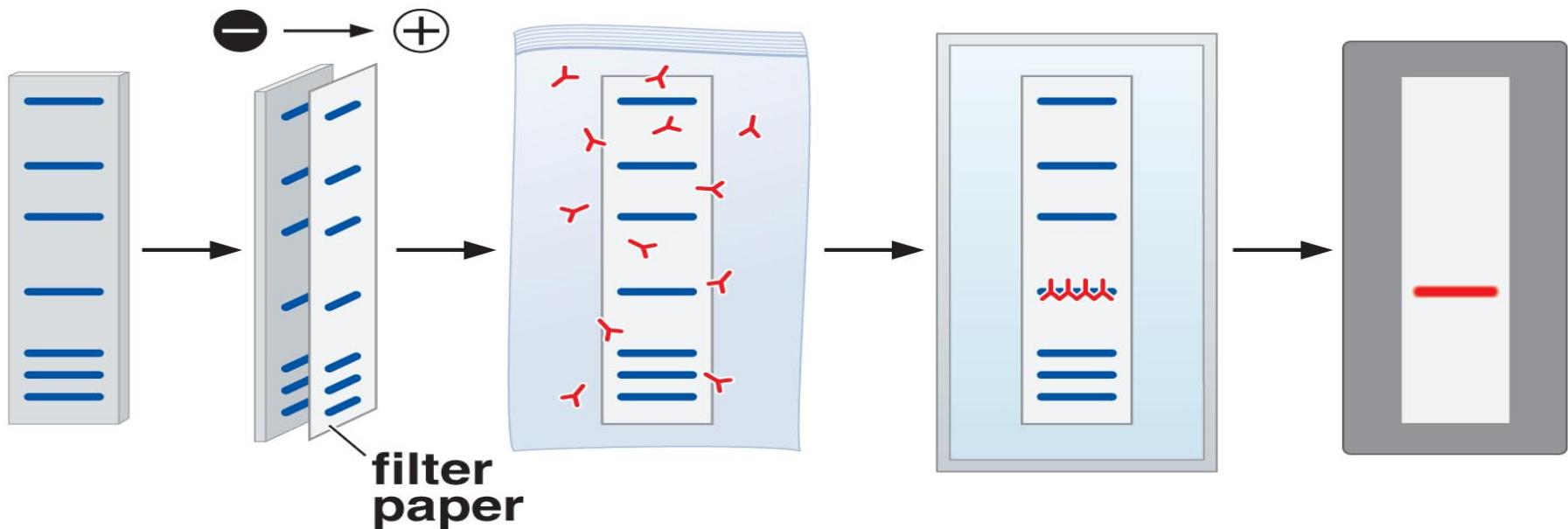


Stained
protein
extracts

Western
Blot

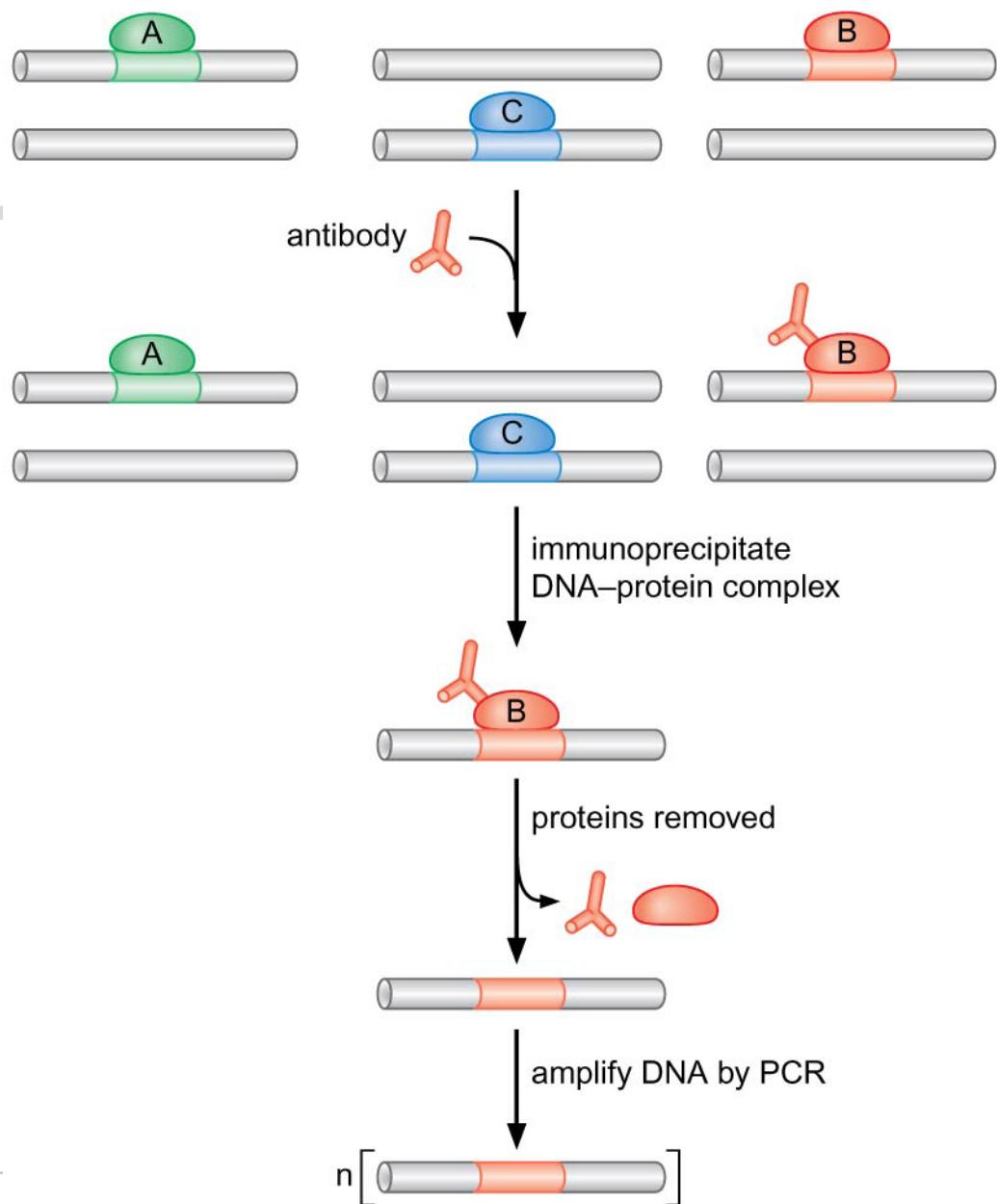


Immunoblot (Western blot)-2



1. Separate protein in a SDS-PAGE gel,
 2. Blot protein bands to a nitrocellulose membrane.
 3. Incubate the membrane with 1st Ab (Ab1) that recognizes the antigen, then the second Ab (Ab2) (or protein A) that recognizes the 1st Ab.
 4. Visualize antigen by either radioactively label of the 2nd Ab or protein A, or a colorimetric reactions catalyzed by the marker enzyme conjugated to the 2nd Ab or protein A.
- Most commonly, a horseradish peroxidase-linked secondary is used to cleave a chemiluminescent agent, and the reaction product produces luminescence in proportion to the amount of protein. A sensitive sheet of photographic film is placed against the membrane, and exposure to the light from the reaction creates an image of the antibodies bound to the blot.

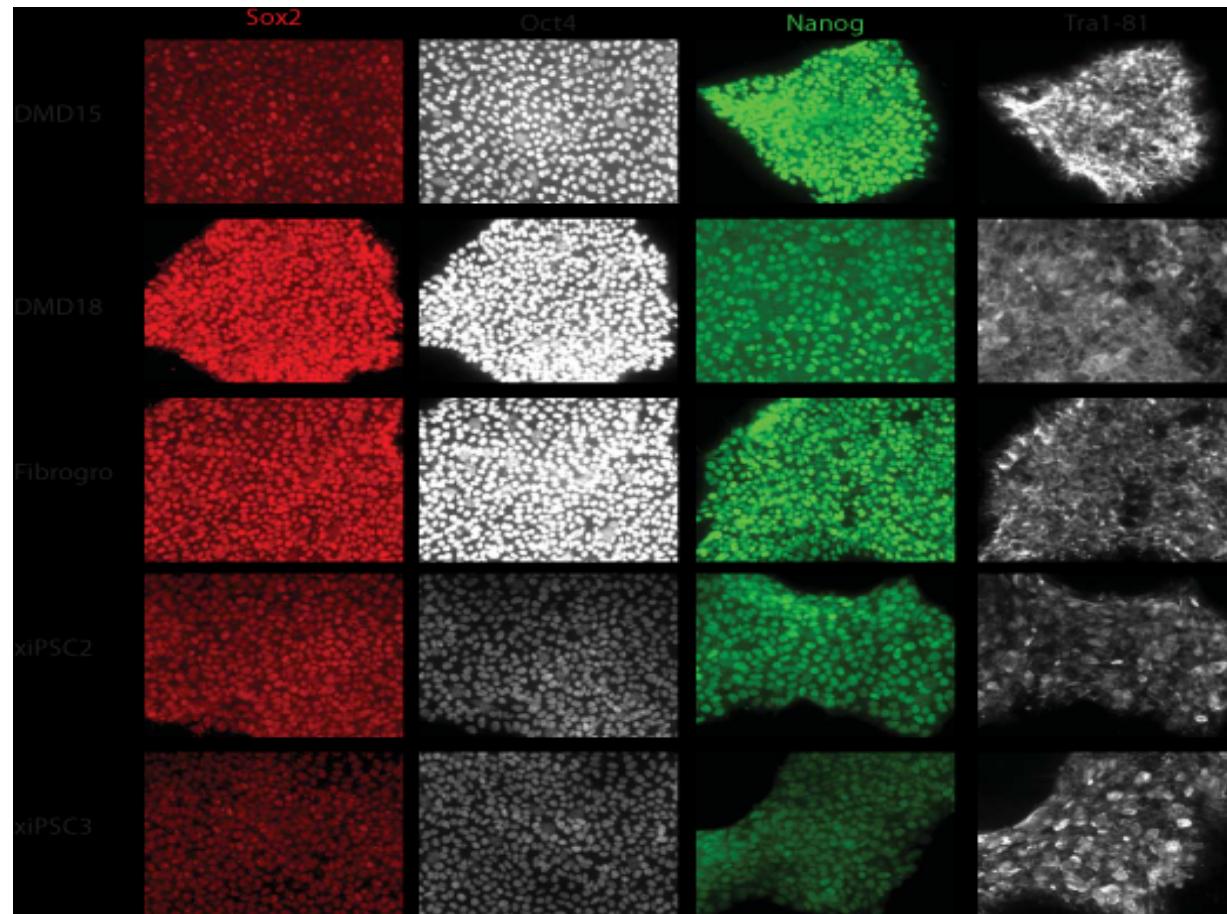
proteins cross-linked
to DNA fragments



Chromatin Immunoprecipitation Can Detect Protein Association with DNA in the Cell

Proteins Can Also be Detected using Antibodies to Specific Proteins and Secondary Antibodies that have a fluorescent tag

DMD 15



DMD 18

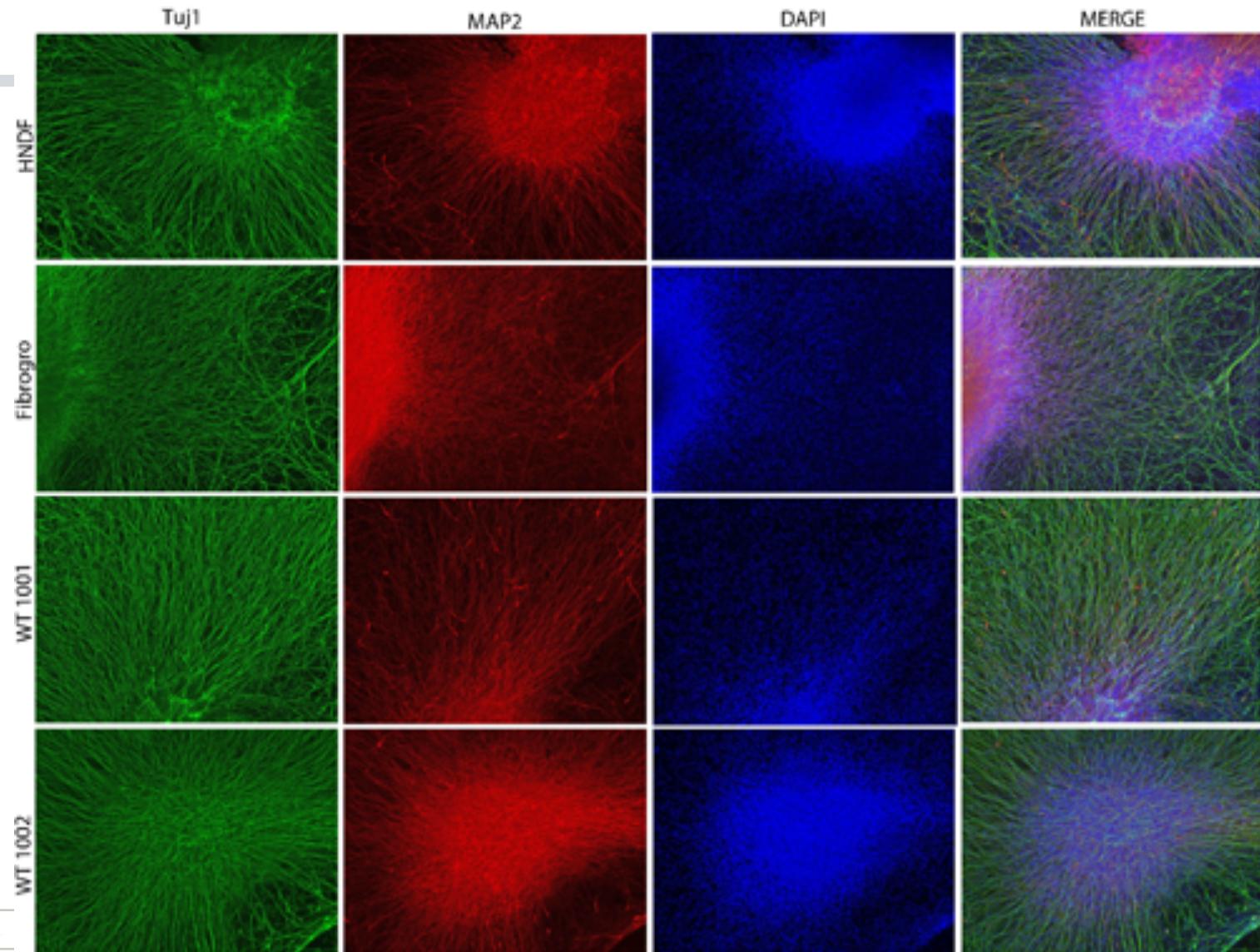
Fibrogro

XiPSC 2

XiPSC 3

Patient Specific Fibroblasts Reprogrammed to hiPSCs!

Patient Derived hiPSCs Differentiate into Neurons!



Integral Membrane Protein with Transmembrane and Cytoplasmic Domain

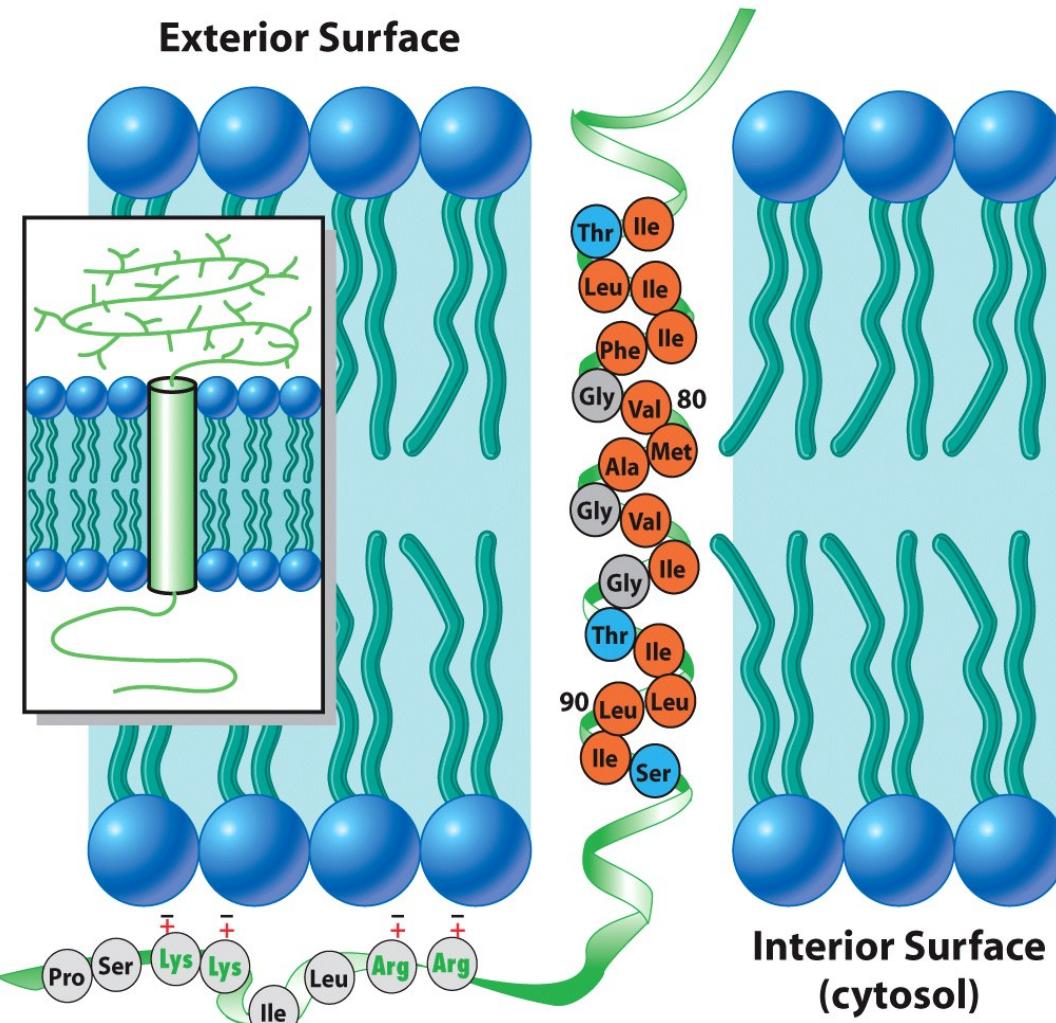


Figure 4-17 Cell and Molecular Biology, 5/e (© 2008 John Wiley & Sons)

These properties of proteins make living systems so dynamic!!

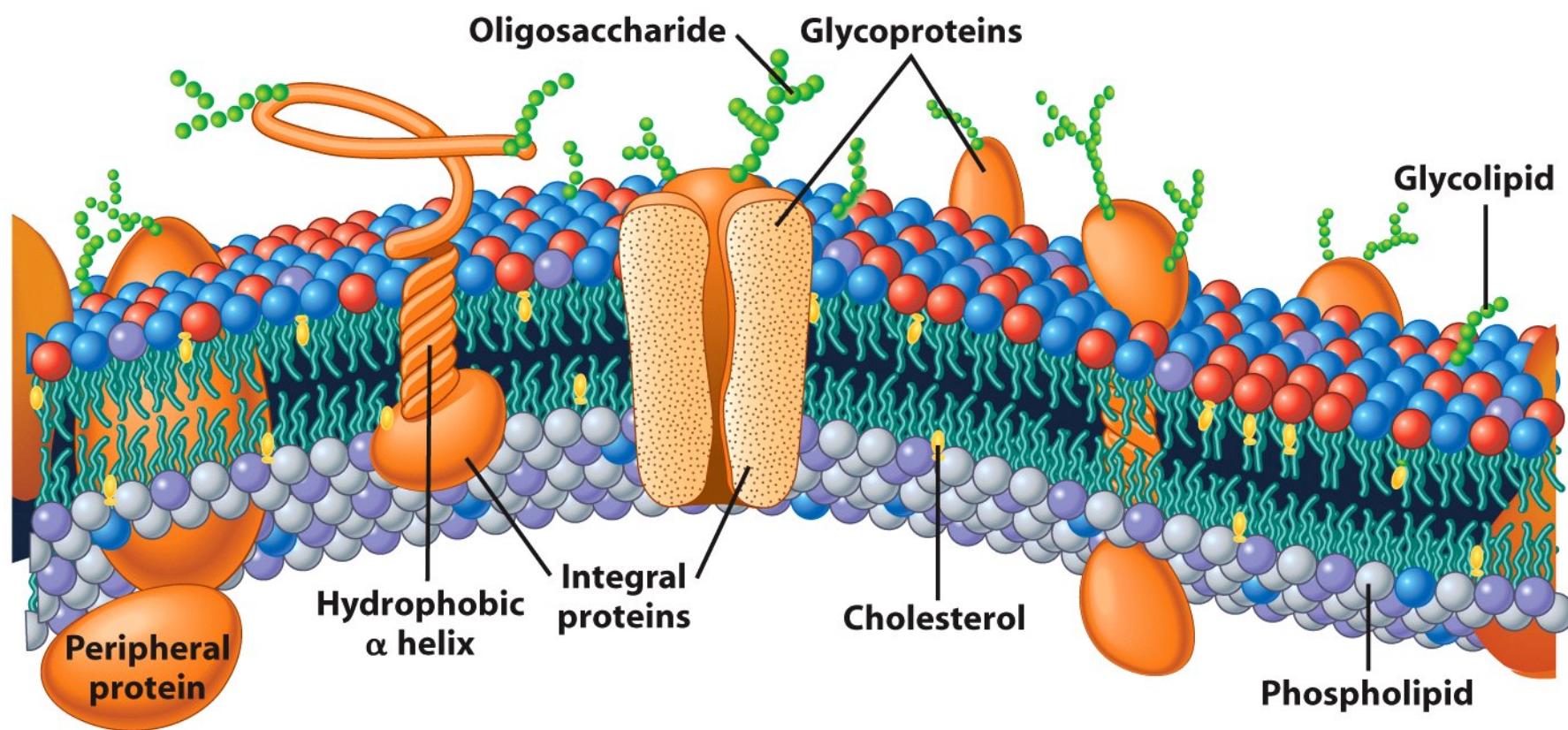
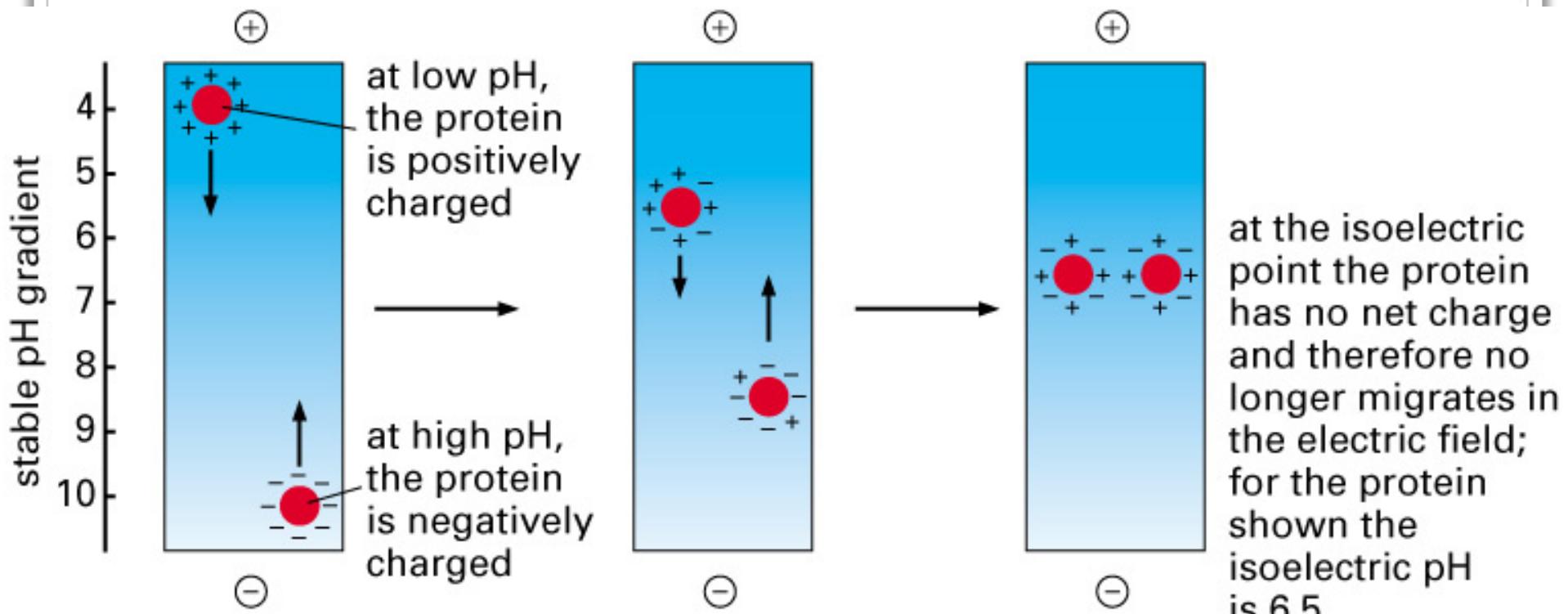


Figure 4-4c Cell and Molecular Biology, 5/e (© 2008 John Wiley & Sons)

Isolation of membrane proteins often require screening of detergents to find the optimal conditions to isolate proteins in tact from membrane

Isoelectronic focusing (IEF) gel electrophoresis

- SDS-page can only separate proteins having large differences in molecular weight.
- IEF gel electrophoresis is based on electric charge, which is determined by the numbers of acidic and basic residues but not the molecular weight of a protein.
- Charged proteins will migrate through the pH gradient until they reach their pI (isoelectric point), the pH at which the net charge of the protein is zero.
- Two unrelated proteins having similar masses are unlikely to have identical net charges.



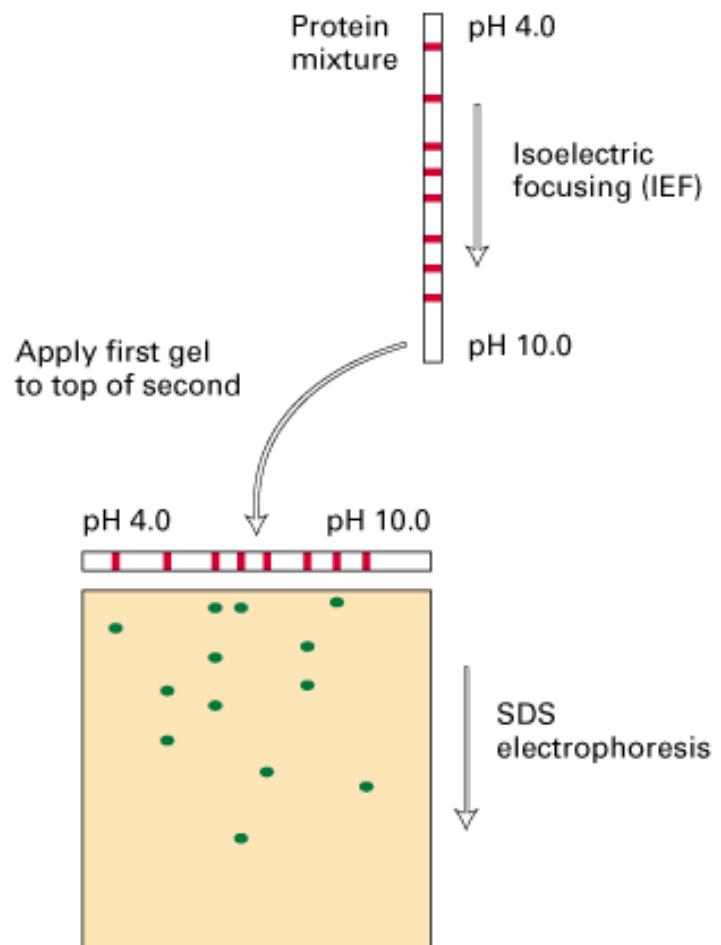
2-D Gel Electrophoresis

First dimension: isoelectric focusing (IEF), in which proteins migrate in a pH gradient until they reach a point where the pH is equal to the isoelectric point (charge=0) of the protein.

Second dimension: SDS-PAGE, in which proteins migrate according to size.

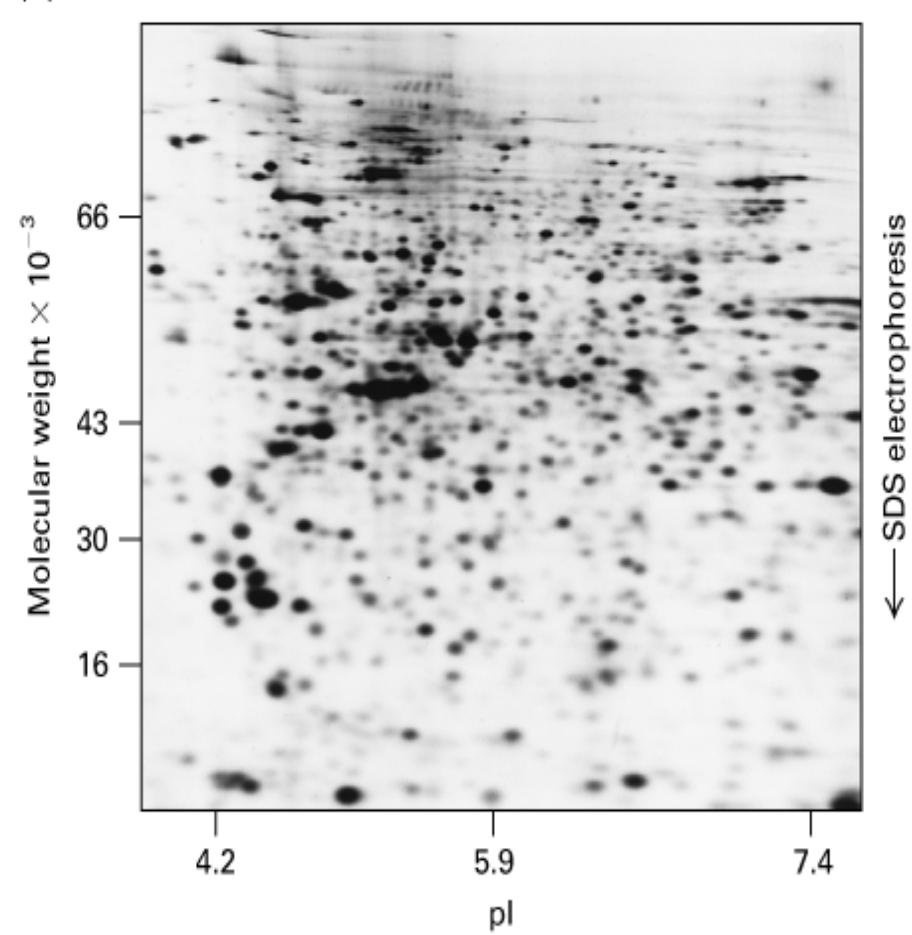
(a)

Separation
in first
dimension
(by charge)



(b)

IEF →



Protein sequence analysis

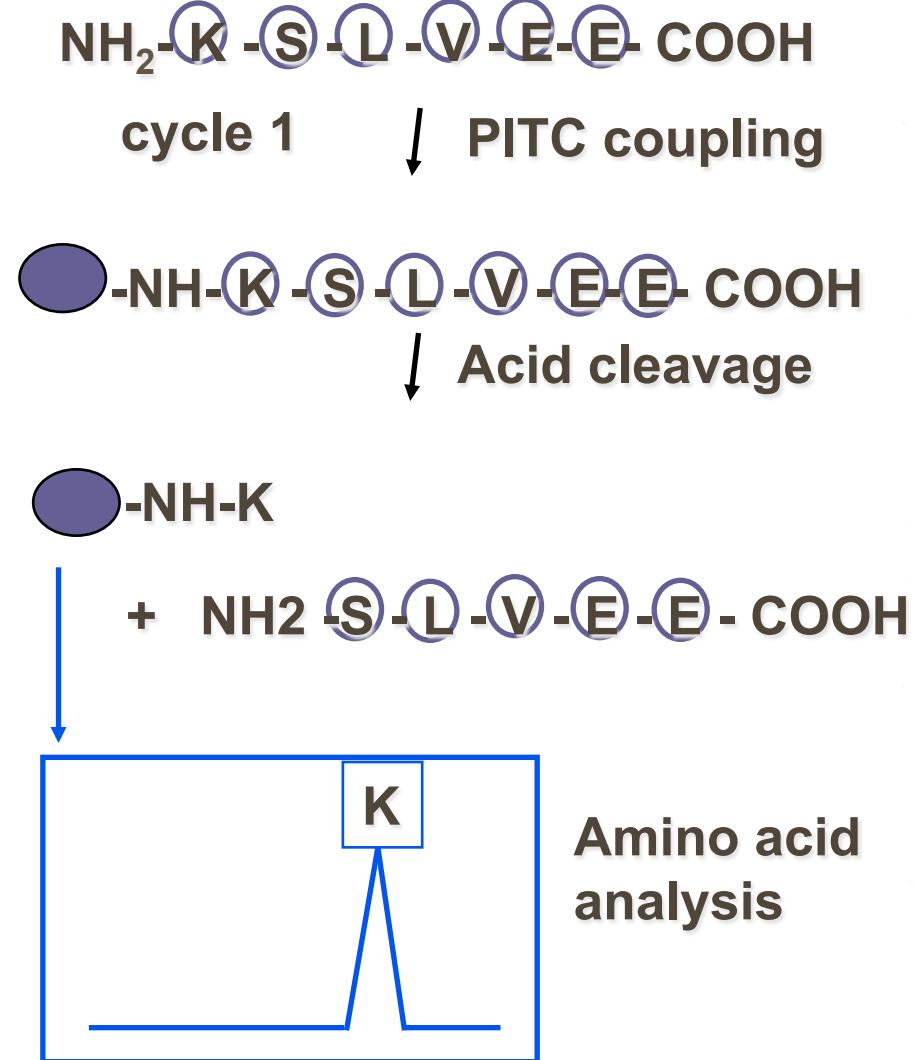
1. Chemical method- **Edman degradation**
2. Mass Spectrometry
3. Deduction from DNA sequence

Edman degradation

1. React with phenyl-isothiocyanate on free amino group
2. Acid cleavage
3. Amino acid analysis
4. Repeat many times

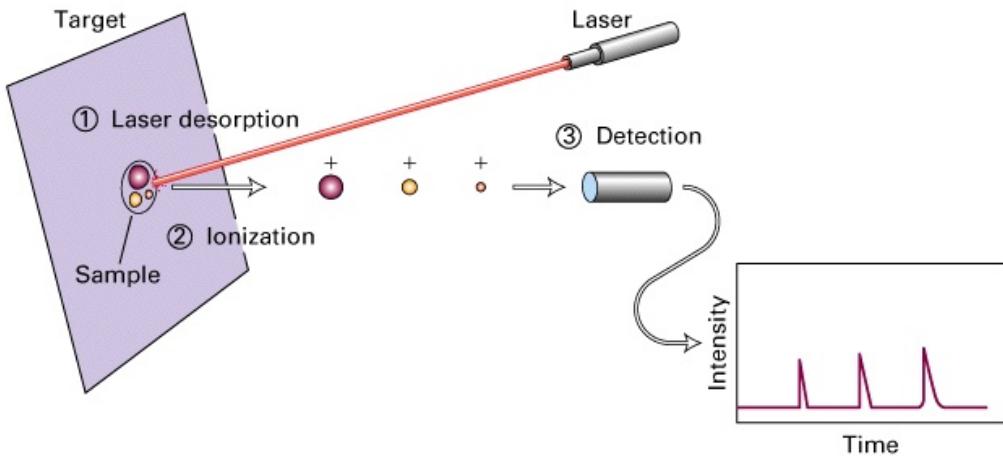
Only good for small peptides generally and non-modified peptides at amino end

Repeat for cycle 2, cycle 3, etc.



Mass Spectrometry

A fast and accurate way to measure molecular weight (MW)
Predicts the ratio of mass to charge which determines MW



Laser desorption/ionisation

The atom is ionised by knocking one or more electrons off to give a positive ion.

This is true even for things which you would normally expect to form negative ions (chlorine, for example) or never form ions at all (argon, for example).

Mass spectrometers always work with positive ions.

Acceleration

The ions are accelerated so that they all have the same kinetic energy.

Deflection

The ions are then deflected by a magnetic field according to their masses.

The lighter they are, the more they are deflected.

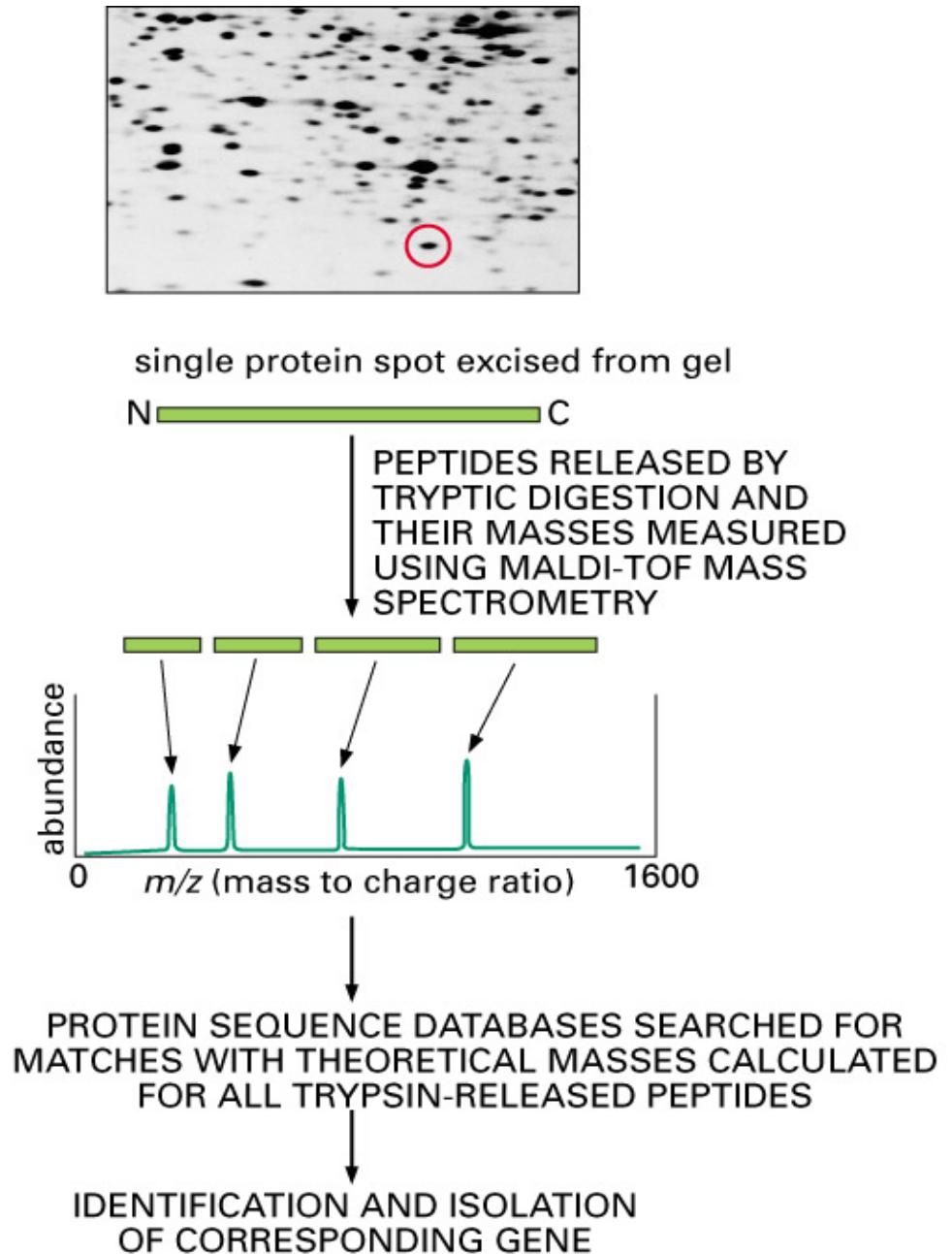
The amount of deflection also depends on the number of positive charges on the ion - in other words, on how many electrons were knocked off in the first stage. The more the ion is charged, the more it gets deflected.

Detection

The beam of ions passing through the machine is detected electrically.

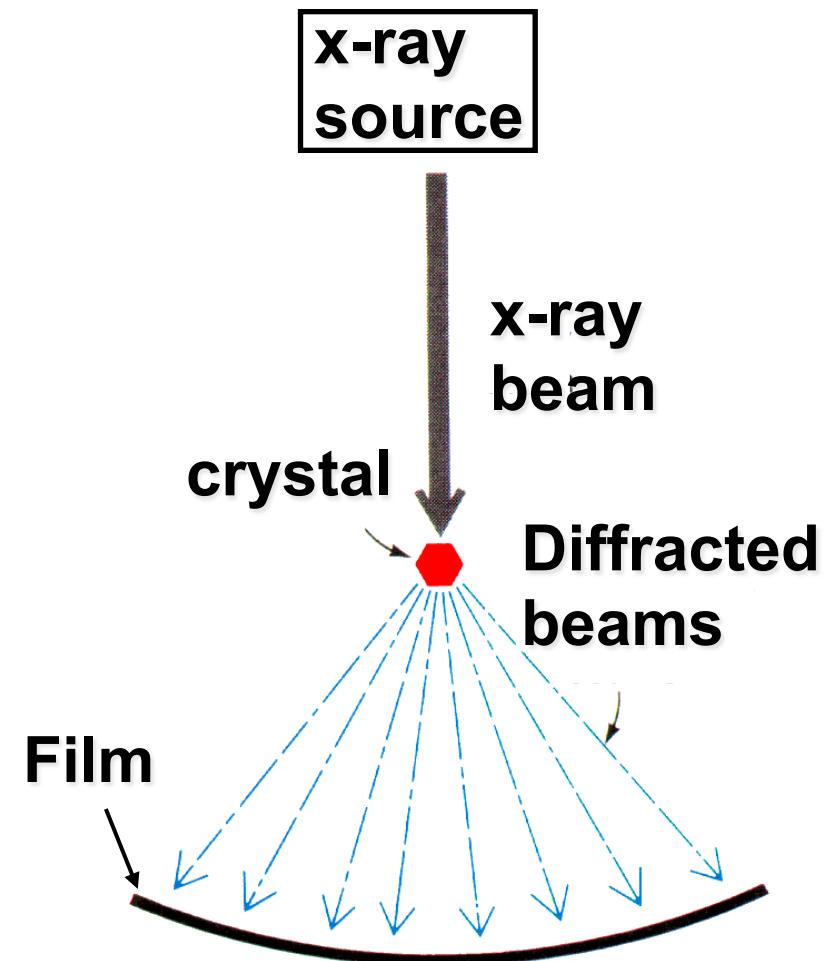
Proteomics:

- Study of all proteins produced in cell or species under a given set of conditions.
- Ex. 2D gel electrophoresis coupled with mass spectrometry to identify MW of protein



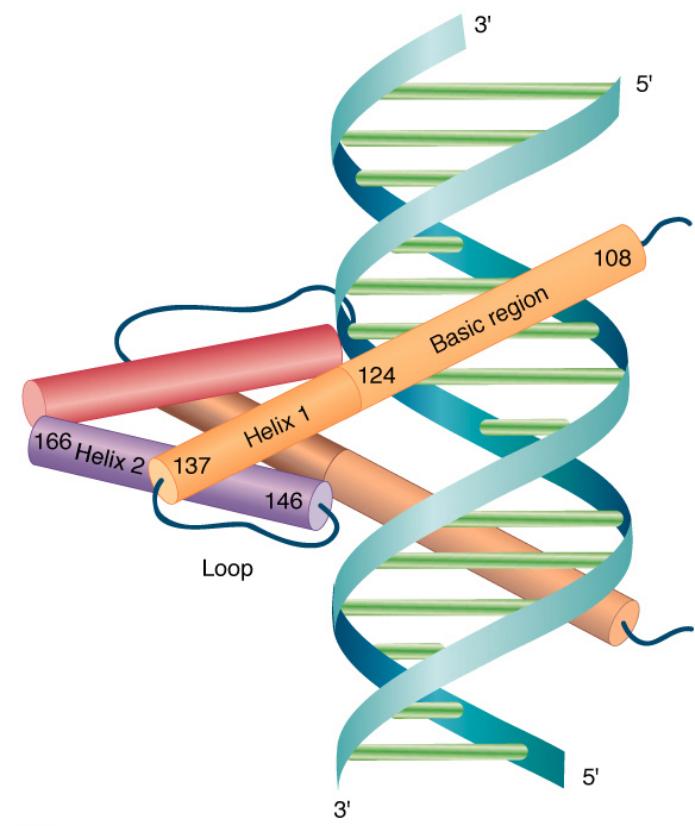
X-ray crystallography

- X-ray diffraction patterns of protein crystals allow determination of protein structure to atomic resolution
- This analysis yields an electron density map, much like a topographical map, of the position and types of atoms



Why study protein structure?

Below: Analysis of 3-D structure of a HLH (helix-loop-helix) type transcription factor helps us to understand how the protein dimer binds to DNA



(b)

Implications and Critical Thinking-3

- Transcription Factors Bind to DNA to activate or repress expression of specific genes
- What would happen if you over expressed proteins such as transcription factors in a cell?
- How could this redirect activity/function of a cell?