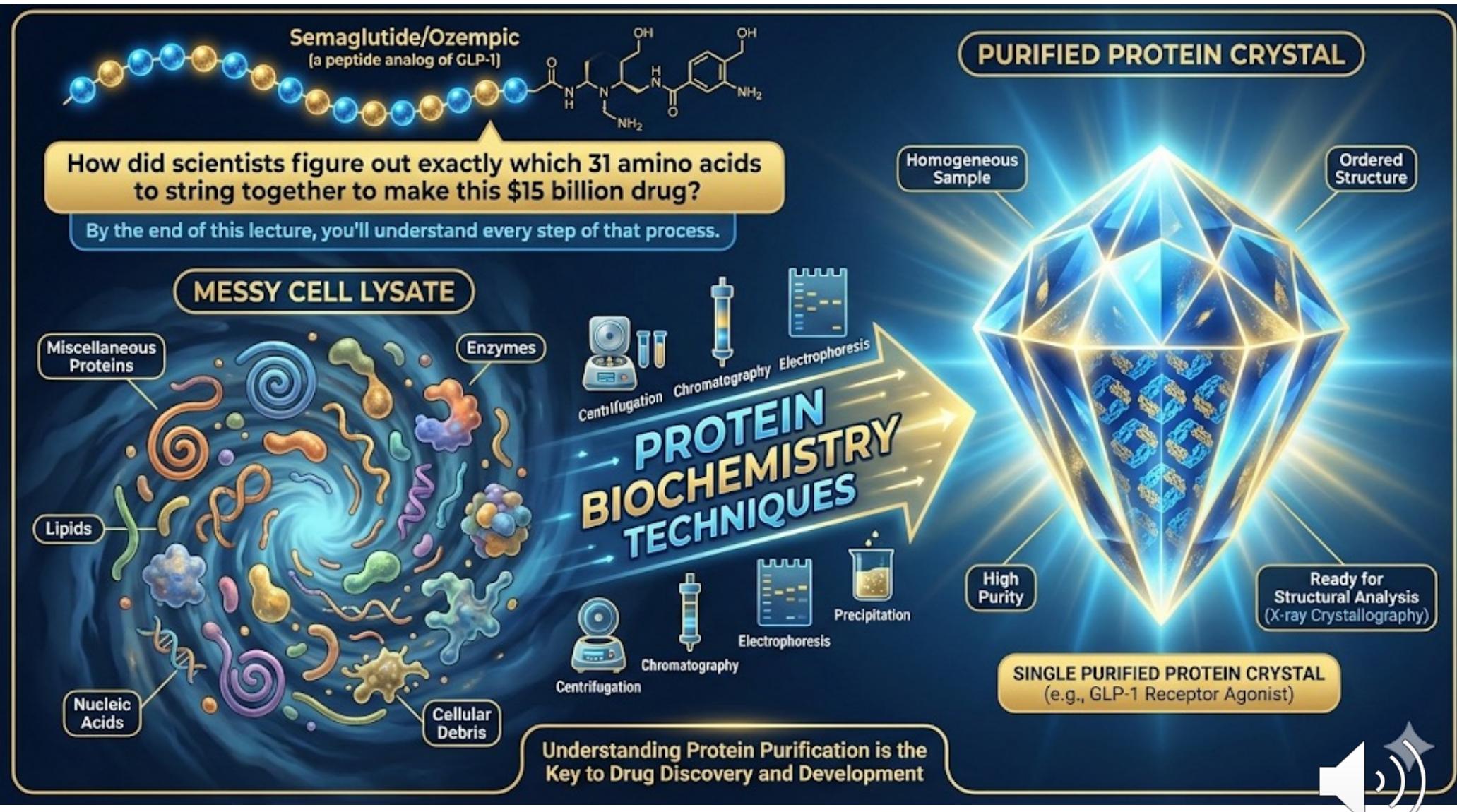


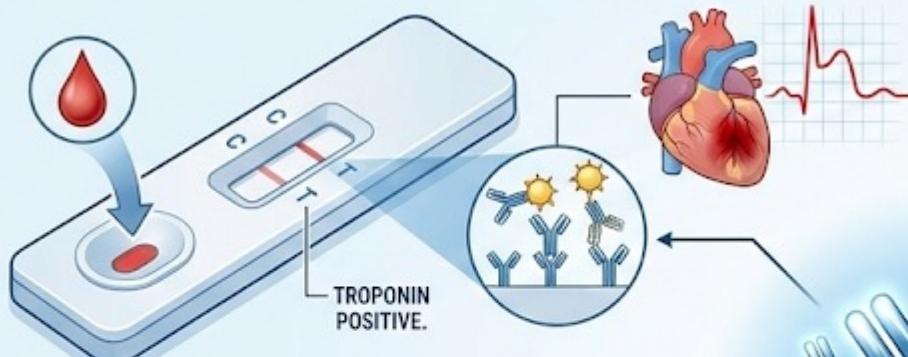
CHAPTER 6

Techniques in Protein Biochemistry

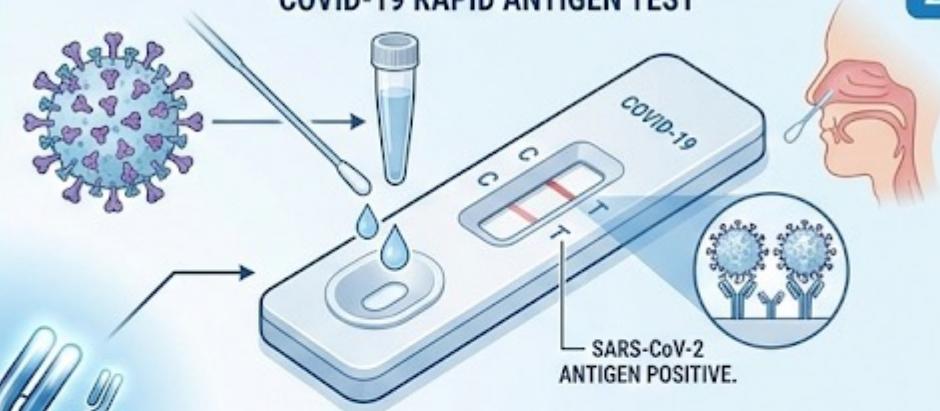


ANTIBODY-BASED DIAGNOSTIC TESTS

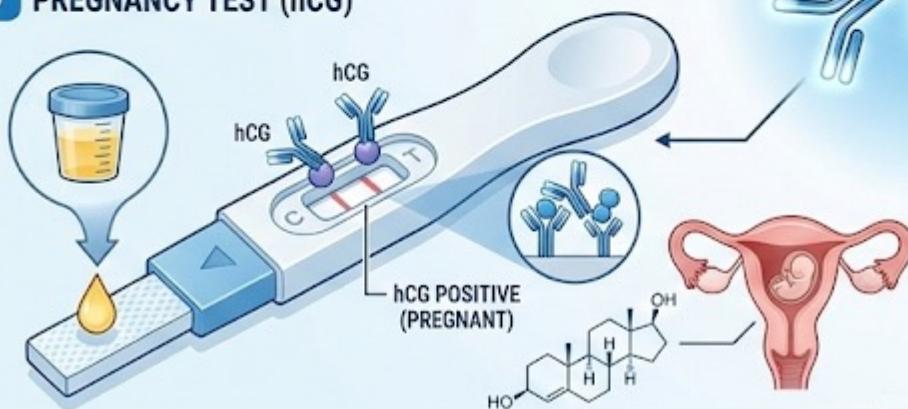
1 TROPONIN BLOOD TEST (HEART ATTACK DIAGNOSIS)



COVID-19 RAPID ANTIGEN TEST

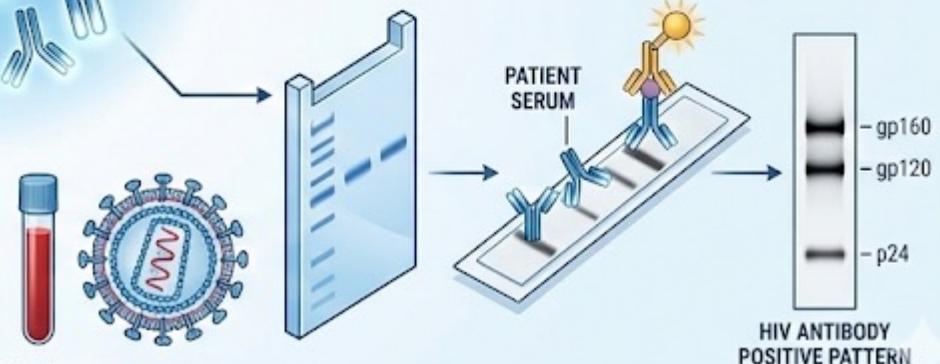


3 PREGNANCY TEST (hCG)



Common Principle:
Specific Antibody-Antigen Binding

WESTERN BLOT (HIV CONFIRMATION) 4



CHAPTER OBJECTIVES: PROTEIN BIOCHEMISTRY



CALCULATE YIELD & PURITY

From purification table data.



PREDICT ELUTION ORDER

Ion-Exchange, Gel Filtration, Affinity.



ANALYZE SDS-PAGE

Determine Molecular Mass.



INTERPRET ANTIBODY ASSAYS

Western Blot & ELISA experiments.



DEDUCE SEQUENCE

Edman & Mass Spectrometry data.

How many different proteins does a human cell make?



Section 6.1 The Proteome Is the Functional Representation of the Genome

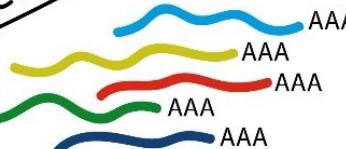
The proteome is the entire set of proteins expressed and modified by a cell under a particular set of biochemical conditions.

Unlike the genome, the proteome is not an unvarying characteristic of the cell.



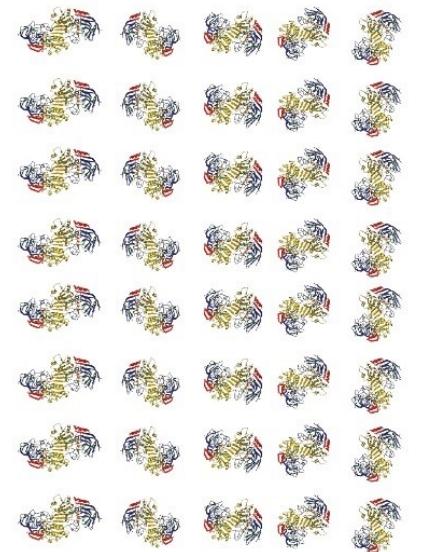
Genome
~20-25,000 genes

Alternative promoters
Alternative splicing
mRNA editing



Transcriptome
~100,000 transcripts

Proteome Complexity



Proteome
>1,000,000 prot.

Post-translational modifications

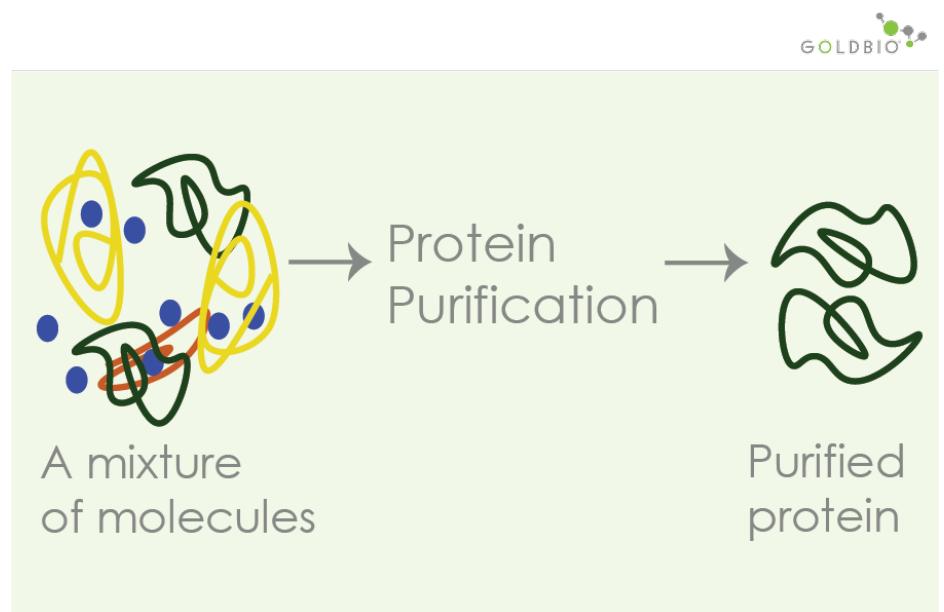
Which two statements are correct?

- A. The genome provides a list of gene products that could be present.
- B. The genome indicates the level of functional information.
- C. The proteome provides a list of gene products that could be present.
- D. The proteome indicates the level of functional information.



Section 6.2 The Purification of Proteins Is the First Step in Understanding Their Function

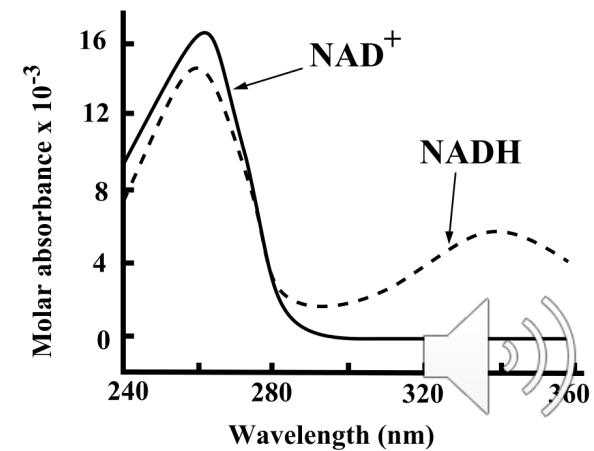
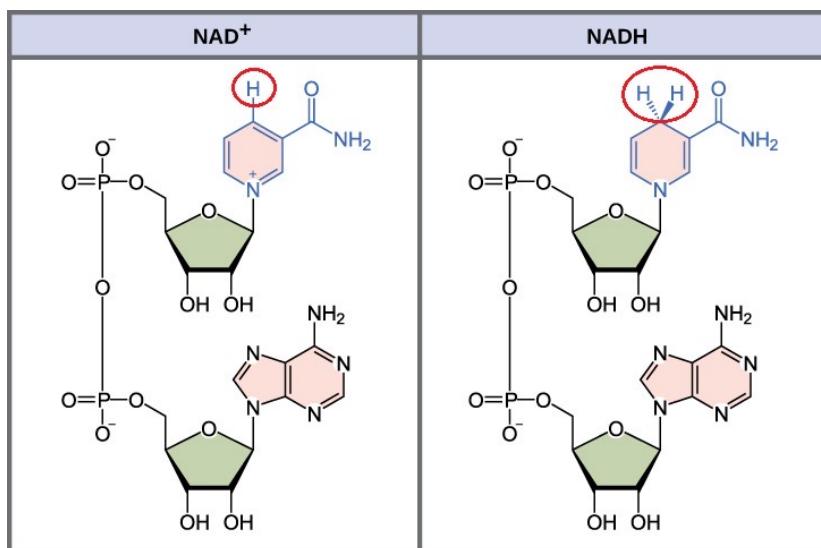
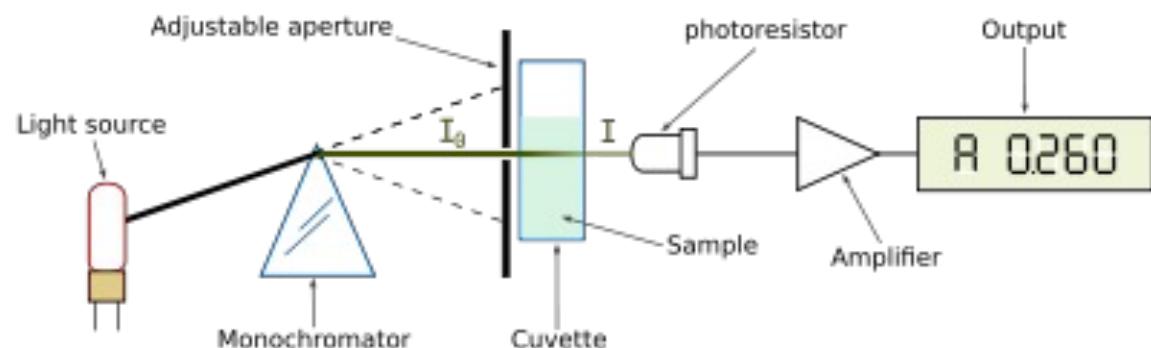
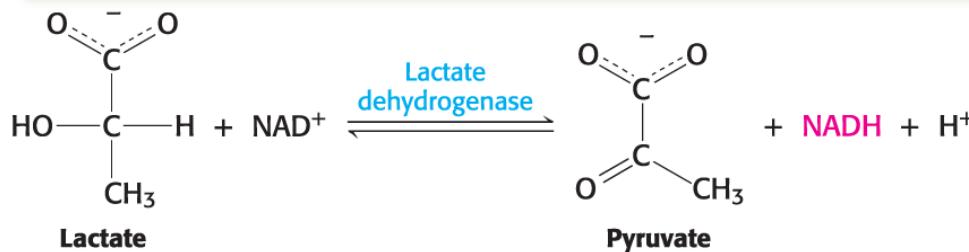
- Proteins can be purified on the basis of differences in their chemical properties.
- Protein purification requires a test, or assay, that determines whether the protein of interest is present.



If I hand you a tube of cell goop, how do you know if your protein is even in there? 

Lactate Dehydrogenase

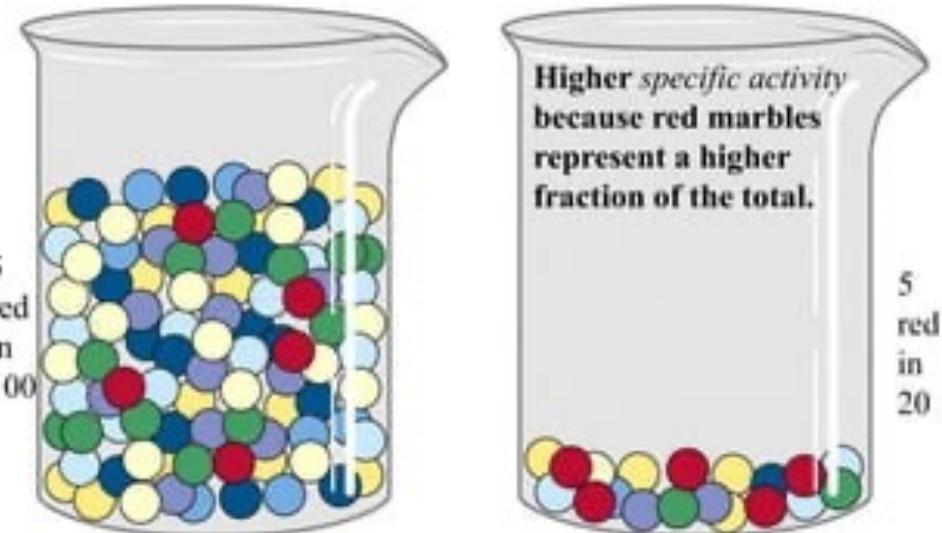
- Proteins can be purified on the basis of differences in their chemical properties.
- An assay for the enzyme lactate dehydrogenase is based on the fact that a product of the reaction, NADH, can be detected spectrophotometrically.



Specific Activity

- Proteins can be purified on the basis of differences in their chemical properties.
- Protein purifications are monitored in part by determining the specific activity of the protein being purified.
- In the case of an enzyme purification, specific activity is the ratio of enzyme activity to protein concentration. Specific activity should increase with each step of the purification procedure.

If the marbles represent proteins, both beakers contain the same *activity* of the protein represented by the red marbles.



SPECIFIC ACTIVITY

$$\text{SA} = \text{Enzyme Units} \div \text{Total Protein (mg)}$$

Measures PURITY — should INCREASE during purification



FOLD PURIFICATION

$$\text{Fold} = \text{Final SA} \div \text{Initial SA}$$

How many times purer? (e.g., 8-fold = 8× purer)



PERCENT YIELD

$$\% \text{ Yield} = (\text{Final Units} \div \text{Initial Units}) \times 100$$

How much enzyme did you RECOVER? (Want high %)



EXAMPLE CALCULATION

START: 1000 units, 100 mg protein → SA = 10 U/mg

END: 800 units, 10 mg protein → SA = 80 U/mg

Fold = 80/10 = 8-fold | Yield = 800/1000 = 80%



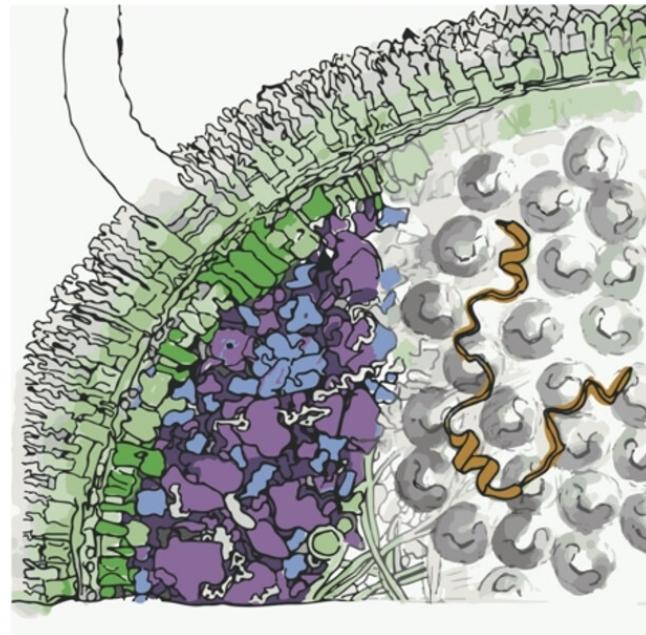


QUICK QUIZ 1

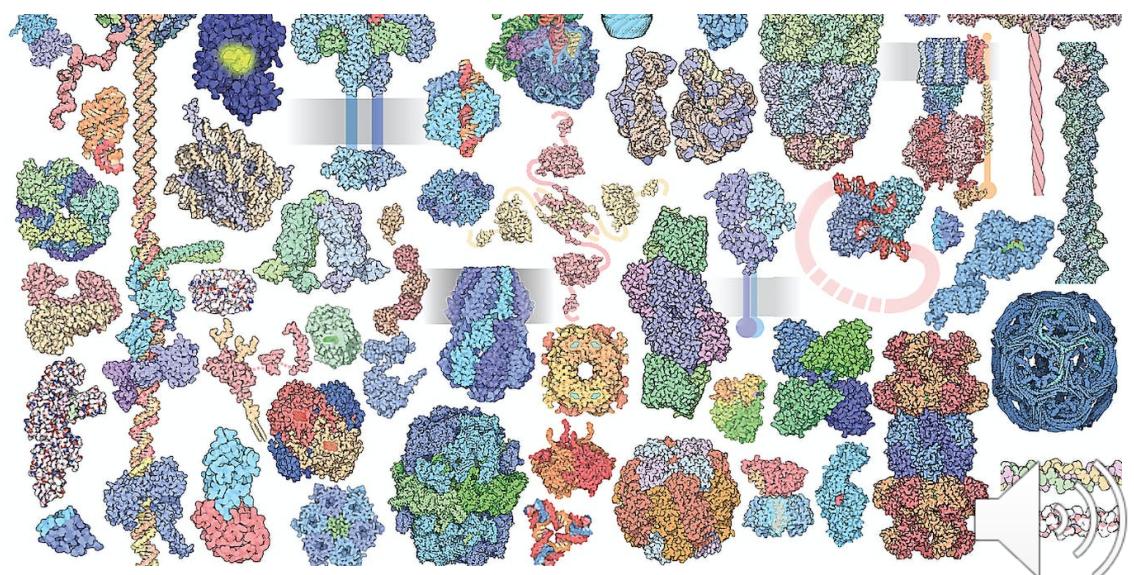
Why is an assay required for protein purification?



How do we get our proteins out of a cell?

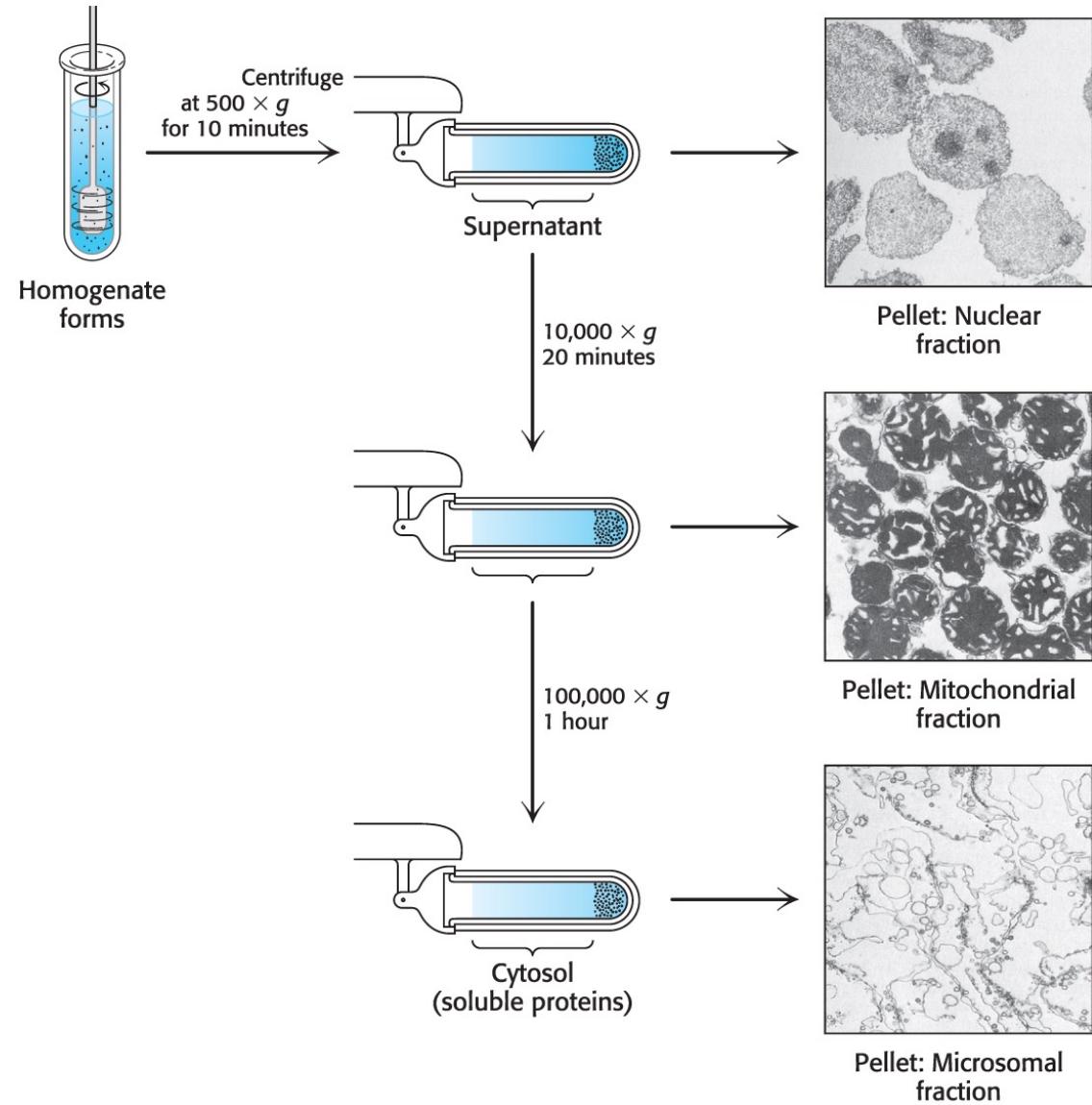


How do we get rid of the garbage? e.g. insoluble material



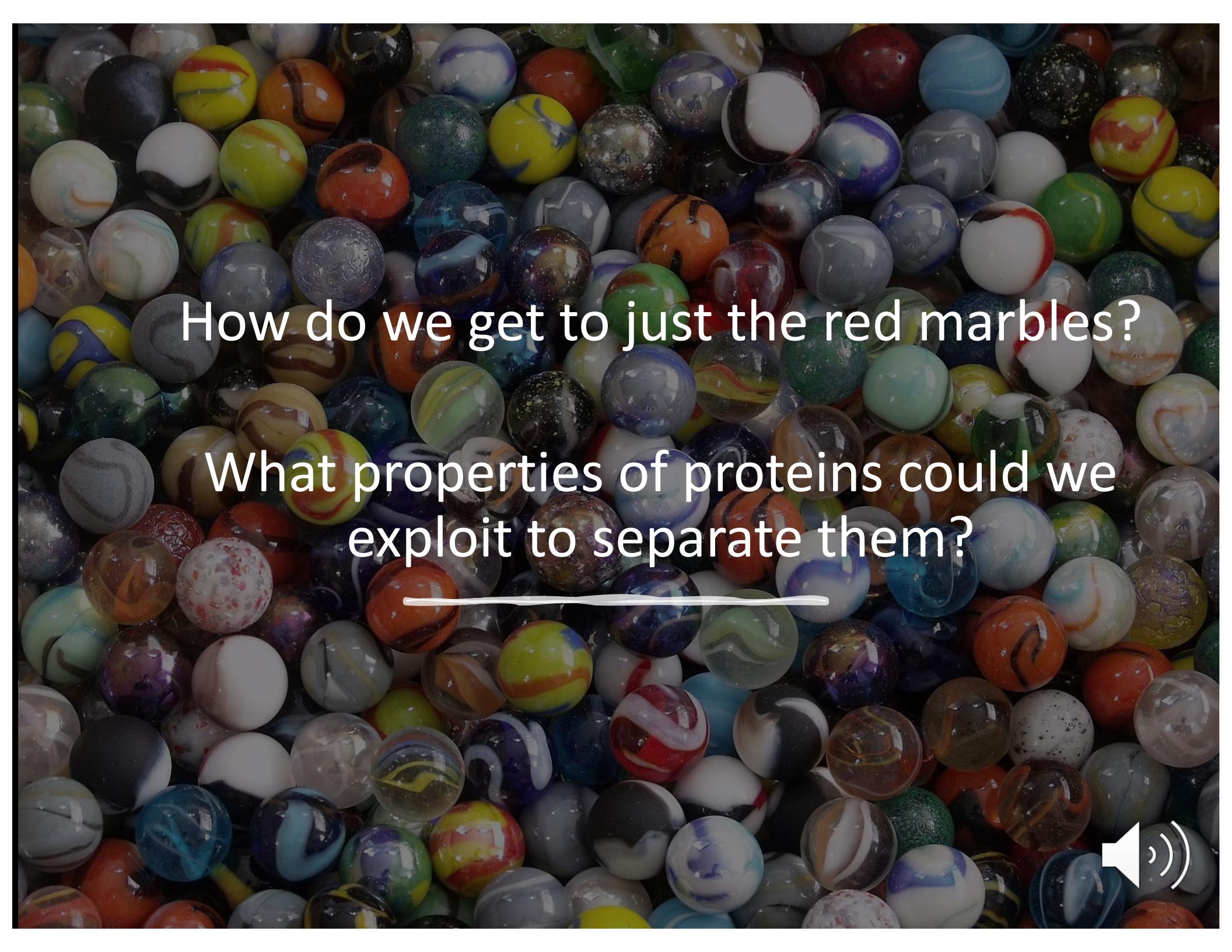
Proteins Must Be Removed from the Cell to Be Purified

- Cells are disrupted to form a homogenate, which is a mixture of all of the components of the cell but no intact cells.
- The homogenate is then centrifuged at low speed to yield a pellet consisting of nuclei and a supernatant. This supernatant is then centrifuged at a higher centrifugal force to yield another pellet and supernatant. Higher speed = smaller particles pellet. Think of it as increasing the 'gravitational' filter. This process, called differential centrifugation, is repeated several more times to yield a series of pellets enriched in various cellular materials and a final supernatant called the cytosol.



Courtesy of S. Fleischer and B. Fleischer.



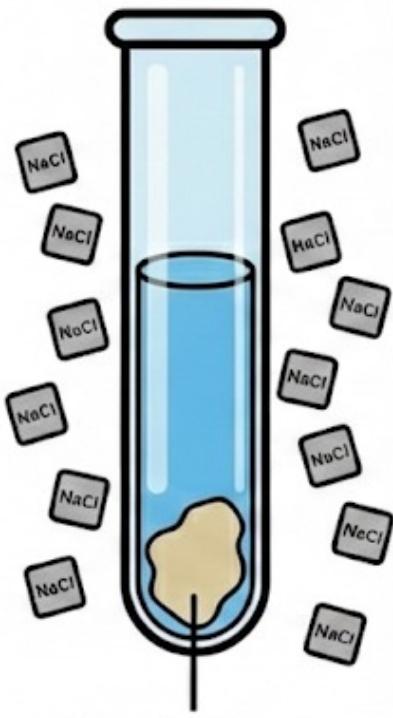


How do we get to just the red marbles?

What properties of proteins could we exploit to separate them?



SOLUBILITY

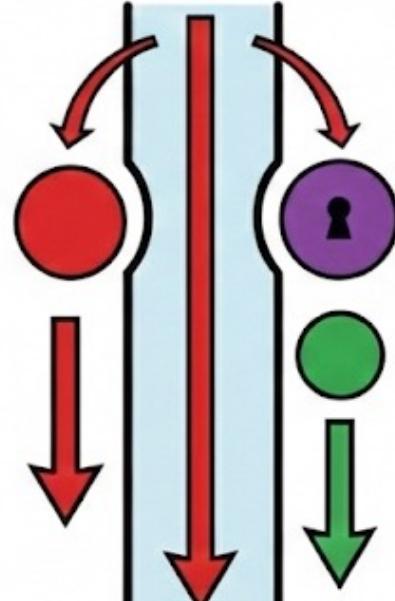


Precipitated Protein

Salting Out

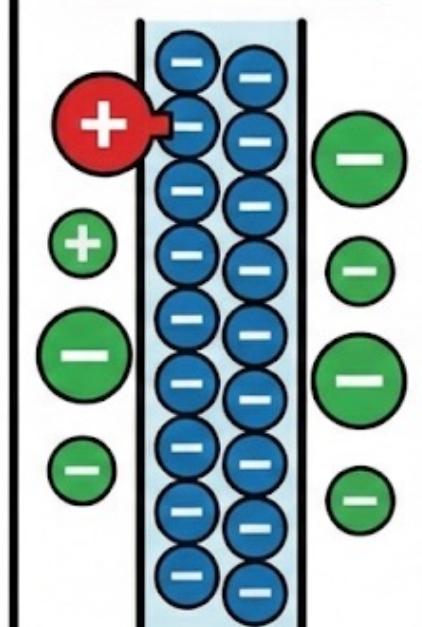
FOUR WAYS TO SEPARATE PROTEINS

BINDING AFFINITY



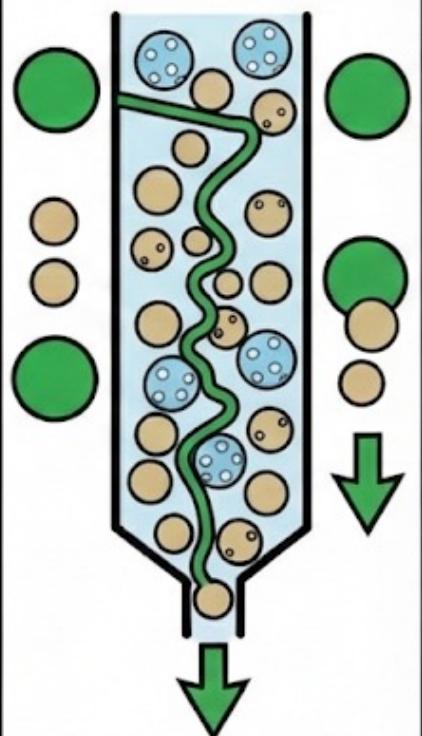
Affinity

CHARGE



Ion Exchange

SIZE



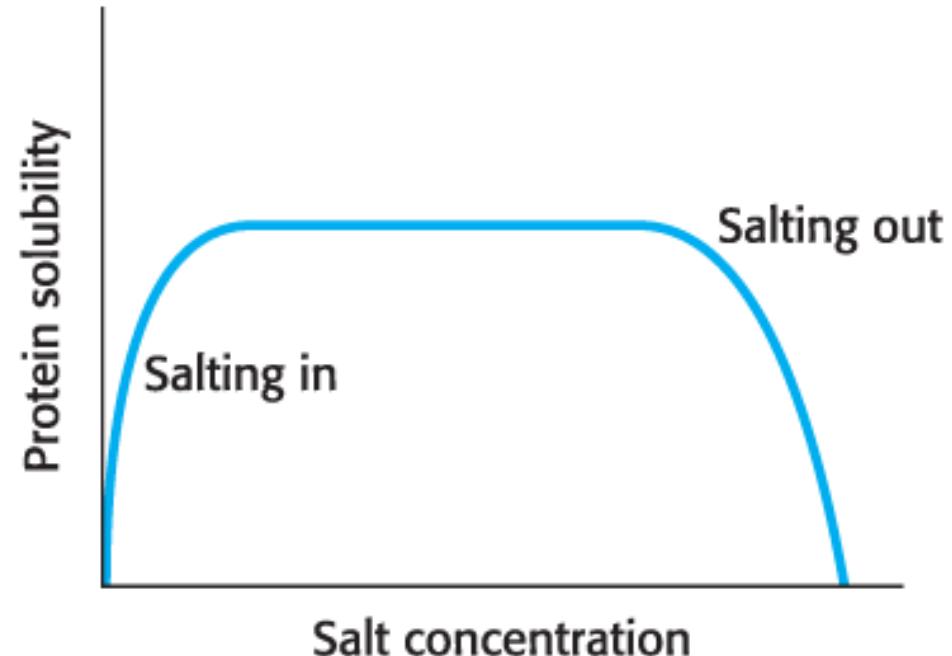
Large protein exits first

- **SOLUBILITY** → Salting out
- **SIZE** → Gel filtration
- **CHARGE** → Ion exchange
- **BINDING** → Affinity chromatography



Proteins Can Be Purified According to Solubility, Size, Charge, and Binding Affinity

- Salting out takes advantage of the fact that the solubility of proteins varies with the salt concentration. Most proteins require some salt to dissolve in water, a process called salting in. As the salt concentration is increased, different proteins will precipitate at different salt concentrations, a process called salting out.



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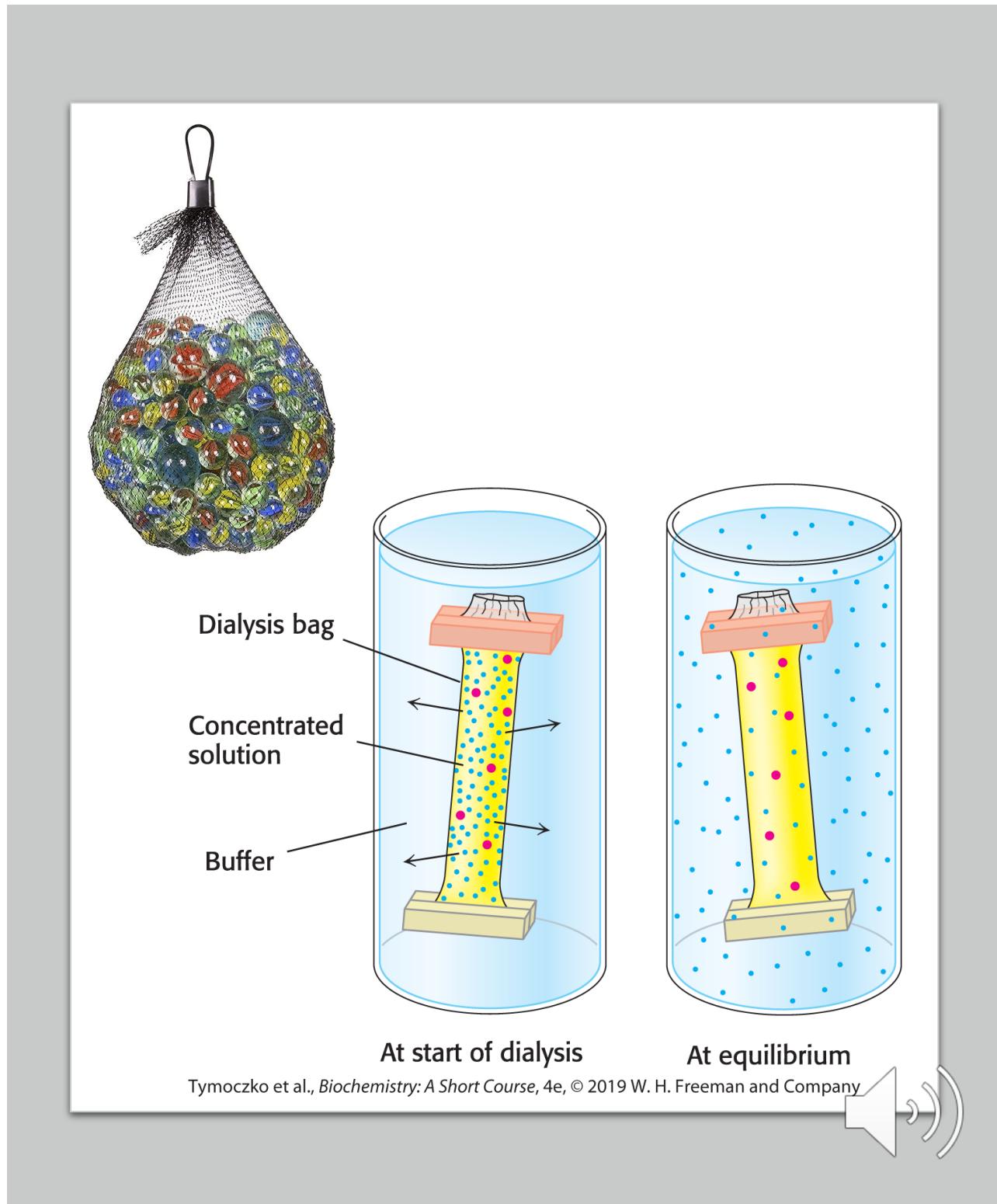


How do we get rid of
the salt?

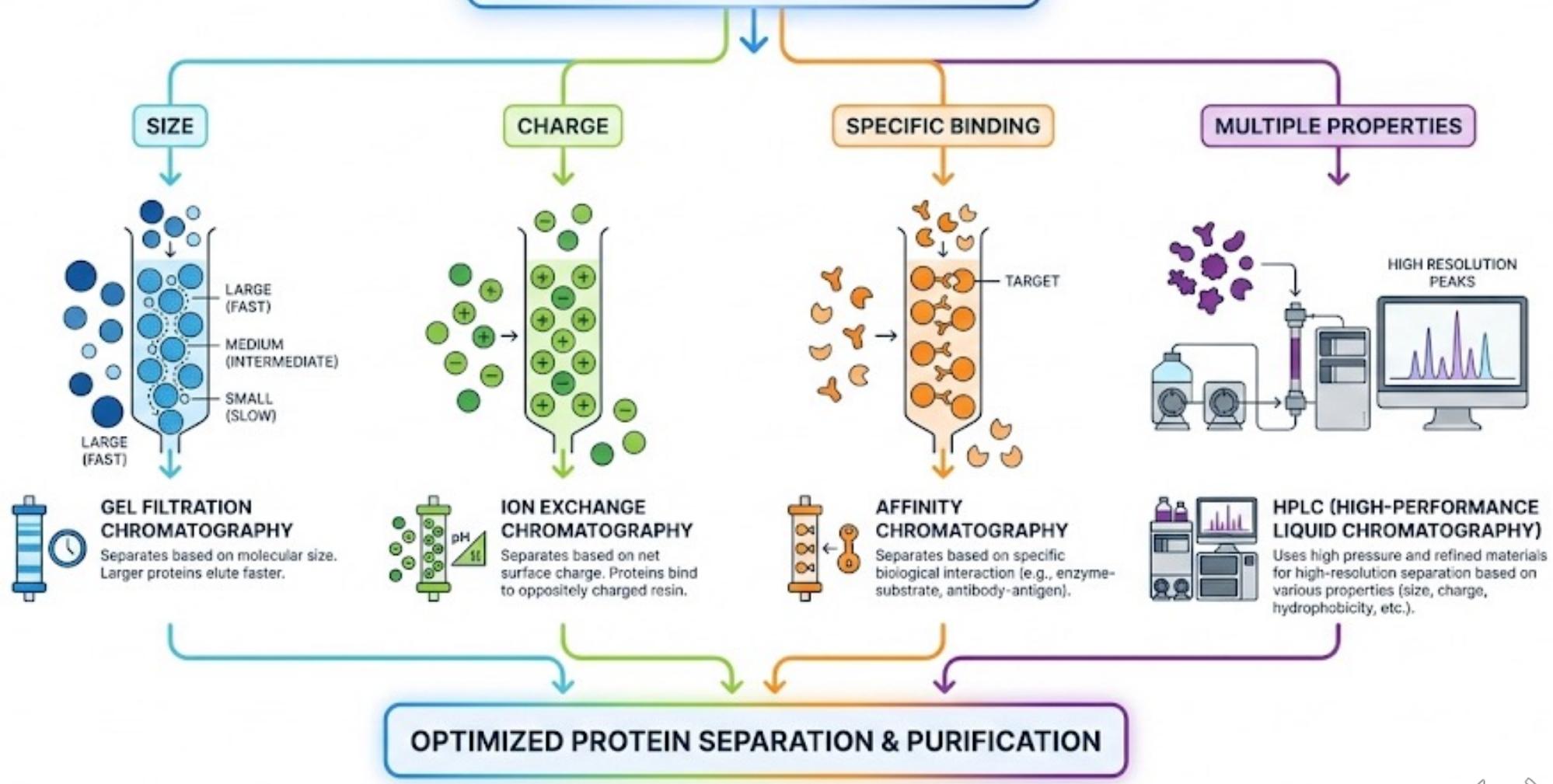


Dialysis

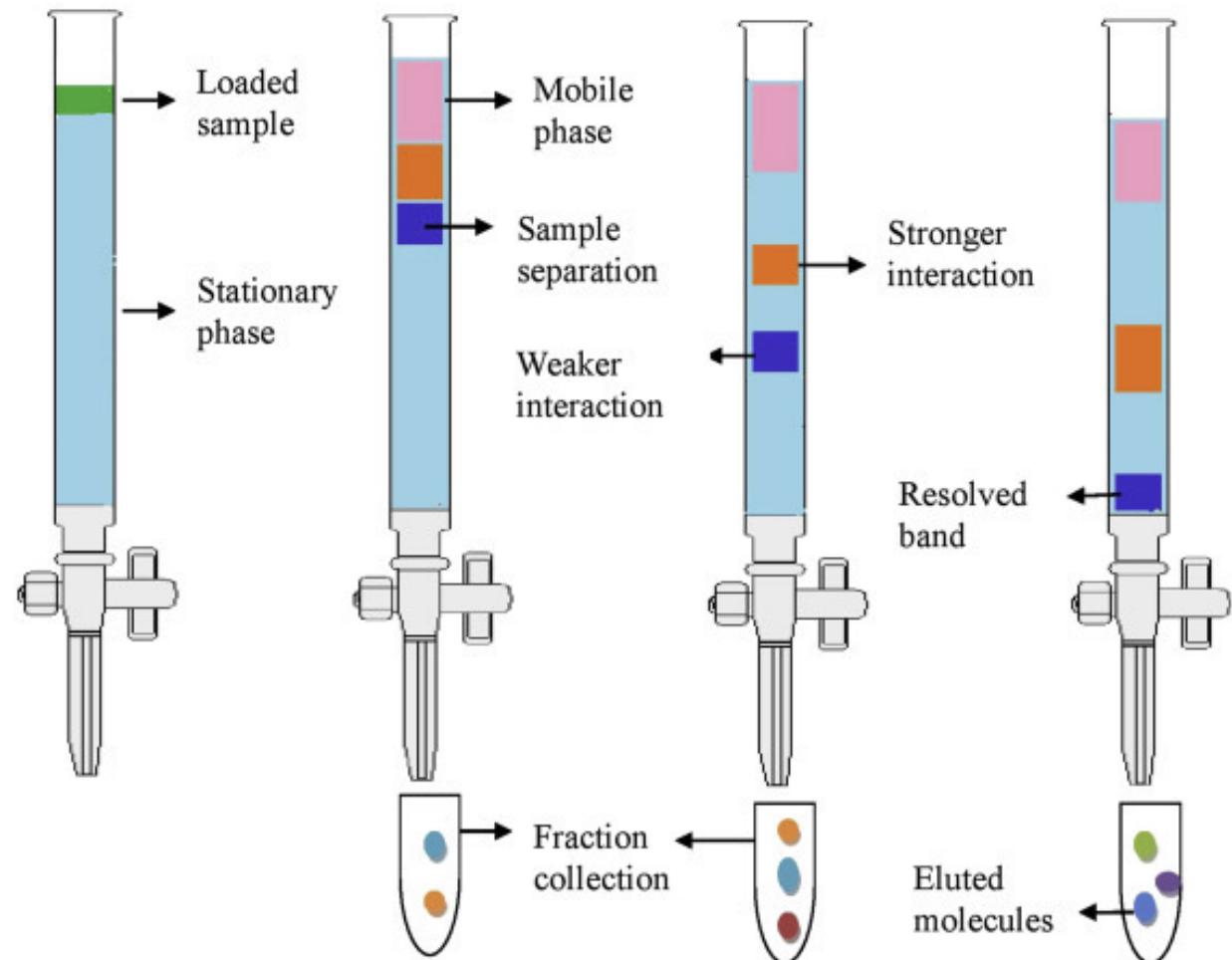
- Proteins can be purified according to solubility, size, charge, and binding affinity.
- The salt can be removed from a protein solution by dialysis. The protein solution is placed in a cellophane bag with pores too small to allow the protein to diffuse but big enough to allow the salt to equilibrate with the solution surrounding the dialysis bag.



What property distinguishes your protein?



A molecular race: Column Chromatography

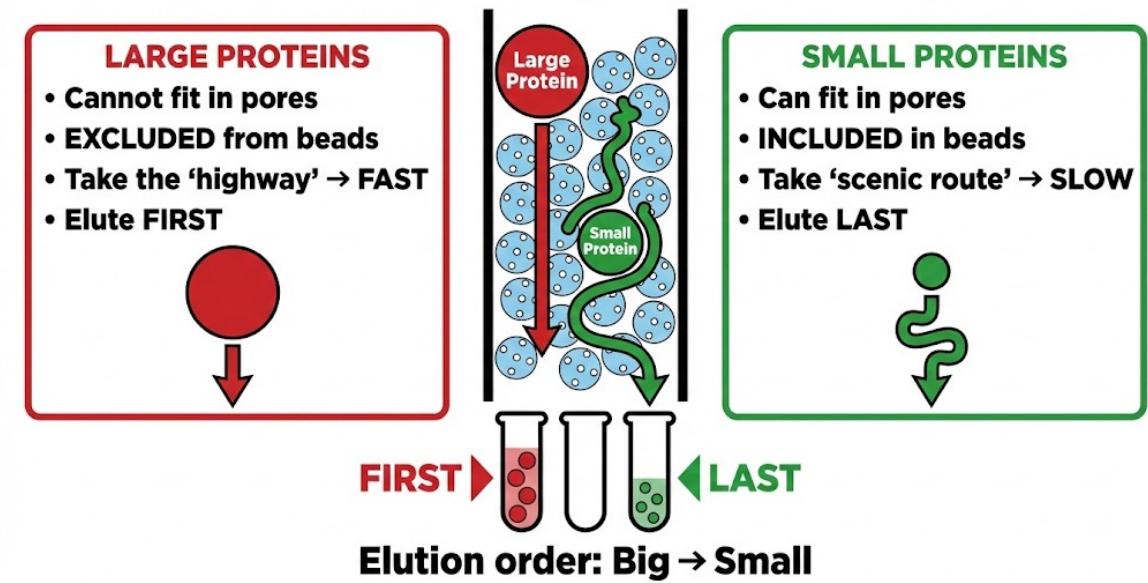


Separation by Size

- Proteins can be purified according to solubility, size, charge, and binding affinity.
- Molecular exclusion chromatography (gel filtration chromatography) allows the separation of proteins on the basis of size. A column is filled with porous beads. When a protein solution is passed over the beads, large proteins cannot enter the beads and exit the column first. Small proteins can enter the beads and thus have a longer path and exit the column last.



GEL FILTRATION: Why Big Comes Out First



Ion-exchange Chromatography

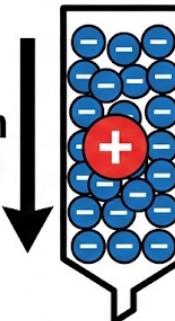
- Proteins can be purified according to solubility, size, charge, and binding affinity.
- Ion exchange chromatography allows separation of proteins on the basis of charge. The beads in the column are made so as to have a charge. When a mixture of proteins is passed through the column, proteins with the same charge as on the column will exit the column quickly. Proteins with the opposite charge will bind to the beads and are subsequently released by increasing the salt concentration or adjusting the pH of the buffer that is passed through the column.

CATION EXCHANGE

pH < pI

Protein is POSITIVE (+)

Protein
BINDS



pH ← → pI

Below pI = + Above pI = -

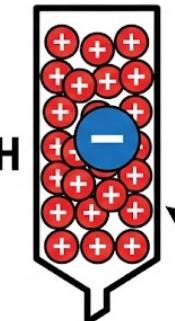
Use CATION EXCHANGE (negative beads)

ANION EXCHANGE

pH > pI

Protein is NEGATIVE (-)

Protein
BINDS



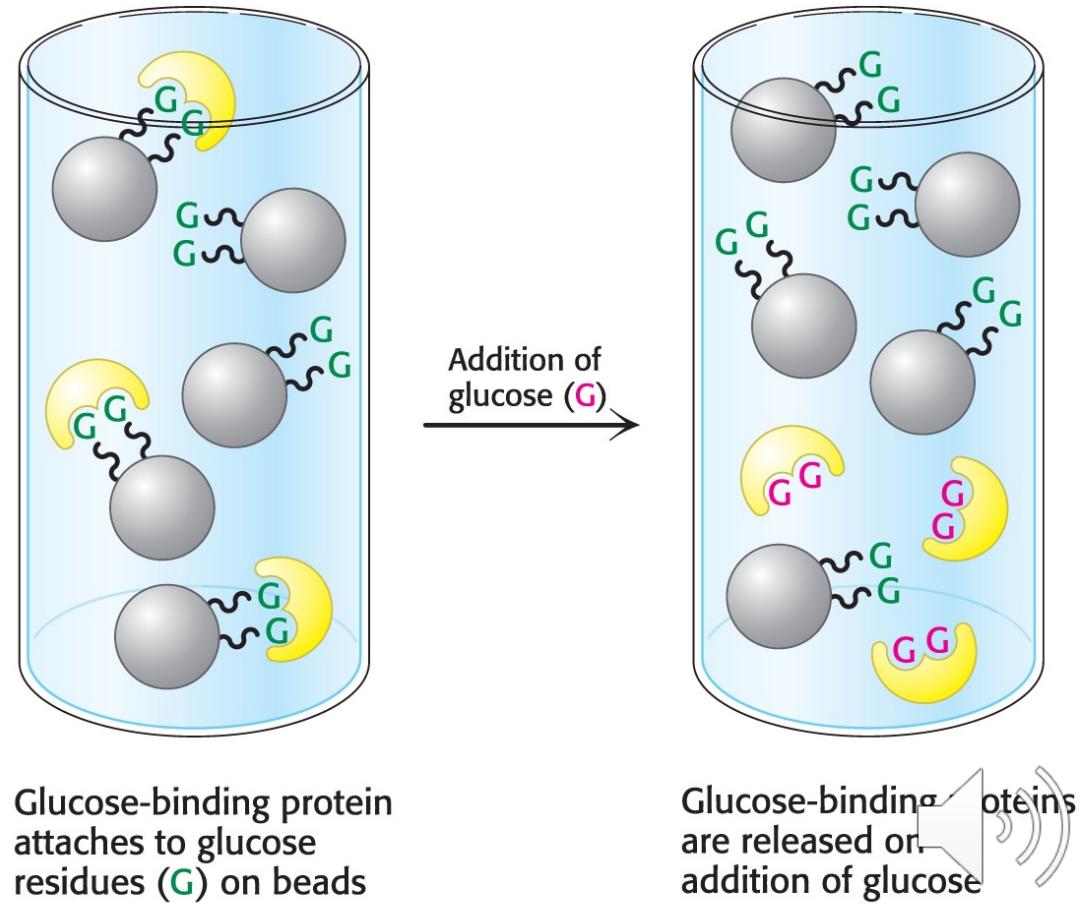
Use ANION EXCHANGE (positive beads)

OPPOSITES ATTRACT: Protein charge binds to opposite bead charge



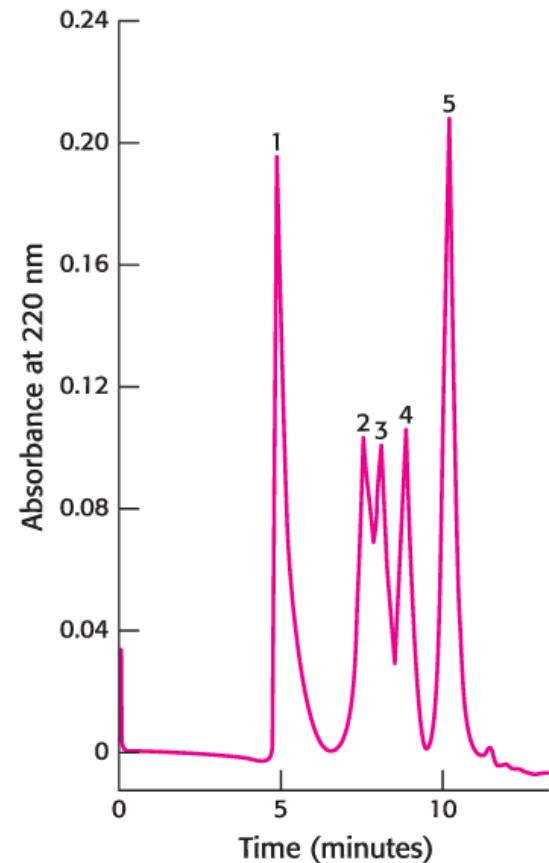
Affinity Chromatography

- Proteins can be purified according to solubility, size, charge, and binding affinity.
 - Affinity chromatography takes advantage of the fact that some proteins have a high affinity for specific chemicals or chemical groups. Beads are made with the specific chemical attached. A protein mixture is passed through the column. Only protein with affinity for the attached group will be retained. The bound protein is then released by passing a solution enriched in the chemical to which the protein is bound.



High-pressure Liquid Chromatography

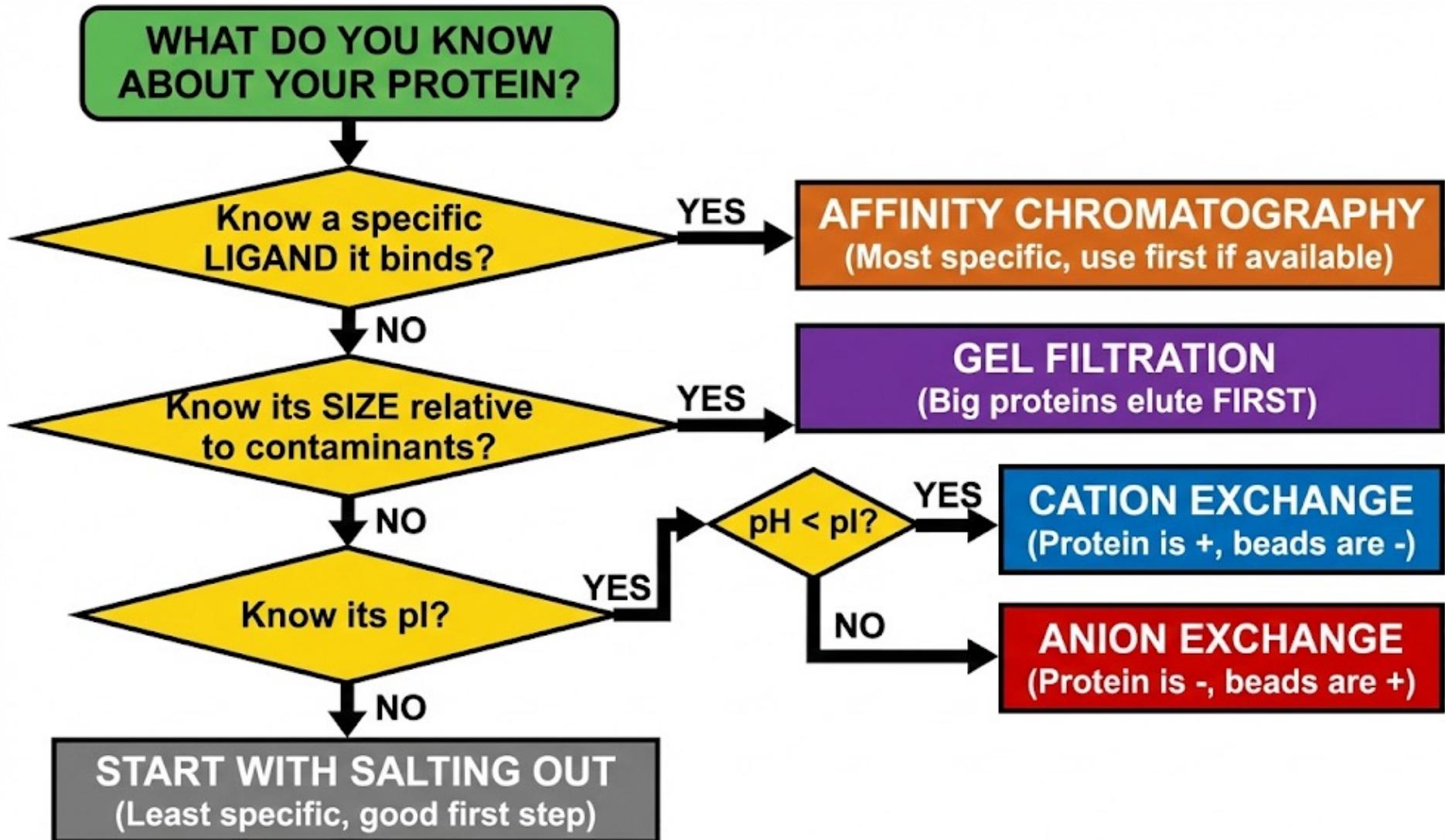
- Proteins can be purified according to solubility, size, charge, and binding affinity.
 - The resolving power of any chromatographic technique is related to the number of potential sites of interaction between the protein and the column beads. Very fine beads allow more interactions and thus greater resolving power, but flow rates through such columns are too slow.
 - High-pressure liquid chromatography (HPLC) uses very fine beads in metal columns and high-pressure pumps to move the liquid through the column. Because of the increased number of interaction sites, the resolving power of HPLC is greater than normal columns.



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Which Column Do I Use? A Purification Decision Tree



Let's practice:

Question 1: You have a kinase that binds ATP. Which column?

Question 2: Your protein has pI of 9, and your buffer is pH 7. Which column?

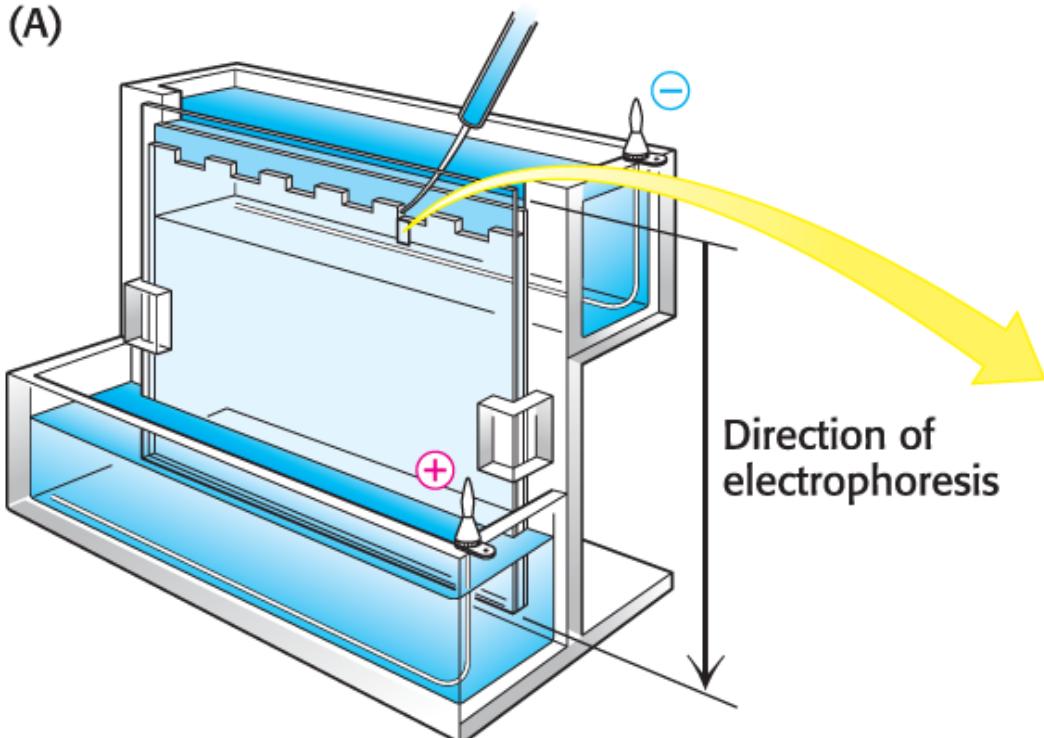
Question 3: You need to separate a 50 kDa protein from a 10 kDa contaminant. Which column?



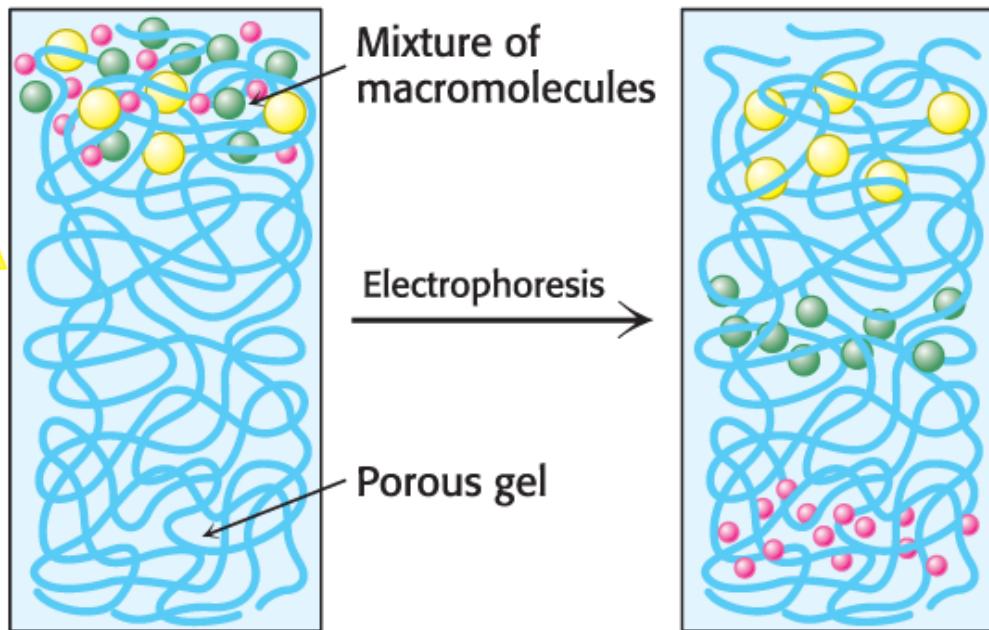
How do we look
at our proteins
after purification?



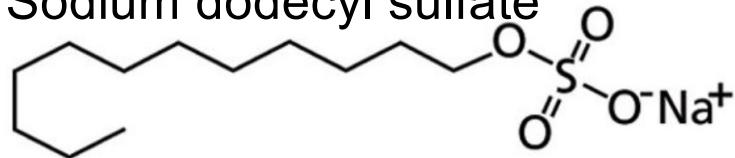
(A)



(B)



Sodium dodecyl sulfate



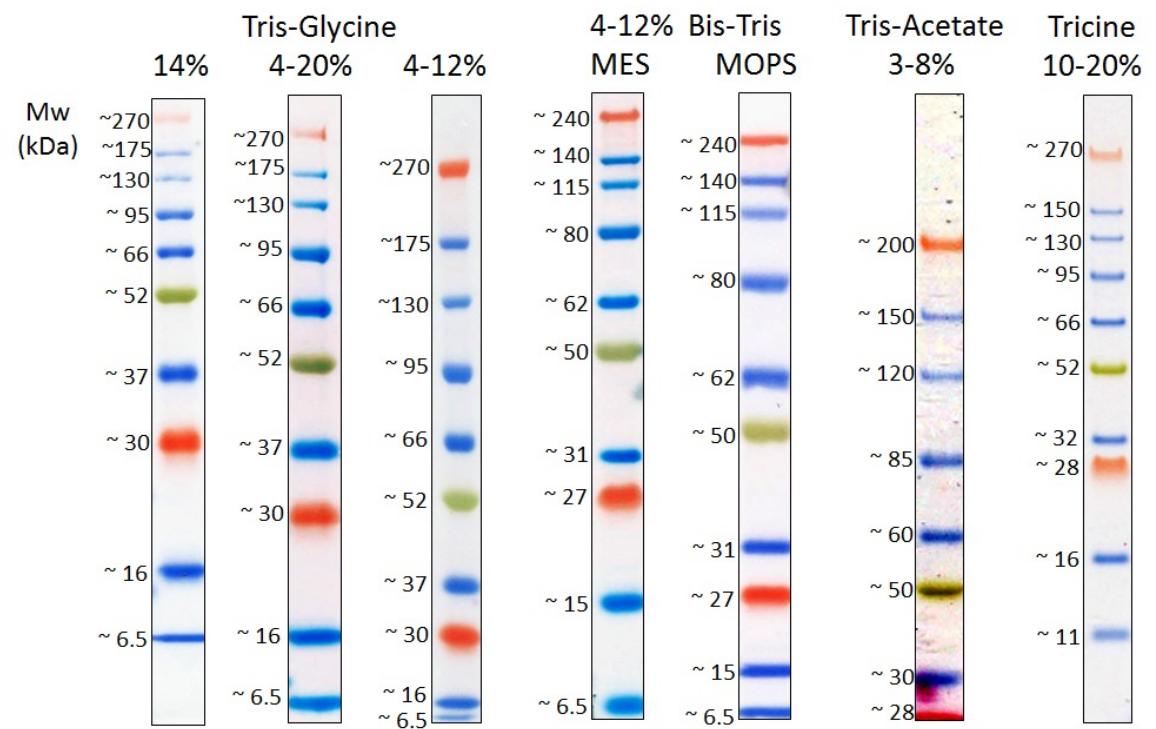
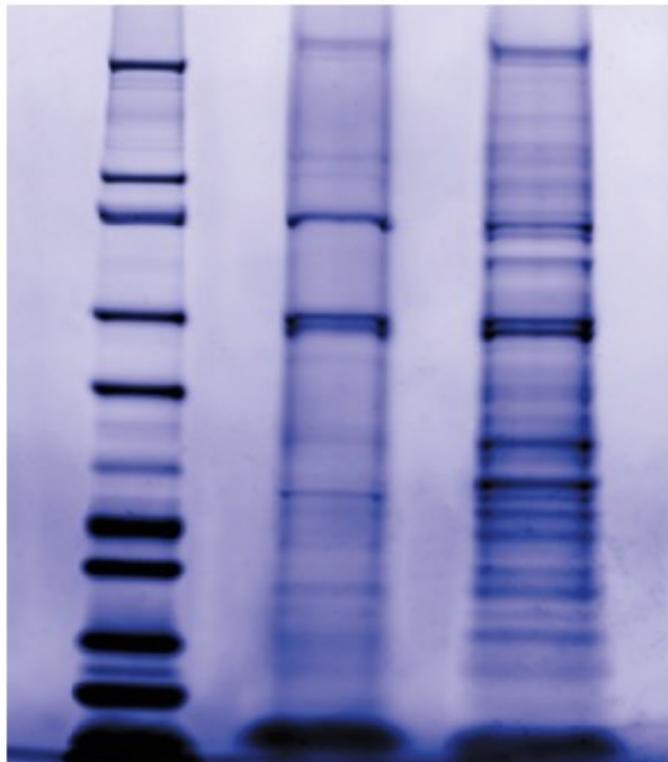
Proteins Can Be Separated by Gel Electrophoresis and Displayed

- Proteins will migrate in an electrical field because they are charged. When the migration occurs in a gel, the process is called gel electrophoresis.
- Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) allows accurate determination of mass. SDS denatures proteins, and for most proteins, 1 molecule of SDS binds for every two amino acids. Thus, proteins have the same charge-to-mass ratio and migrate in the gel on the basis of mass only.



Staining the Gel with Dyes to Visualize Protein

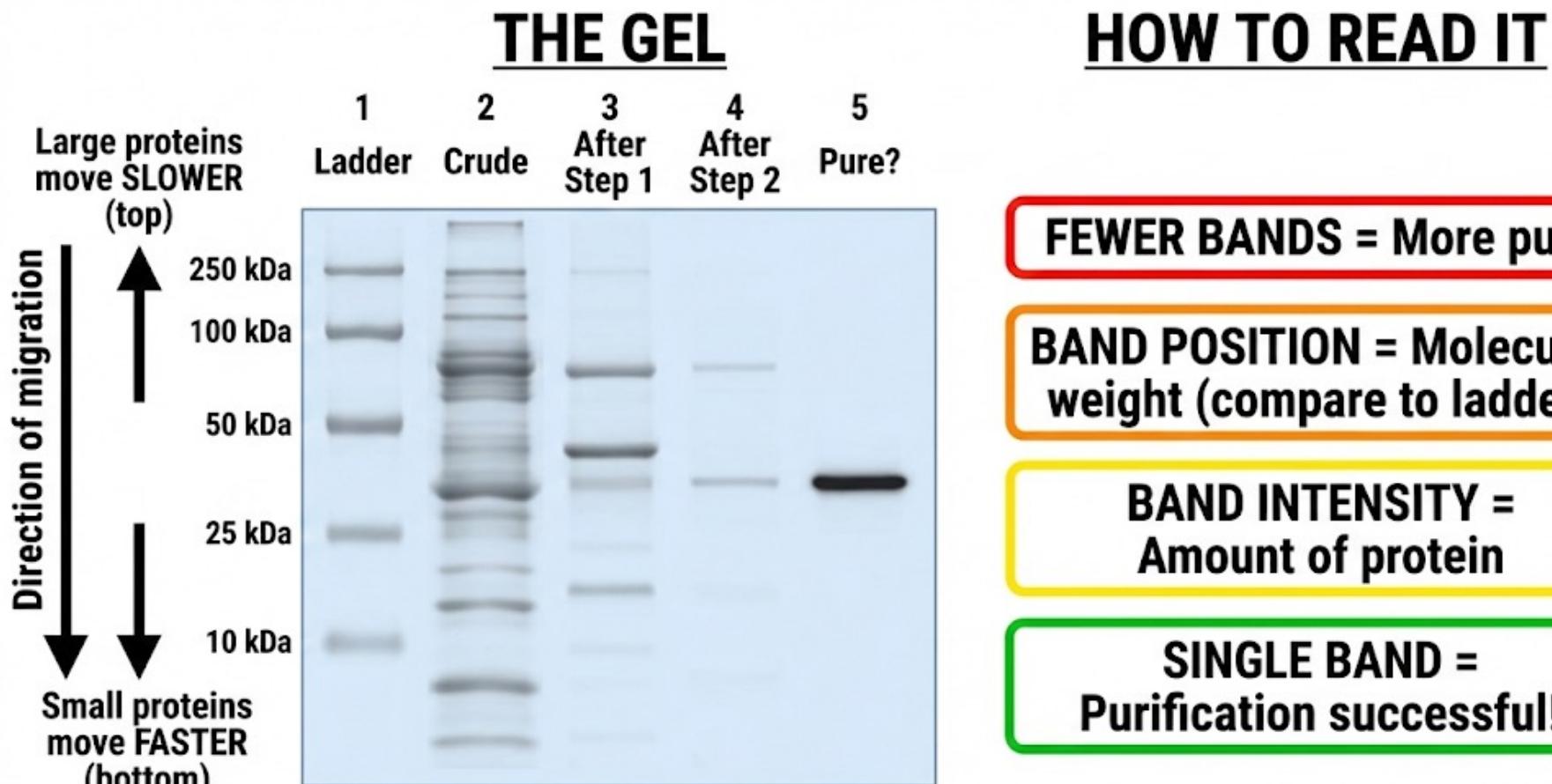
- Proteins can be separated by gel electrophoresis and displayed.
- Proteins separated by SDS-PAGE are visualized by staining the gel with dyes such as Coomassie blue.



It's still difficult to see all the proteins in a complex mixture



SDS-page interpretation guide.

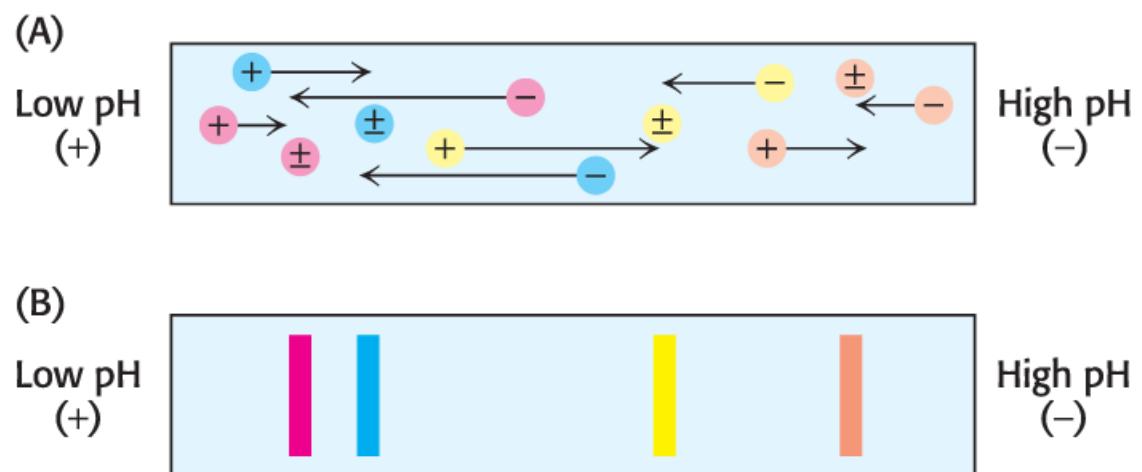


SDS gives all proteins equal charge → Separation by SIZE ONLY



Isoelectric Focusing

- Proteins can be separated by gel electrophoresis and displayed.
 - Isoelectric focusing allows separation of proteins in a gel on the basis of their relative amounts of acidic and basic amino acids. If a mixture of proteins is placed in a gel with a pH gradient and an electrical field is applied, proteins will migrate until they reach their isoelectric point (pl), the pH at which they have no net charge.

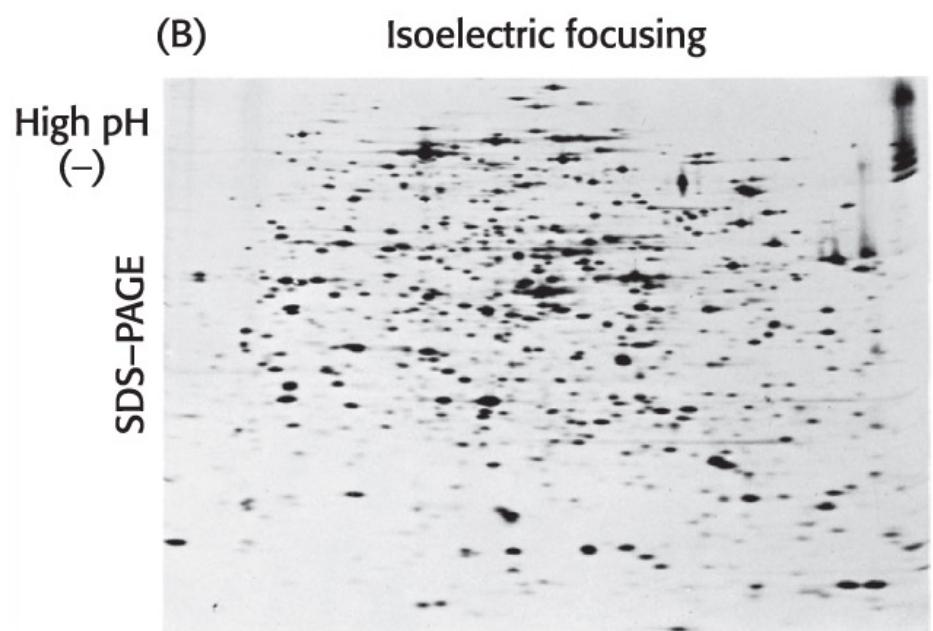
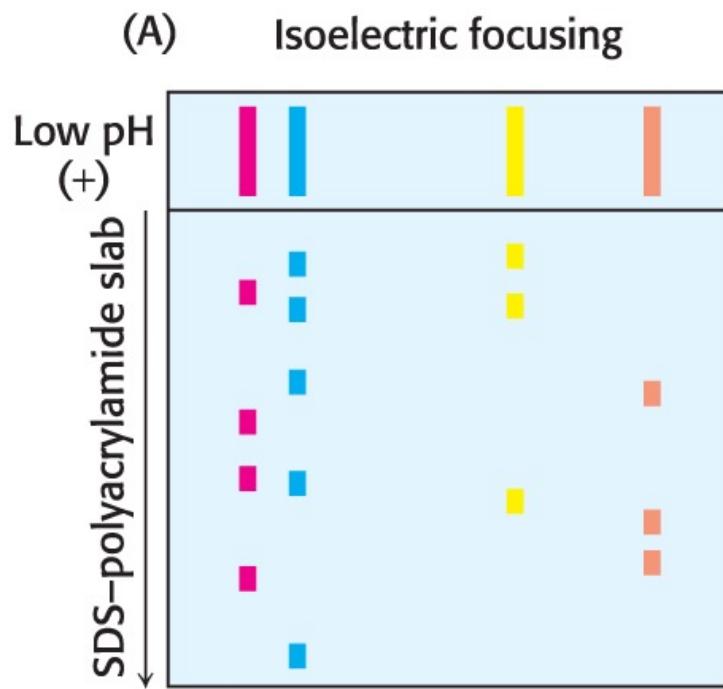


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Two-dimensional Gel Electrophoresis

- Proteins can be separated by gel electrophoresis and displayed.
- In two-dimensional gel electrophoresis, proteins are separated in one direction by isoelectric focusing. This gel is then attached to an SDS-PAGE gel, and electrophoresis is performed at a 90° angle to the direction of the isoelectric focusing separation.

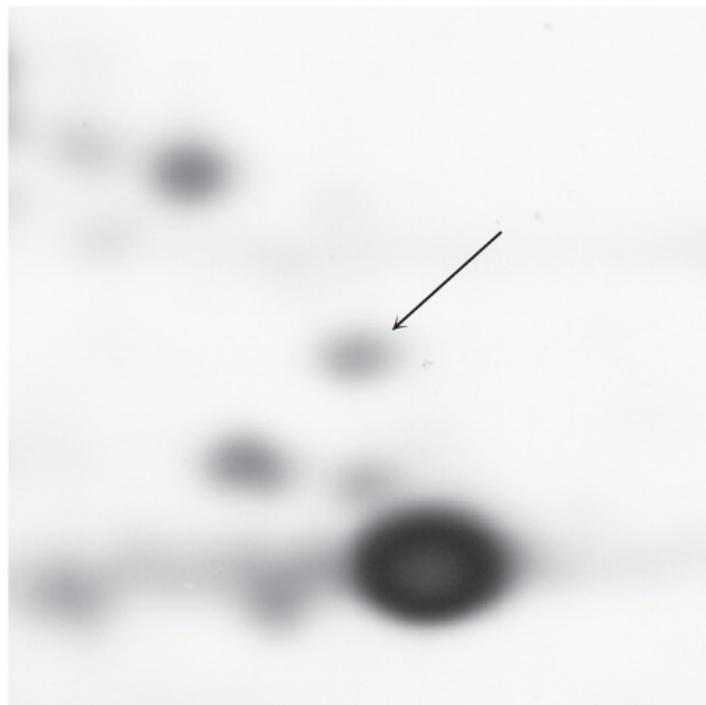


(B) Courtesy of Dr. Patrick H. O'Farrell.



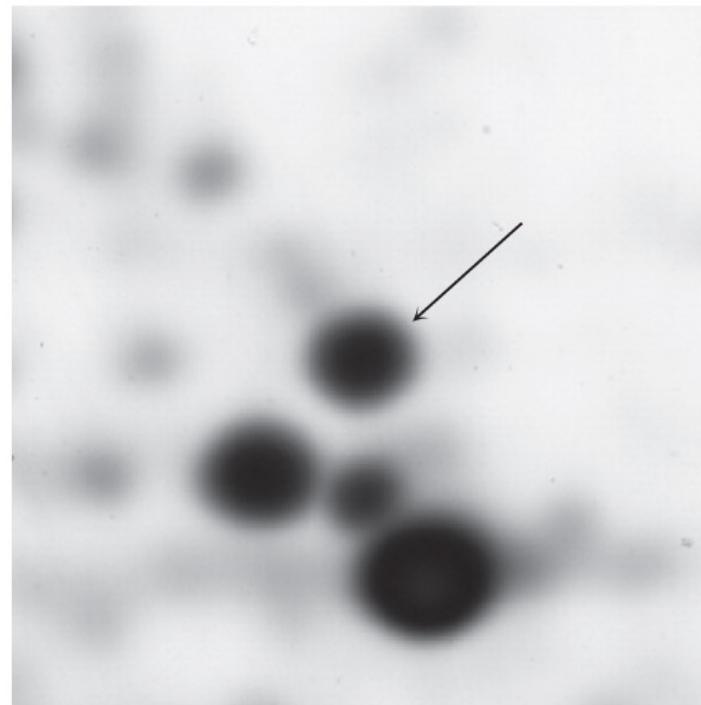
Images of Alteration in Proteins Levels Detected by Two-dimensional Gel Electrophoresis

(A)



Normal colon mucosa

(B)



Colorectal tumor tissue

Images courtesy Qingsong Lin © 2006 The American Society for Biochemistry and Molecular Biology.



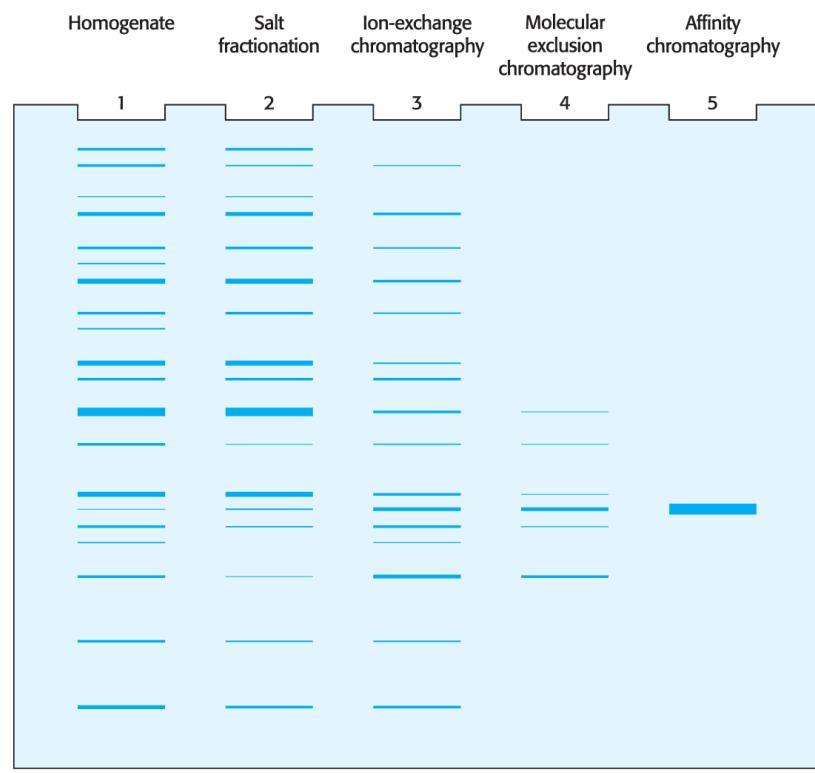
A Purification Scheme Can Be Quantitatively Evaluated

- The effectiveness of a purification scheme is measured by calculating the **specific activity** after each separation technique.
- SDS-PAGE allows a visual evaluation of the purification scheme.

Step	Total protein (mg)	Total activity (units)	Specific activity (units mg ⁻¹)	Yield (%)	Purification level
Homogenization	15,000	150,000	10	100	1
Salt fractionation	4600	138,000	30	92	3
Ion-exchange chromatography	1278	115,500	90	77	9
Molecular exclusion chromatography	68.8	75,000	1100	50	110
Affinity chromatography	1.75	52,500	30,000	35	3000

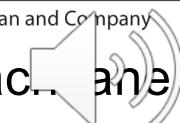


This is the column that matters most.



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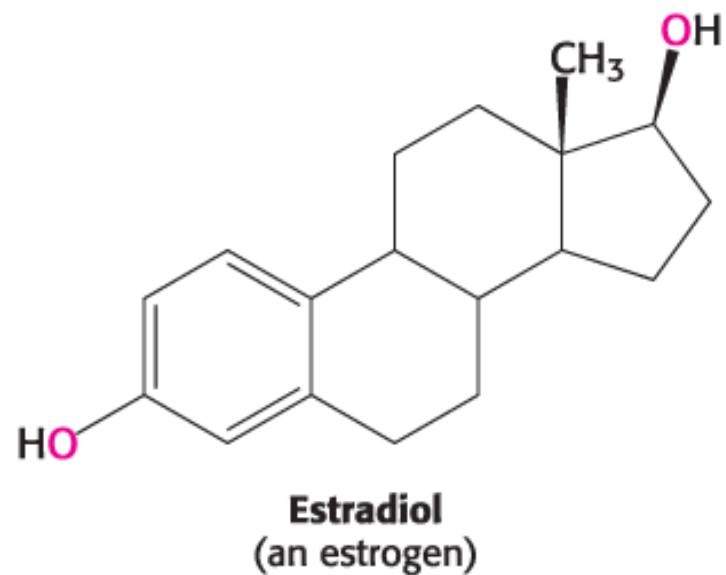
50ug of sample in each lane



How would you purify the estrogen receptor?

Learning objective 5: Explain how immunological techniques can be used to purify and identify proteins.

- Estrogen is crucial for regulating reproductive function, bone density, and cardiovascular health, and its dysregulation is implicated in conditions such as breast cancer and osteoporosis.
- The estrogen receptor binds the steroid hormone estradiol tightly and with great specificity.
- The estrogen receptor has no enzymatic activity but can be purified by immunological techniques and the use of gradient centrifugation.



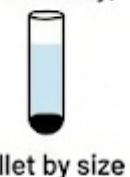
Back in the day they knew what the ligand/hormone was but not the receptor



Separate, fractionate, assay: the binding peak reveals the target

Centrifugation: 3 common flavors

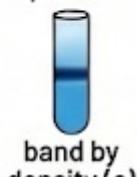
Differential
(pellet/clarify):



Rate-zonal
gradient (THIS):



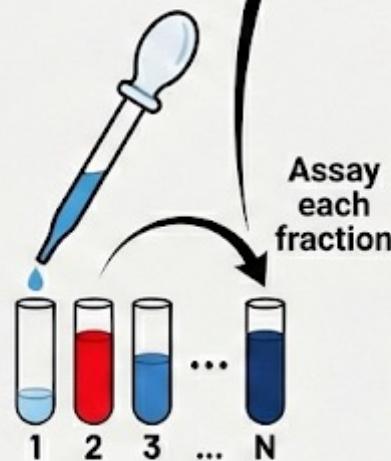
Isopycnic
equilibrium:



THE METHOD



Higher S
sediments
faster

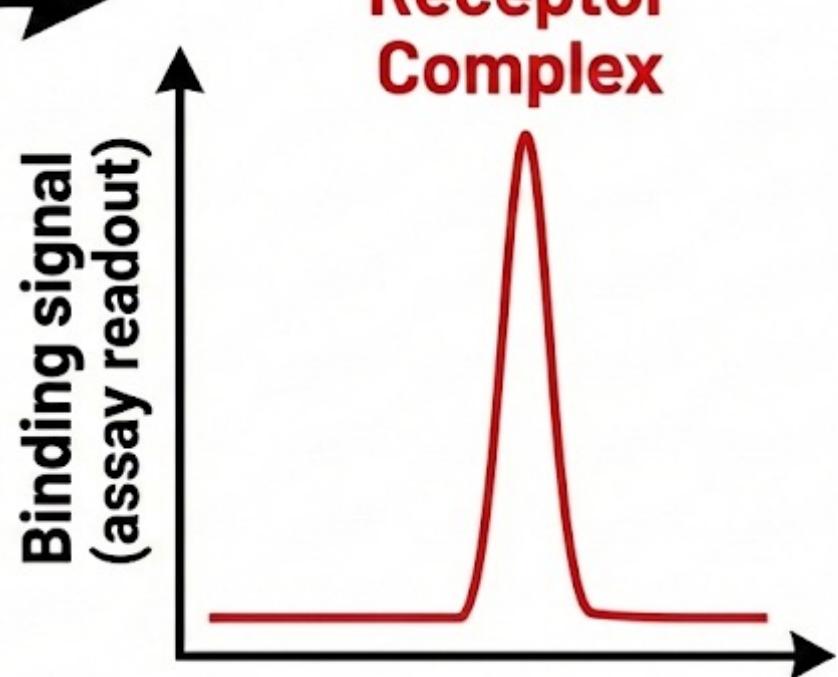


Fractionate
(collect fractions 1...N)

S = sedimentation coefficient

THE RESULT

Receptor
Complex



Sedimentation
coefficient (S)



Break!!



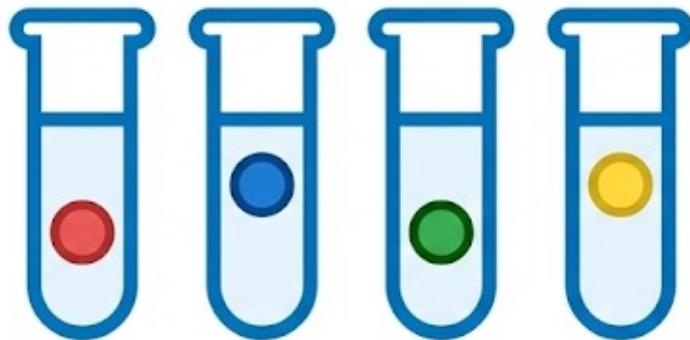
QUICK QUIZ 2

What physical differences among proteins allow for their purification?



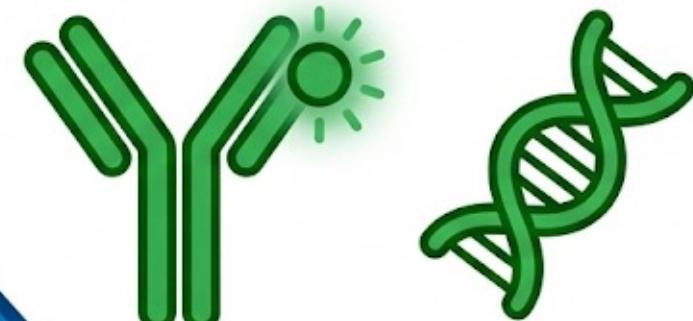
From Separation to Identification: The next step

We've **SEPARATED** proteins



Proteins are now in
different tubes

Now we **IDENTIFY** our target



NEXT
STEP

But which tube has OUR protein?

1. ANTIBODIES:
Quick yes/no answer
2. SEQUENCING:
Definitive identification
**Confirmation that we got
the RIGHT protein**

PURE ≠ IDENTIFIED – You must CONFIRM what you purified

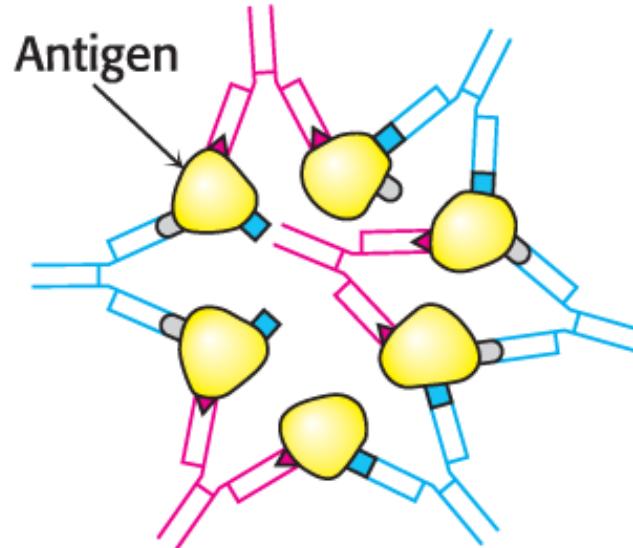
- Purification gives you a PURE sample
- But pure ≠ identified
- Two strategies:
- **Antibodies:** Quick confirmation (Western, ELISA)
- **Sequencing:** Definitive identification (Mass spec)



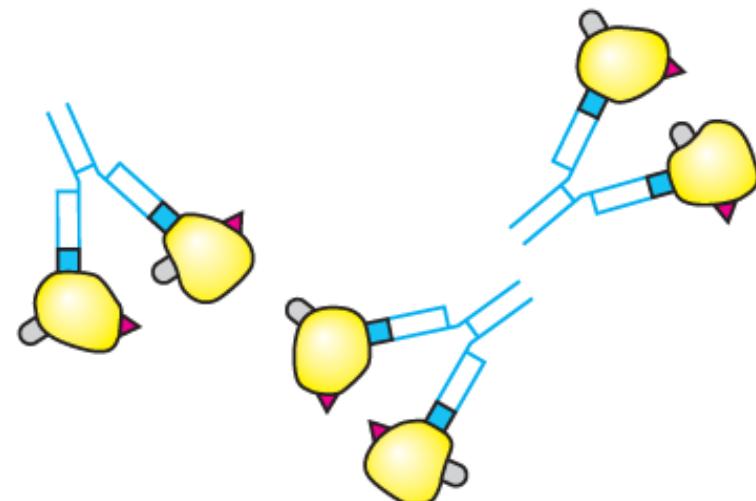
Monoclonal vs Polyclonal Antibodies

- Antibodies to specific proteins can be generated.
 - Any antibody-producing cell synthesizes antibodies that recognize only one epitope. Each antibody-producing cell thus synthesizes a monoclonal antibody.
 - Any antigen may have multiple epitopes. The antibodies produced to the antigen by different cells are said to be polyclonal.

Polyclonal antibodies



Monoclonal antibodies

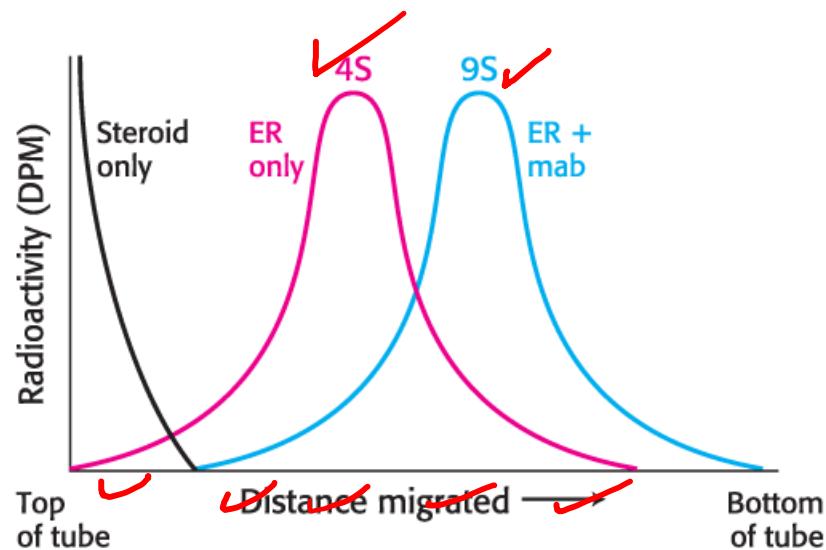


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The Estrogen Receptor Can Shift Sedimentation during centrifugation.

- A monoclonal antibody for the estrogen receptor can be isolated by searching for cell lines that produce an antibody that binds to the receptor.
- If an antibody for the receptor is present, it will bind to the receptor and alter the sedimentation constant of the receptor.



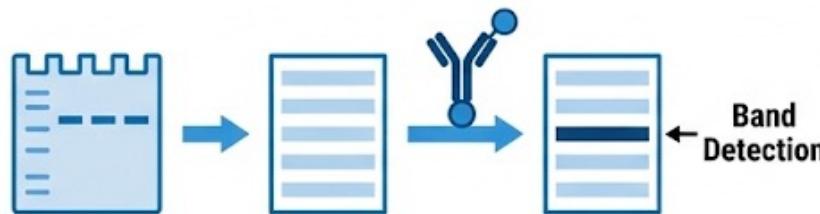
Tymoczko et al., *Biochemistry: A Short Course*, 4e, © 2019 W. H. Freeman and Company

What else can antibodies be used for?



Some cool stuff you can do with antibodies

WESTERN BLOTH



Detect specific protein in a mixture

Confirms protein IDENTITY



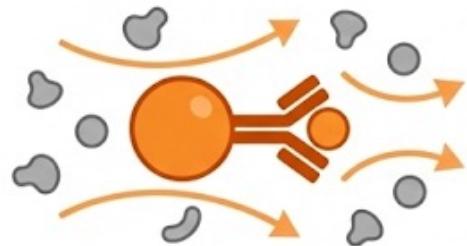
ELISA



Quantify protein amount

Pregnancy test, COVID test

IMMUNOPRECIPITATION



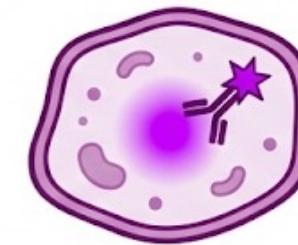
Purify specific protein

Pull down your target

ANTIBODIES =

FLUORESCENCE MICROSCOPY

Molecular Detectives



Locate protein in cell

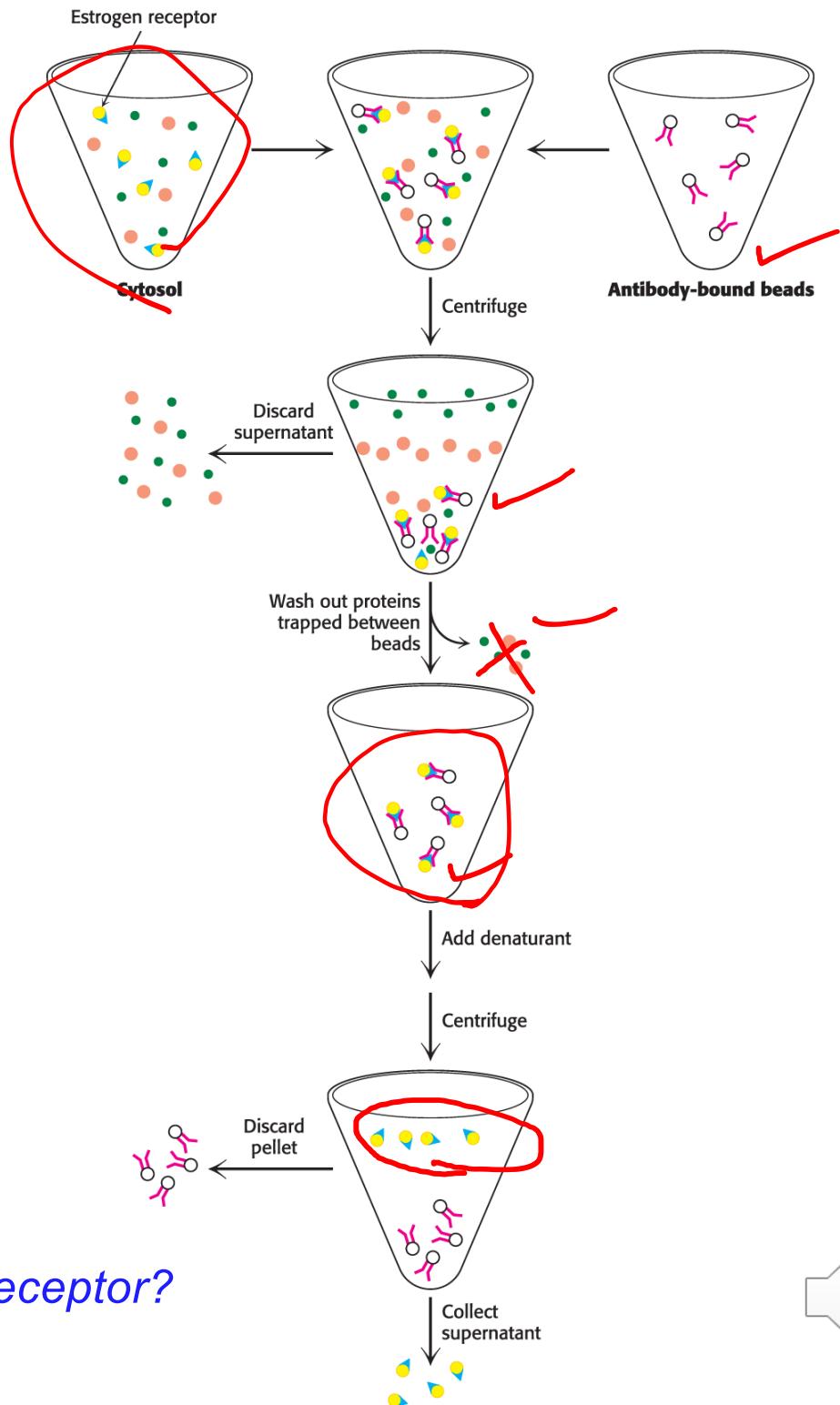
See **WHERE** protein is

[Back to estrogen](#)



Antibody Can be Used to Purify the Estrogen Receptor

- The estrogen receptor can be purified by immunoprecipitation
- Once the monoclonal cell line is isolated, the antibody can be used to purify the estrogen receptor.

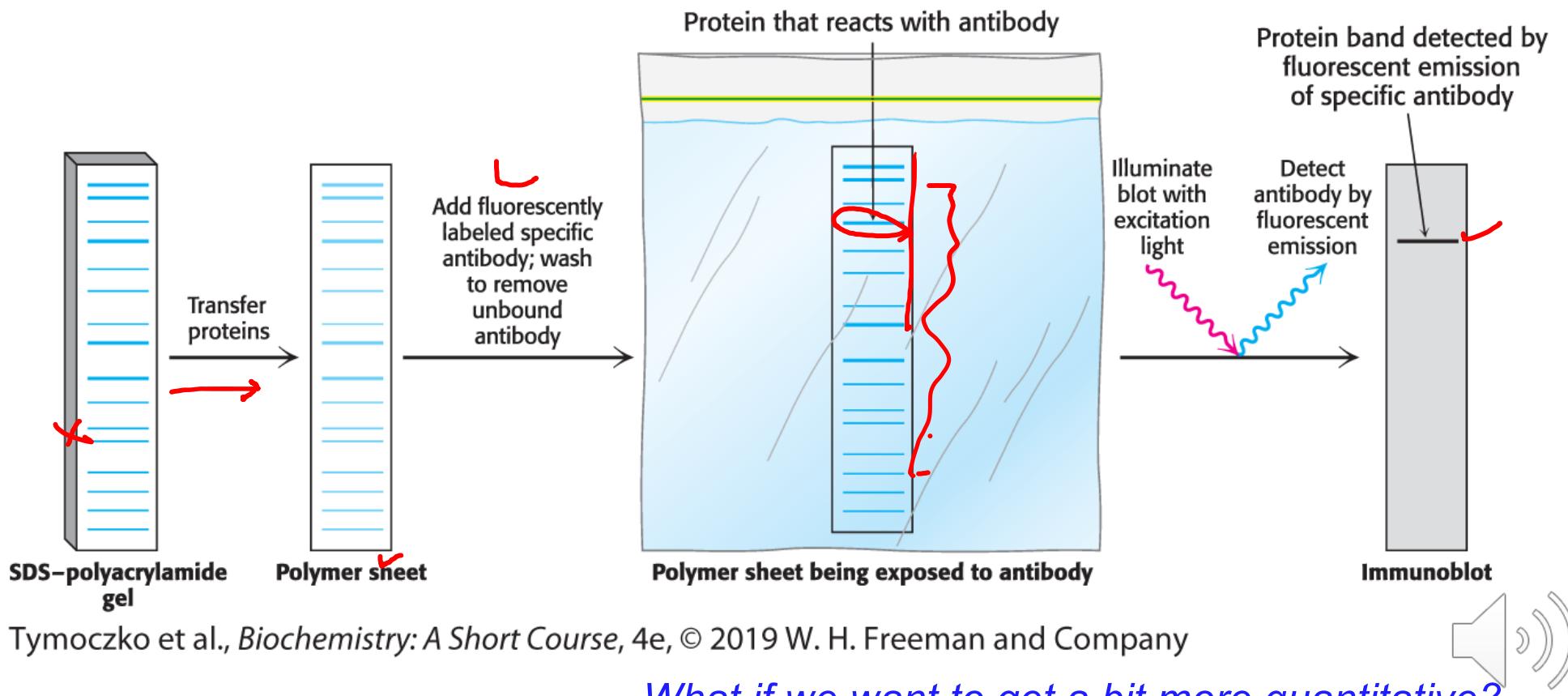


How can we tell if we purified estrogen receptor?

Western Blotting

Permits the Detection of Proteins Separated by Gel Electrophoresis

- In western blotting or immunoblotting, proteins are separated in an SDS-PAGE gel, transferred to a sheet of polymer, and then stained with a fluorescent antibody.



Tymoczko et al., *Biochemistry: A Short Course*, 4e, © 2019 W. H. Freeman and Company

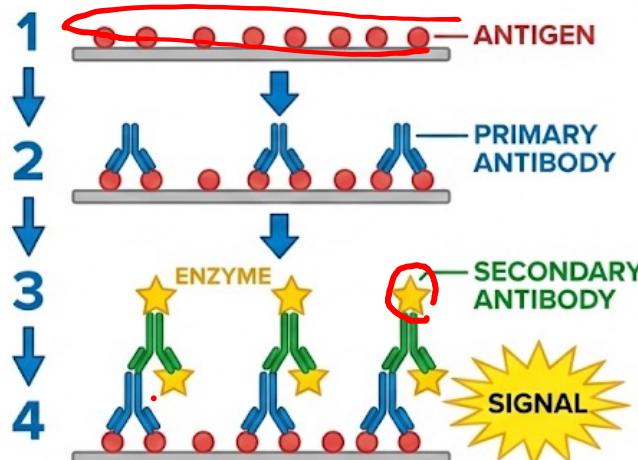
What if we want to get a bit more quantitative?



Proteins Can Be Detected and Quantified with the Use of an Enzyme-Linked Immunosorbent Assay

- Antibodies are used as a reagent to determine the amount of a protein or other antigen present. Enzyme-linked immunosorbent assay (ELISA) quantifies the amount of protein present because the antibody is linked to an enzyme whose reaction yields a readily identified colored product.

INDIRECT ELISA

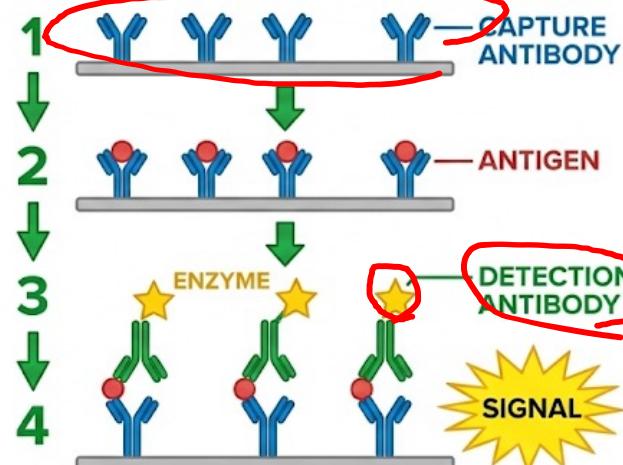


Detects: ANTIBODIES in sample

Example: HIV antibody test

INDIRECT: Looking for antibodies
(immune response)

SANDWICH ELISA



Detects: ANTIGENS in sample

Example: Pregnancy test (hCG)

SANDWICH: Looking for antigens
(the protein itself)

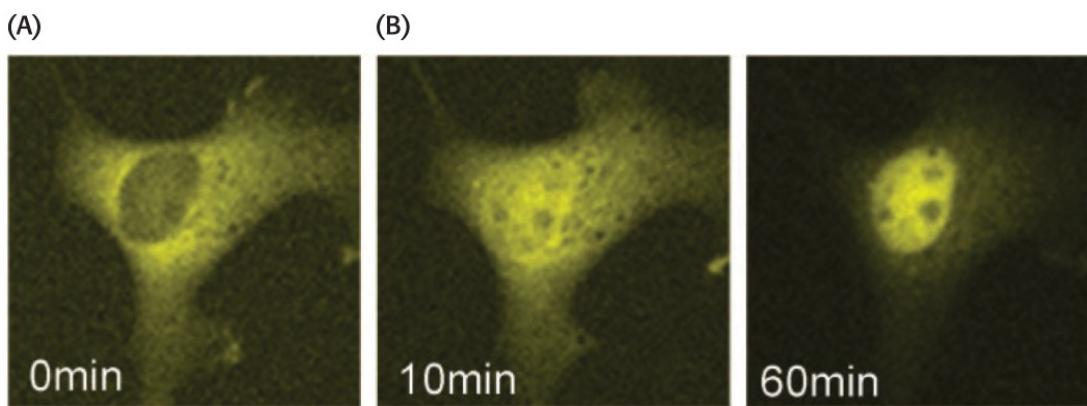


Fluorescence Microscopy

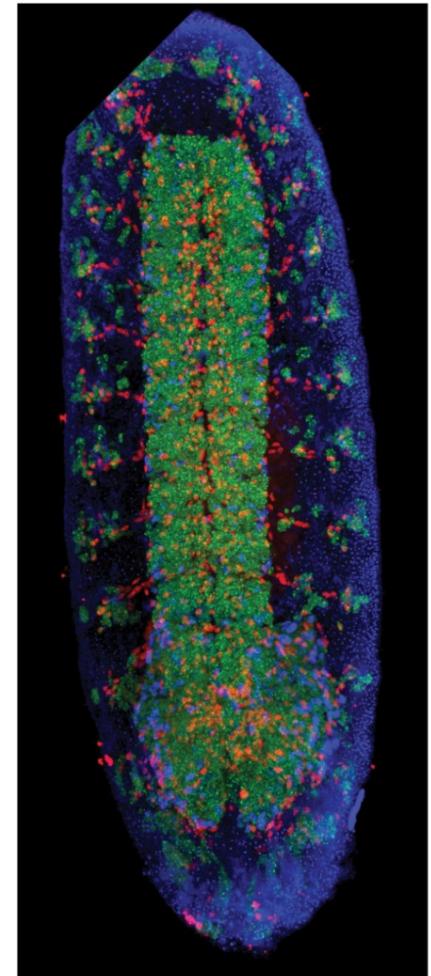
Cells can be stained with fluorescence-labeled antibodies and examined by **fluorescence microscopy** to reveal the location of a protein of interest.

By tracking protein location, fluorescent markers also provide clues to protein function.

The highest resolution of fluorescence microscopy is about $0.2 \mu\text{m}$, the wavelength of visible light.



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Immunoelectron Microscopy

Immunoelectron microscopy can define the position of antigens to a resolution of 10 nm (100 Å) or finer.

This technique is electron microscopy with the antibodies tagged with electron-dense markers.



Courtesy of Dr. Peter Sargent



Now that we've fished out our estrogen binding protein, how do we know what it is?

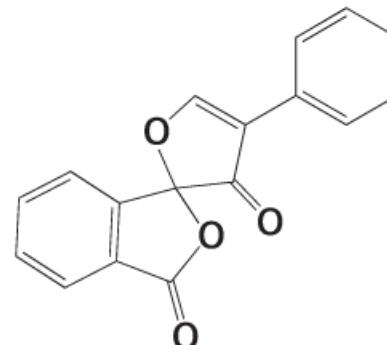


First let's figure out the amino acid composition

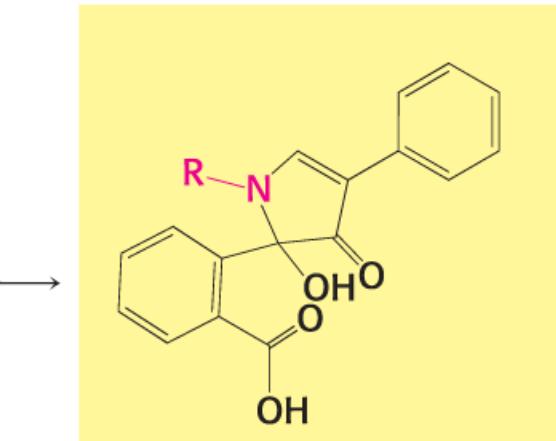


Section 6.4 Determination of Primary Structure Facilitates and Understanding of Protein Function

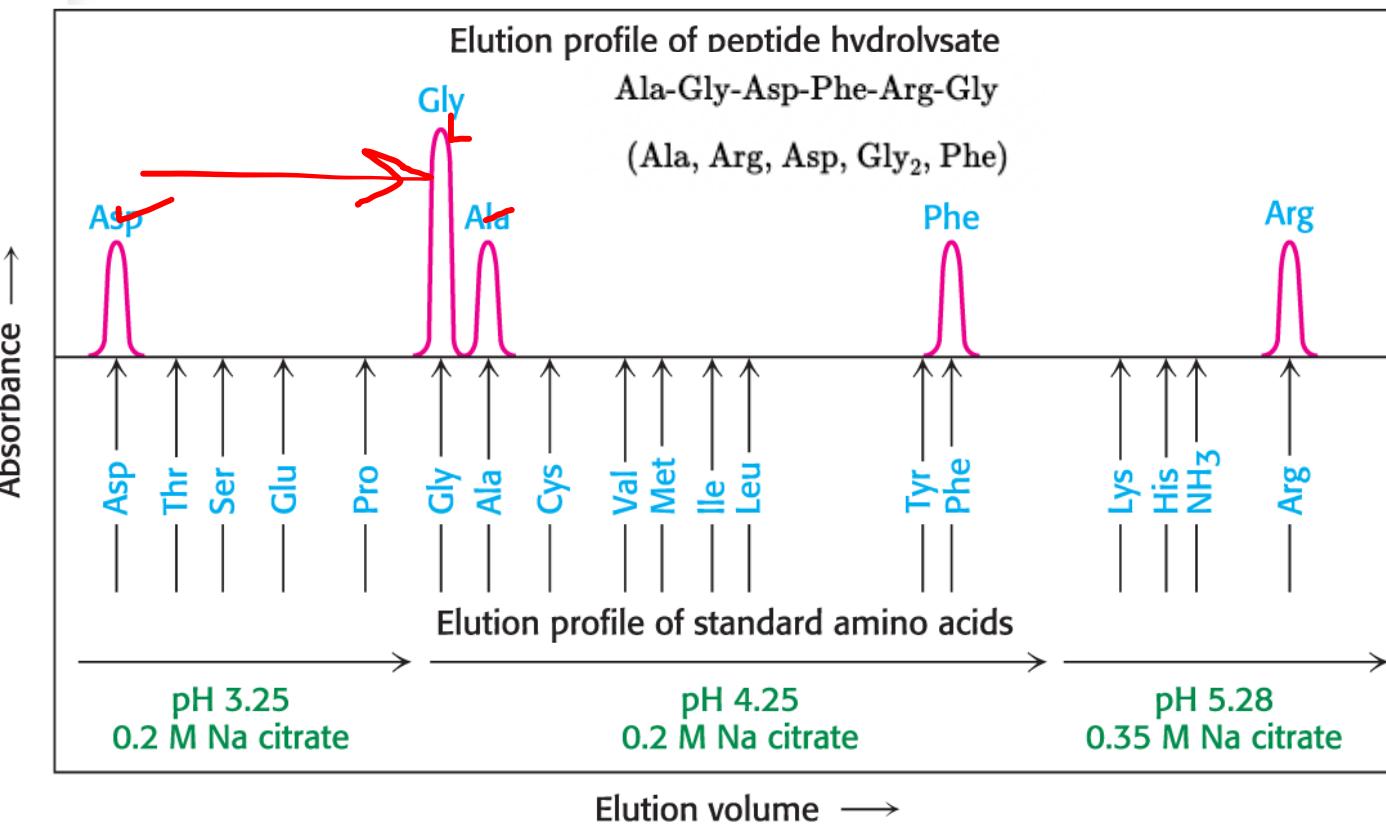
- A key step in understanding protein function is to determine the primary structure, or the amino acid sequence, of the protein. A preliminary step is to determine the amino acid composition of the protein.
- The protein is hydrolyzed, and the constituent amino acids are separated on an ion-exchange column. The amino acids are visualized by reaction with fluorescamine.



Fluorescamine



Amine derivative



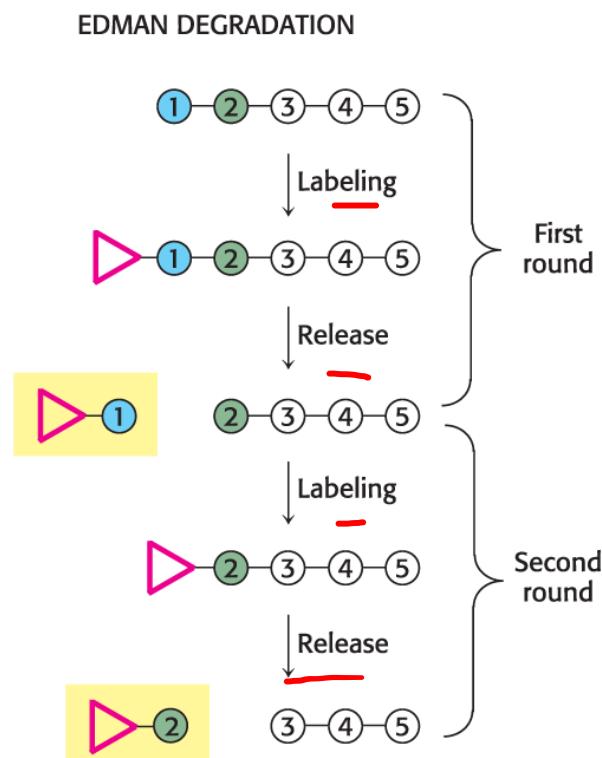
Tymoczko et al., *Biochemistry: A Short Course*, 4e, © 2019 W. H. Freeman and Company

Now let's figure out the sequence

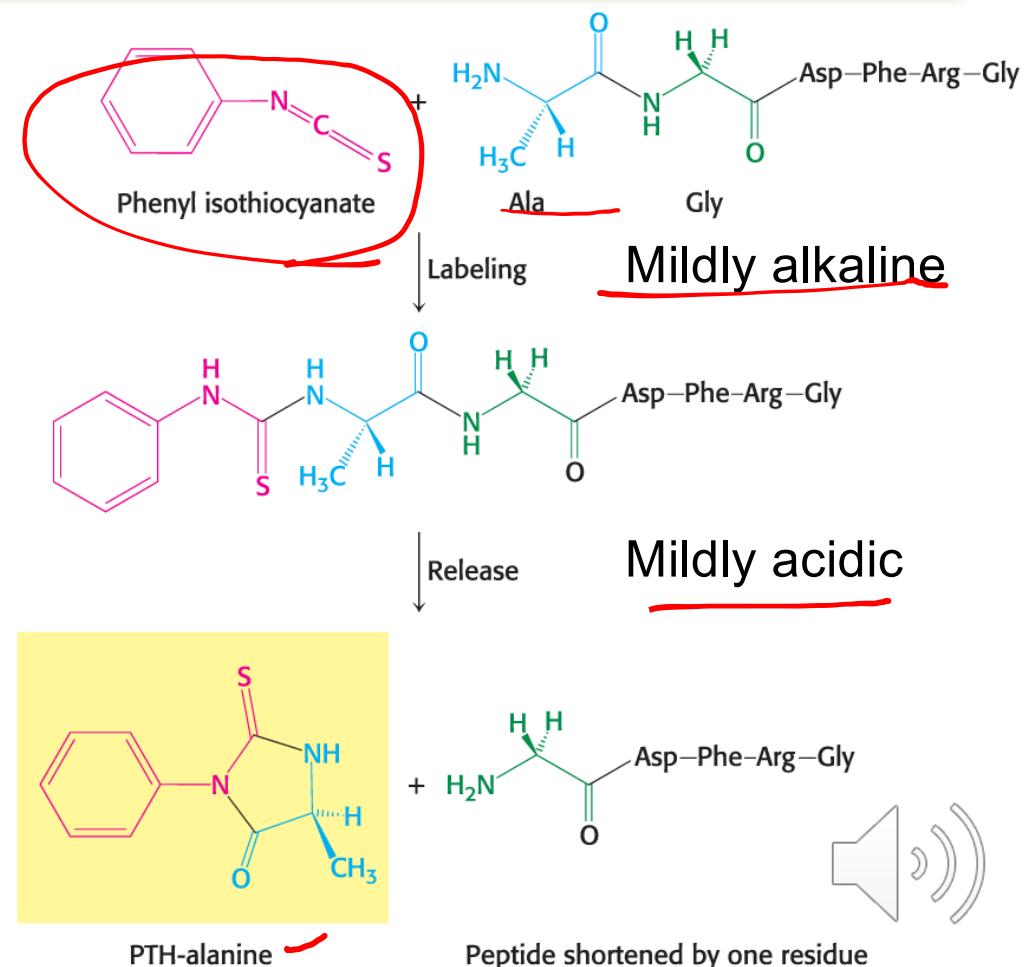


Edman Degradation

- The amino acid sequence can be determined by Edman degradation. The protein is exposed to phenyl isothiocyanate (PTH), which reacts with the N-terminal amino acid to form a PTH-derivative. The PTH-amino acid can be released without hydrolyzing the remainder of the protein, and the degradation is subsequently repeated.



Only good for 50 amino acids but most proteins are bigger than that



DETERMINING PROTEIN SEQUENCE WITH OVERLAP PEPTIDES

TOP PROBLEM - THE PROBLEM

Full Protein: Too long to sequence directly

Edman degradation only works for ~ 50 amino acids

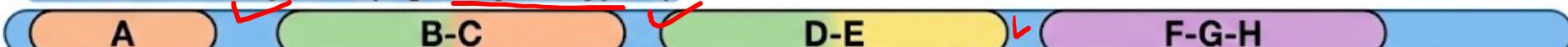
THE SOLUTION

A. Cut with Enzyme 1 (e.g., Trypsin)



We know sequence within each piece, but not the ORDER

B. Cut with Enzyme 2 (e.g., Chymotrypsin)



These fragments OVERLAP with the first set

THE OVERLAP

THE OVERLAP



Final Assembled Sequence

Overlapping regions reveal the ORDER

Like solving a puzzle: overlapping pieces show how fragments connect

- Because the reactions of the Edman degradation procedure are not 100% effective, it is not possible to sequence polypeptides longer than 50 amino acids.
- In order to sequence the entire protein, the protein is chemically or enzymatically cleaved to yield peptides of fewer than 50 amino acids.
- The peptides are then ordered by performing a different cleavage procedure in order to generate overlap peptides.

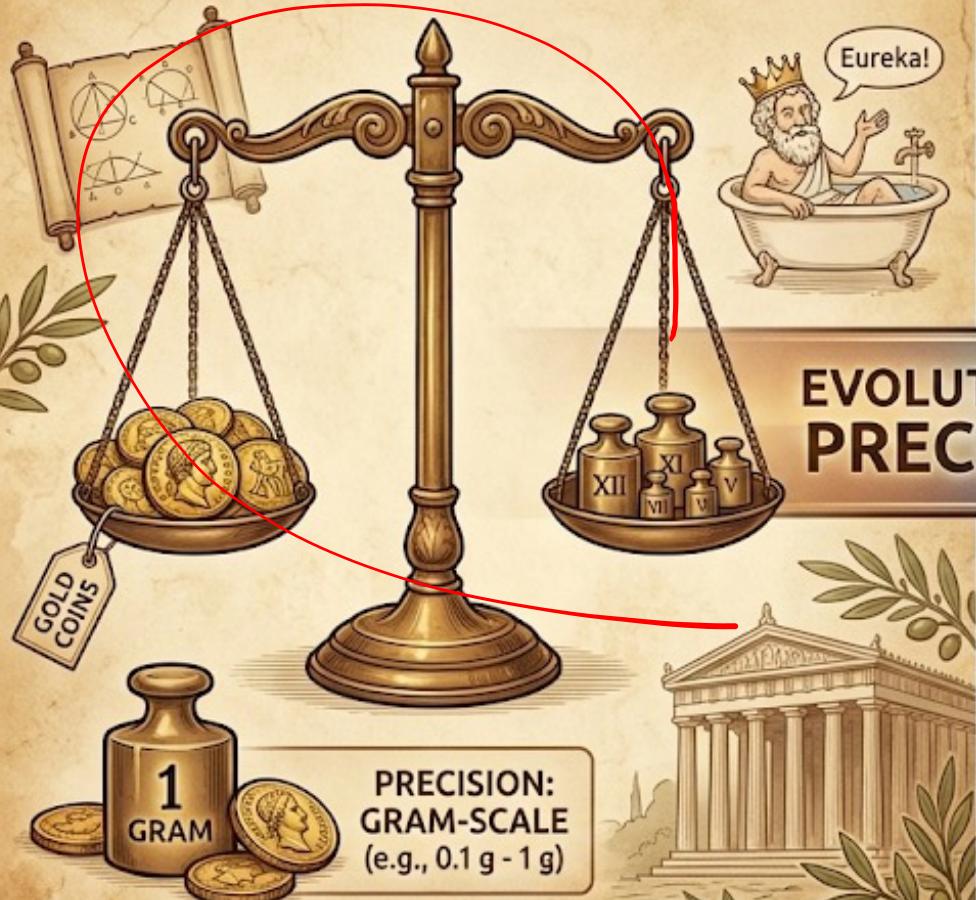


A close-up photograph of a vintage-style mechanical scale. The scale has a silver-colored metal frame and a circular weighing platform with a white face and black markings. The numbers visible on the scale are 20, 40, 60, 80, 100, 120, 140, 160, 180, 200, 220, 240, 260, 280, and 300. A small number '0' is also present near the center. The scale is resting on a red and white vertically striped cloth. The background is a plain, light-colored wall.

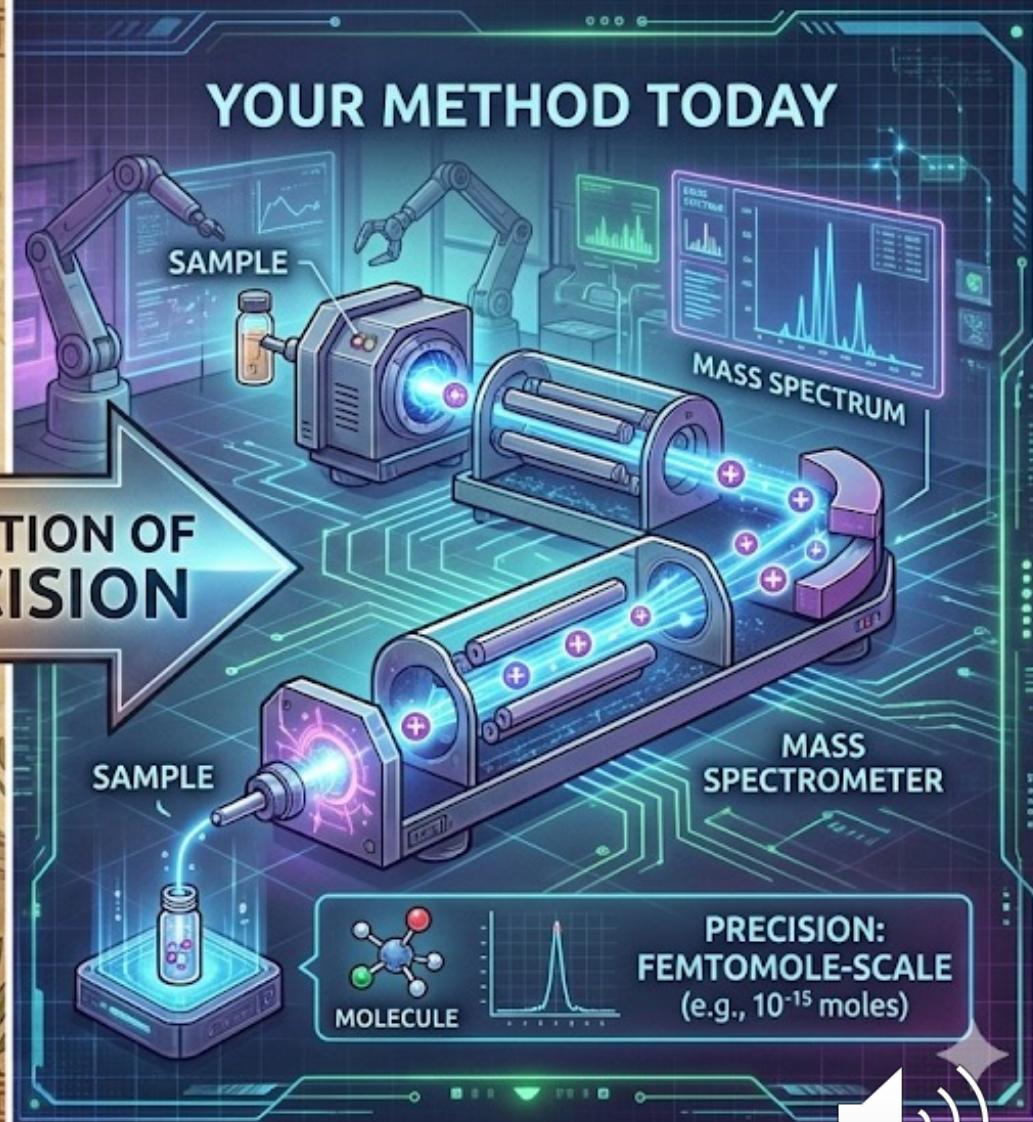
Edman Degradation is
the old school. Mass
spec is the new school



ARCHIMEDES' METHOD ~250 BC



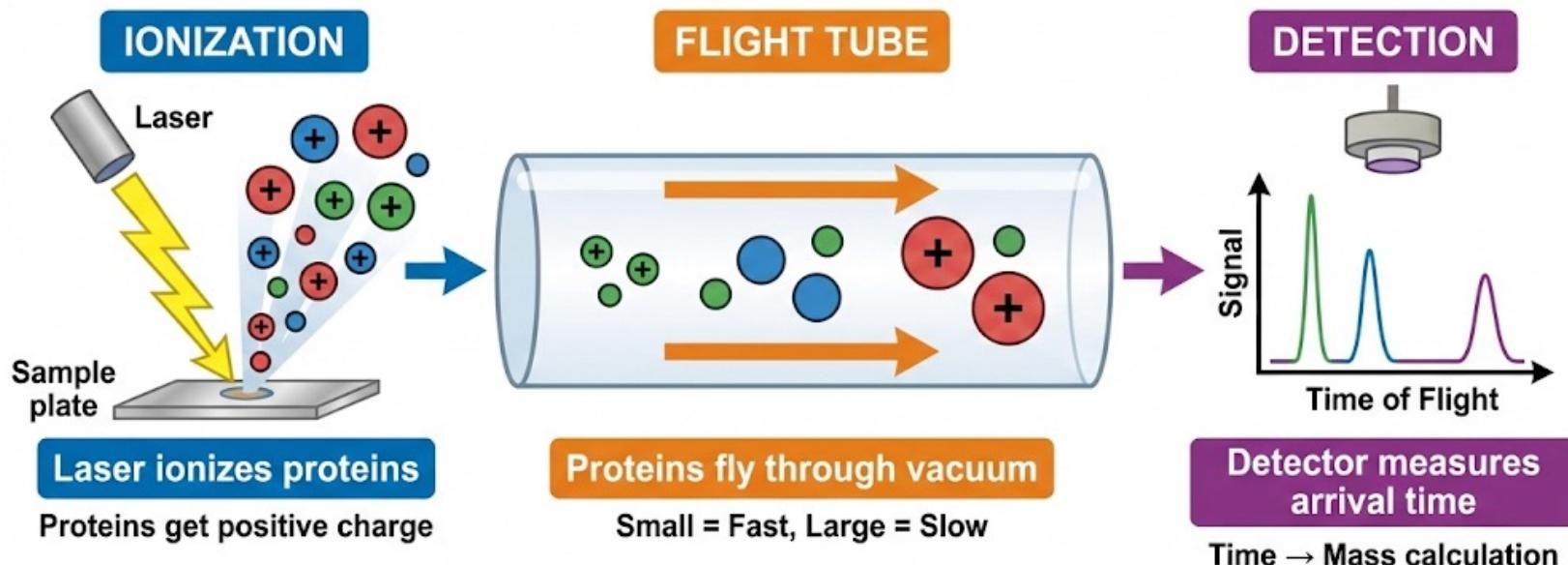
YOUR METHOD TODAY



Mass Spectrometry Can Be Used to Determine a Protein's Mass, Identity, and Sequence

- Two mass spectrometry techniques can be used to determine a protein's mass: matrix-assisted laser desorption (MALDI) and electrospray ionization (ESI). In MALDI, proteins are precipitated onto a matrix and a laser flash releases positively charged ions.
- Time of flight (TOF) analysis is used to measure how rapidly the ions move toward a detector.
- Only picomoles or femtomoles of a protein are required for MALDI-TOF analysis.

MALDI-TOF MASS SPECTROMETRY

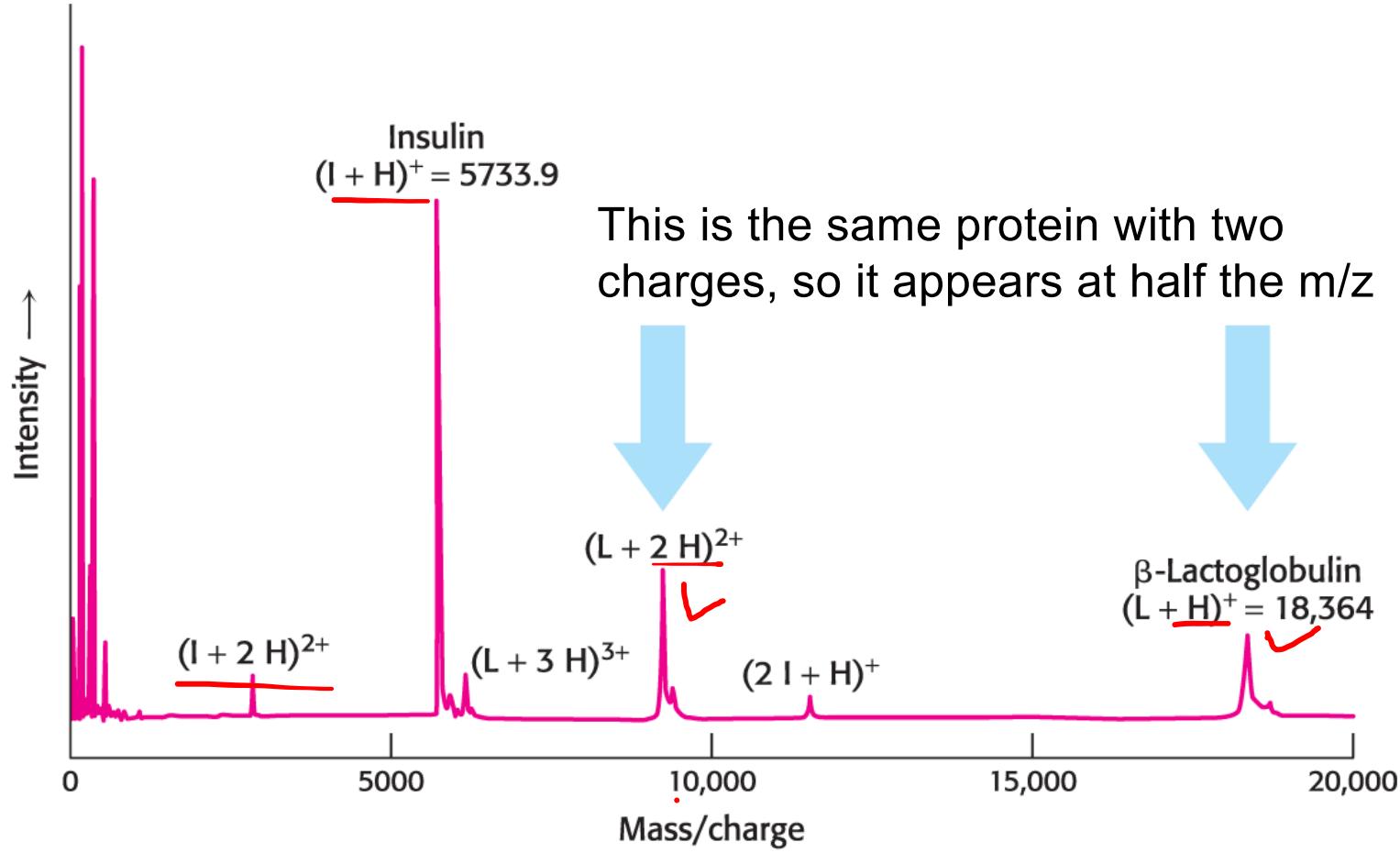


KEY TAKEAWAYS

- Measures EXACT protein mass
- Needs only femtomoles of sample
- Can analyze proteins >100 kDa



MALDI-TOF Mass Spectrum

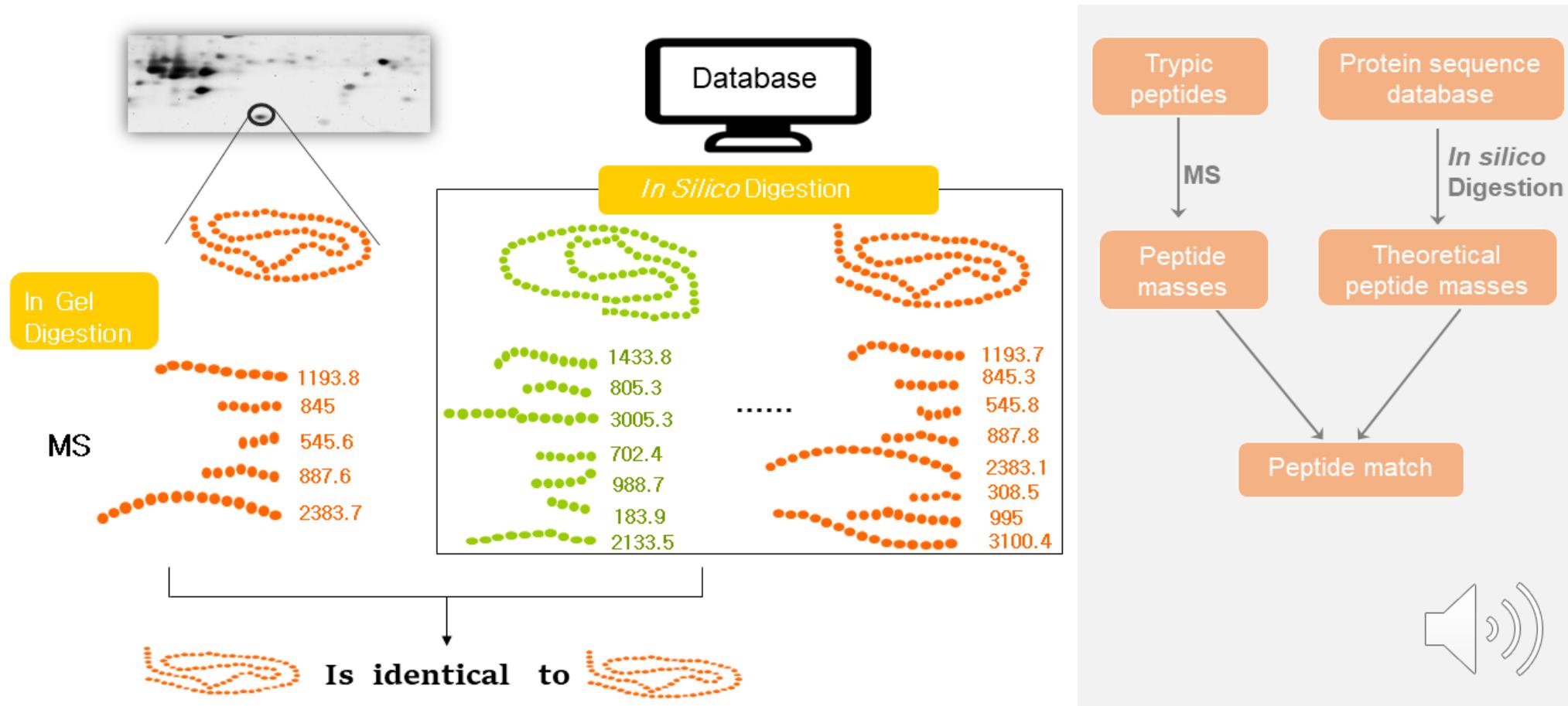


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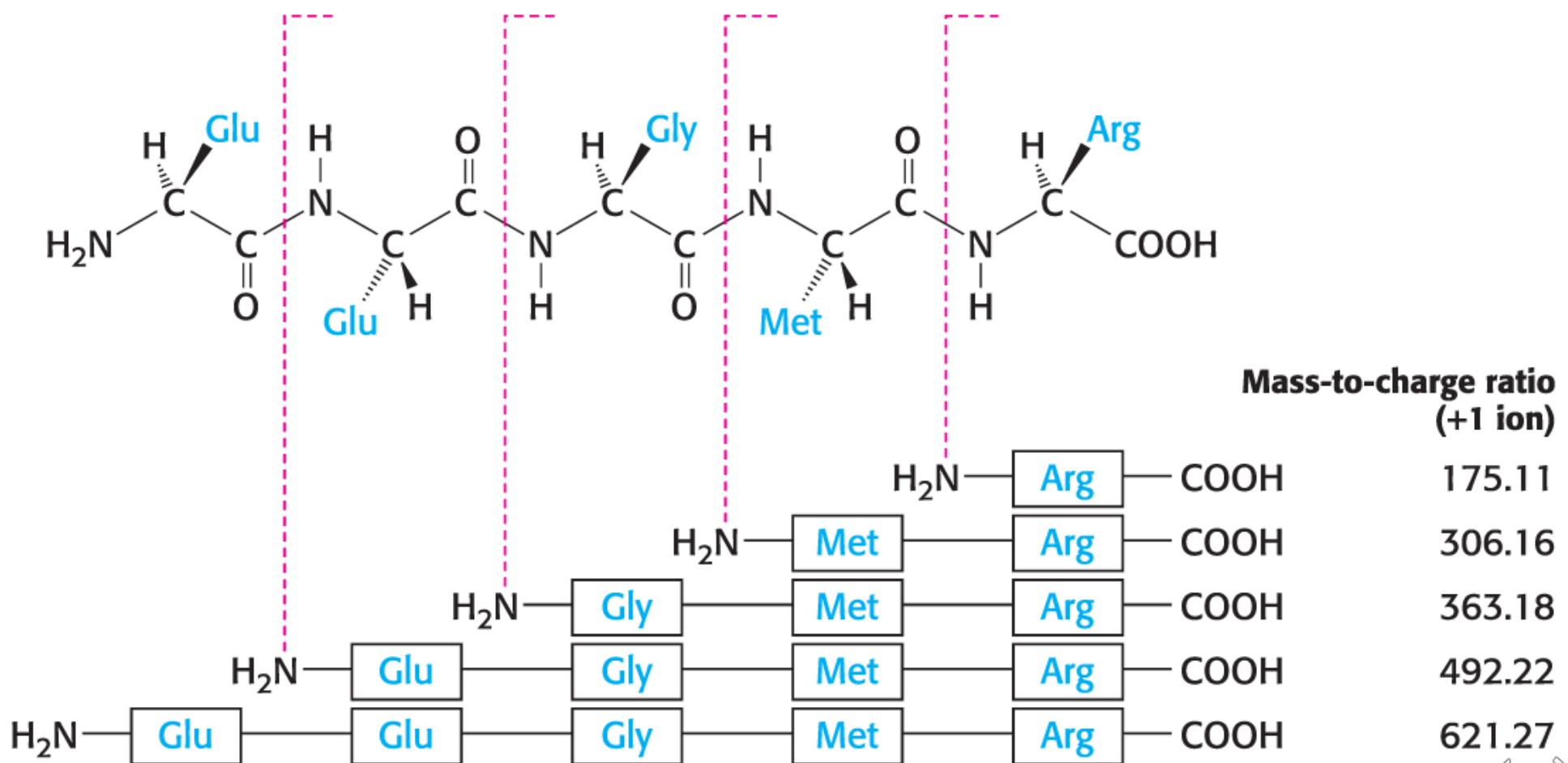
Protein Identity

- Mass spectrometry allows determination of a protein's identity. For instance, an unknown protein visible in a two-dimensional gel can be removed from the gel.
- The protein is then cleaved in some fashion and subjected MALDI-TOF, revealing a series of peptides with known masses.
- These peptide masses are then compared to proteins in a database and are "electronically cleaved" by a computer using the same cleavage technique used to generate the protein fragments.



Protein Sequence

A protein's sequence can be determined with the use of tandem mass spectrometry.





QUICK QUIZ 4

Differentiate between amino acid composition and amino acid sequence.



SEQUENCE DETECTIVE

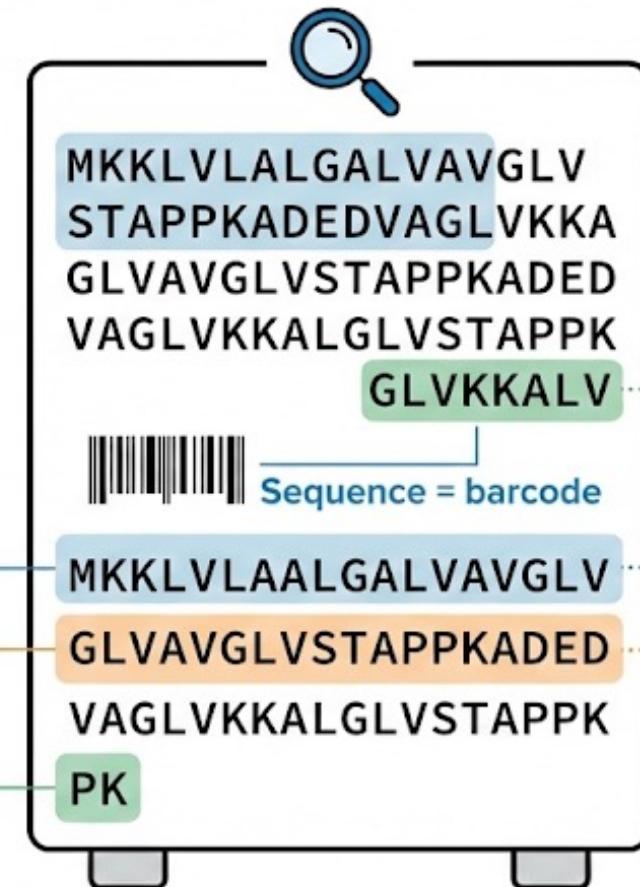
A sequence is a barcode.
What can you read from it?



Signal peptide?

Transmembrane?

Motif / active site?



What can you infer?



Function
(motifs/domains)



Evolution
(homology)



History
(repeats)



Location
(signals/TM)



Disease
(mutations)



Find the
gene

Choose one. Defend it.

Purify → Sequence → Infer: function, location, evolution, disease.



ACCELERATING PROTEIN SCIENCE: A HISTORY OF BREAKTHROUGHS

Timeline showing the exponential acceleration of protein structure and therapeutic capabilities.

1951
INSULIN SEQUENCE
(Sanger)
First protein sequence determined, pioneering molecular biology.

Gly-Ile-Val-Glu-Gln-Cys-Cys-Ala-Ser-Val-Cys-Ser-Leu-Tyr-Gln-Leu-Glu-Asn-Tyr-Cys-Asn

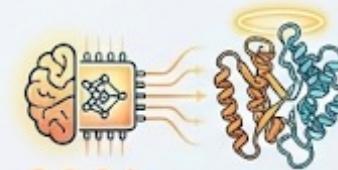
1984
FIRST THERAPEUTIC MONOCLONAL ANTIBODY
(Muromonab-CD3)

Approval of first monoclonal antibody drug, opening new therapeutic era.



2020
COVID SPIKE PROTEIN STRUCTURE SOLVED
(In Weeks)

Rapid structural determination using cryo-EM, enabling vaccine development.



2024

AI PROTEIN STRUCTURE PREDICTION
(AlphaFold 3 & Beyond)

Artificial Intelligence predicts nearly all known protein structures with high accuracy, revolutionizing biology.

EXponential Acceleration of Capability

Visualizing the future of biological discovery

