

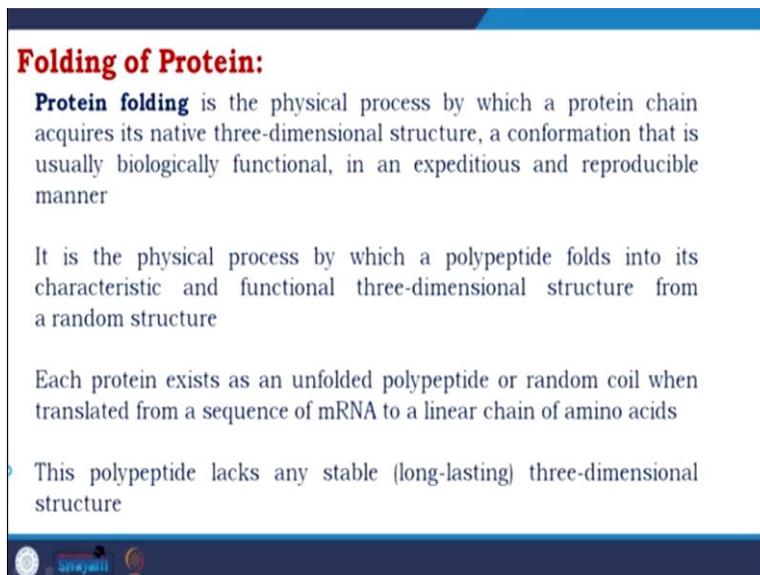
Structural Biology
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Lecture -10

**Protein: Folding of Protein, Thermodynamics and Kinetics of protein folding,
Characterization of Proteins**

Hi everyone, welcome again. So, today we are in the last class of the protein module. And here, we will discuss protein folding, Thermodynamics, and Kinetic parameters of protein folding.

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Folding of Protein:

Protein folding is the physical process by which a protein chain acquires its native three-dimensional structure, a conformation that is usually biologically functional, in an expeditious and reproducible manner

It is the physical process by which a polypeptide folds into its characteristic and functional three-dimensional structure from a random structure

Each protein exists as an unfolded polypeptide or random coil when translated from a sequence of mRNA to a linear chain of amino acids

- This polypeptide lacks any stable (long-lasting) three-dimensional structure

Protein folding is the physical process by which a protein chain acquires its native 3-dimensional structure, a conformation that is usually biologically functional in an expeditious and reproducible manner. Protein folding is a process by which protein sequences make trial and error to reach a form where the protein is compact and functional.

It is the physical process by which a polypeptide folds into a characteristic and functional 3-dimensional structure from a random structure. Each protein exists as an unfolded polypeptide or random coil when translated from an mRNA sequence to a linear chain of amino acids.

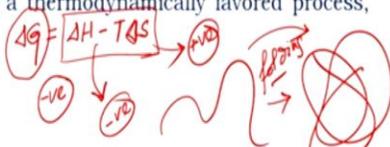
Nature allows that primary sequence to become a protein good enough to make those non-covalent interactions with the disulfide bond. So get a meaningful structure shape so that either

they could work as a structural protein like a fibrous protein or a functional protein like a globular protein.

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The Thermodynamics of Folding:

Folding of a globular protein is a thermodynamically favored process, i.e. ΔG must be negative



The folding process involves going from a multitude of random-coil conformations to a single folded structure

The folding process involves a decrease in randomness and thus a decrease in entropy $-\Delta S$ and an overall positive contribution to ΔG . This decrease in entropy is termed “conformational entropy”

An overall negative ΔG : a result of features that yield a large negative ΔH or some other increase in entropy on folding

The Folding of a globular protein is a thermodynamically favored process, that is ΔG must be negative,

$$\Delta G = \Delta H - T \Delta S$$

where ΔG is the free energy change, H is the enthalpy change, and ΔS is the entropy change.

The folding process involves going from a multitude of random coil conformation to a single folded structure from a random coil to a fold. The folding process involves a decrease in randomness and, thus, a decrease in entropy and an overall positive contribution to ΔG . These decreases in entropy are called conformational entropy. The folding process involves a decrease in randomness. This decrease in entropy is called conformational entropy. And overall, negative ΔG results from features that yield a large negative ΔH (enthalpy change) or some other increase in entropy on folding.

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Protein Folding : Enthalpic Contribution

Formation of secondary structure is an enthalpy driven process

Energy derived from the formation of many van der Waals and H-bonding interactions as well as the alignment of dipoles overcomes the loss of entropy associated with the formation of the peptide backbone conformation

Formation of tertiary structure is enthalpically unfavorable

Energy loss in the burying of ion-pairs (~1 kcal/mol) and the breaking of shorter, stronger H₂O bonds

Though some energy is gained from van der Waals packing, very little is gained from the formation of internal H-bonds because as many H-bonds with water are broken as are formed in the process of folding a protein

Free energy associated with solvation of an ion is ~ -60 kcal/mol

An ion will NOT be buried in the hydrophobic interior of a protein

The formation of a secondary structure is an enthalpy-driven process. Why? If you remember, so many hydrogen bonds are developed mainly on the main chain. So, energy derived from the formation of many van der Waals and hydrogen bonding interactions and alignment of dipoles overcomes the loss of entropy associated with the formation of peptide backbone conformation.

The alpha helix, and the dipole formation all favor the enthalpy. Formation of tertiary structure is enthalpically unfavorable energy loss in burying ion pairs and breaking up shorter, stronger H₂O bonds. Some energies are gained from van der Waals and hydrophobic packing, and very little is gained from forming internal hydrogen bonds. Free energy associated with the solvation of an ion is -60 kcal/mol.

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Protein Folding : Entropy Driven Process

Upon protein folding

hydrophobic residues move to the interior of the protein

caged H₂O molecules are released

Enthalpy is gained : unfavorable ($\Delta H +$)

entropy is also gained ($\Delta S +$) : extremely favorable

Increase in entropy of water compensates for the loss of conformational entropy of the protein and drives the protein folding process

$$\Delta G = \Delta H - T\Delta S$$

Handwritten notes: $\Delta H \uparrow$ (unfavorable), $-T\Delta S \uparrow$ (favorable), $\Delta G \downarrow$ (favorable)

Protein folding is called an entropic-driven process. Upon protein folding, the hydrophobic residues move to the protein's interior. When caged water molecules are released, then enthalpies are gained. Entropy is also gained, which is extremely favorable. Increasing the entropy of water compensates for the loss of conformational entropy of the protein and drives the protein folding process.

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Free energy of folding:

Difference in energy (free energy) between folded (native) and unfolded (denatured) state is small, 5-15 kcal/mol

Enthalpy and entropy differences balance each other, and ΔG is a small positive number

Small ΔG is necessary because too large a free energy change would mean a very stable protein, one that would never change

However, structural flexibility is important to protein function, and proteins need that for switching

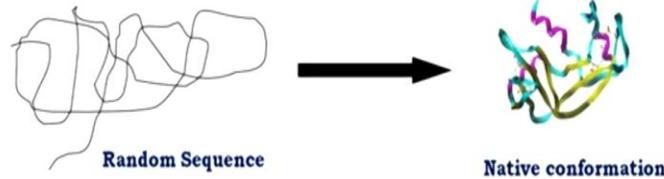
Handwritten notes: ΔG is circled in red with an arrow pointing to the text "Process of protein folding". Below it, another arrow points to the text "flexibility is not much".

The difference in the free energy between the folded native and unfolded state is small, 5 to 15 kcal/mol. Enthalpy and entropy differences balanced each other, which we discussed, and ΔG is a small positive number. So, when we say that the delta G would be small, what that means? It is necessary because too large a free energy change would mean a very stable protein. If the protein is very stable, what will happen, is it would not be able to change its conformation frequently, and because of that, you know, as we discussed, when a protein has to be functional, it changes. Small non-coherent forces like hydrogen bond hydrophobic interactions are altered, and there is a clear scenario of change of protein conformation.

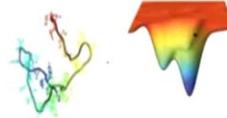
But, if one protein conformation is very stable, the alteration would not happen. Let us say a substrate is coming. The protein has to be opening up which is a change of conformation. If the protein is very stable, that conformation change will not happen. However, structural flexibility is important to protein function, and proteins need that for switching.

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Protein Folding:



- What are the forces that guide this process?
- What are the Steps Involved?
- How Fast Can this Happen?



So, protein folding, as we discussed, from a random sequence to a native conformation, as you see the changes happening here, you see that the protein is attaining the minima. So, what forces guide this process, what are the steps involved, and how fast can this happen? These are questions for millions of years, especially lasting over 100 years.

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The Thermodynamic Hypothesis:

“The native, folded structure of a protein, under optimal conditions, is the most energetically stable conformation possible”
Christian Anfinsen, 1972

Most of the information for determining the three-dimensional structure of a protein is carried in its amino acid sequence

Protein folding is a biological phenomenon

Anfinsen, C.B. Principles that govern the folding of protein chains. *Science* **181**, 223-30 (1973).

We are doing intense research about this. And one of the breaks I have to talk about is from Christian Anfinsen in 1972, who told that the native folded structure of a protein under optimal conditions is the most energetically stable conformation possible. And most of the information for determining the 3-dimensional structure of a protein is carried in its amino acid sequence.

Before this, the protein was thought to be a biological molecule, and the protein folding depends on the system.

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Ribonuclease Refolding Experiment:

Ribonuclease is a small protein that contains 8 cysteines linked via four disulfide bonds. *Expt → (S) / folded*

Urea in the presence of 2-mercaptoethanol fully denatures ribonuclease. *Expt → (P) / No color*

When urea and 2-mercaptoethanol are removed, the protein spontaneously refolds, and the correct disulfide bonds are reformed. *Expt → (S) / folded*

The sequence alone determines the native conformation

Quite "simple" experiment, but so important it earned Chris Anfinsen the 1972 Chemistry Nobel Prize. *functional nucleus for 20*

The diagram illustrates the experimental process. It starts with the native, catalytically active state of ribonuclease, which is a compactly folded protein with four disulfide bonds. The addition of urea and 2-mercaptoethanol leads to the unfolded state, which is inactive because the disulfide cross-links are reduced to free cysteine residues. Upon the removal of urea and 2-mercaptoethanol, the protein spontaneously refolds into its native, catalytically active state, with the disulfide cross-links correctly re-formed. Handwritten annotations in red ink include 'Expt → (S) / folded' next to the native state, 'Expt → (P) / No color' next to the unfolded state, and 'functional nucleus for 20' written vertically on the right side.

Now, he has performed some experiments called the Ribonuclease refolding experiment. So, ribonuclease is the protein with alpha helix and beta sheets. Now interestingly, the proteins have several cysteines, around 8 cysteines. So, Christian Anfinsen chose ribonuclease not because of these but because it is used to develop an assay where a colorless solution is converted to red color.

So, ribonuclease is a small protein containing 8 cysteines linked via 4 disulfide bonds. So, he purified the protein, did the assay, and showed that the protein could generate red color, which means the protein is folded.

Then he used urea in the presence of 2-mercaptoethanol, which fully denature the protein. So, from folded, he brings it to the unfolded state, proving that the protein is unfolded because it cannot convert the colorless solution to red. When urea and 2-mercaptoethanol are removed, the proteins spontaneously fold, and the correct disulfide bonds are reformed.

It is quite a simple experiment. But it was so crucial that he was awarded Nobel Prize.

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Levinthal Paradox:

Enormous number of the possible conformations of a protein can be possible

calculation:

Assume that there are **three conformations for each amino acid** (ex. α -helix, β -sheet and random coil). If a protein is made up of 200 amino acid residues, a total number of conformations is

$$3^{200} = 2.6561398887587475e+95$$
$$\approx 26 \times 10^{95}$$

If 100 picosec (10^{-10} sec) were required to convert from a conformation to another one, a random search of all conformations would require

$$26 \times 10^{95} \times 10^{-10} \text{ sec} \approx 3.2 \times 10^{30} \text{ years.}$$

However, folding of proteins takes place in mili-sec to sec order.

Therefore, proteins fold not via a random search but a more sophisticated search process.

Handwritten notes: Protein conformation is decided by physics & chemistry. Size, charge, distance, interaction.

Protein could fold using physics and chemistry. A sequence remembers where to fold, so it folds not by biology but by remembering the size, charge, distance, and interaction. I told, it is folding by physics and chemistry instead of biology. When Christian Anfinsen performed this experiment, critics told that this was successful for probable ribonuclease and this would not be successful for other proteins. Unfortunately, Christian Anfinsen has no other experimental way to do that because protein folding was impossible to instrumental or experimentally demonstrate display at that time. But Christian Anfinsen got a huge backup from Levinthal.

Levinthal told to assume that there are 3 confirmations of each amino acid. So, consider the minimum number of conformation for each amino acid. Now, if you take an average protein, its size range from 200 to 300 amino acids. So, he took the lowest one, a protein with 200 amino acids. So, the total number of conformation is 3^{200} is equal to 26×10^{95} . Now, if consider 100 picoseconds which is 10^{-10} seconds is required to convert from one confirmation to another, a random search of all confirmation would require $26 \times 10^{95} \times 10^{-10}$ seconds, which is 3.2×10^{30} years. However, we all know that proteins take time to fold from millisecond to second order.

So what Levinthal proved is that protein folding is not a random phenomenon. The protein is not randomly searching for a stable interaction and then finding a stable interaction. Rather, it is a sophisticated search process. It is a thermodynamically driven process.

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In the in-vitro situation, if you see the protein is in the much-diluted situation, maintaining proper temperature and buffering condition, you are making a dilute protein. Whereas when we look at an in-vivo situation, a protein does not have that much facility. Because, in the in-vivo situation in the presence of other proteins, proteolytic enzymes, and a load of other physiological problems, the protein must get a different folding mechanism

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Protein folding:

For any given protein, there is one conformation that has significantly lower free energy than any other state

Achieved through kinetic pathway of unstable intermediates (not all intermediates are sampled)

Handwritten red text: "most stable"

Handwritten red diagram: A jagged red line representing a protein folding pathway, starting from a high energy state and ending in a lower energy state. The word "most stable" is written vertically next to the final state.

Faint text at the bottom of the slide: "As soon as the protein is folded, it will spend most of its time in the most stable state."

So, for any given protein, there is one conformation with significantly lower free energy than any other state. It would be achieved through the kinetic pathway of unstable intermediates, from unfolded protein to fold in between other states.

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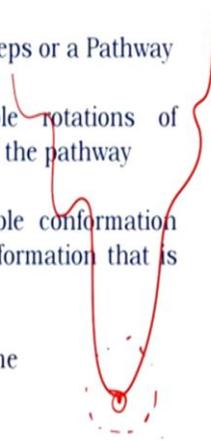
The Kinetic Theory of Protein Folding:

Folding proceeds through a definite series of steps or a Pathway

A protein does not try out all possible rotations of conformational angles, but only enough to find the pathway

The final state may NOT be the most stable conformation possible, but it could be the most stable conformation that is accessible in a reasonable amount of time

This is also the biologically important time frame



The folding process is through a definite series of steps or a pathway. A protein does not try out all possible rotations of conformational angles but only enough to find the pathway. The final state may not be the most stable conformation possible, but it could be the most stable conformation accessible in a reasonable amount of time.

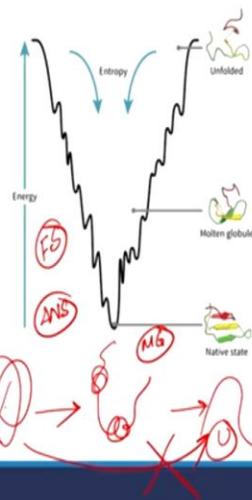
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Folding Pathways:

Protein folding is initiated by reversible and rapid formation of local secondary structures

Secondary structures then form domains through the cooperative aggregation of folding nuclei

Domains finally form the final protein through "Molten globule" intermediates.



Protein folding is initiated by the reversible and rapid formation of local secondary structures, alpha helix and beta sheets and secondary structure, then from domains through the cooperative aggregation of folding nuclei. And domains finally formed the final protein through the molten globule intermediate molten globules are where the hydrophobic collapse happened.

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Models for Protein Folding:

1. **Hydrophobic collapse.** Formation of a 'molten globule'
2. **Framework model.** Secondary structure forms first, perhaps including super secondary structure
3. **Nucleation.** Domains fold independently, and sub-domains serve as 'structural kernels'



There are different proposed models for protein folding. Hydrophobic collapse formation of molten globule; In Framework models, the secondary structure forms, perhaps including super secondary structures, and then goes to the final structure, or nucleation model in which domains fold independently and sub domains are best structural kernels to help the protein domains fold.

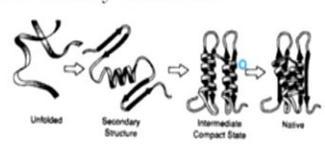
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Framework model:

Local interactions are the main determinants of protein structures

Interactions as the ones responsible for forming secondary structural elements, alpha-helices and beta-sheets

Isolated helices/sheets form early in the protein folding pathway, then assemble in the native tertiary structure



It is the local interaction, and they are the main determinants of protein structure. The interactions are responsible for forming secondary structural elements, isolated helices and sheets from early in the protein folding pathway, then assembled in the native tertiary structures.

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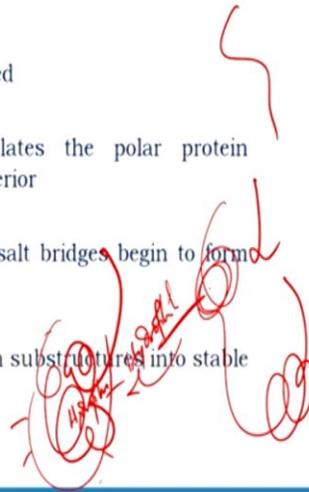
What's really happening?

Hydrophobic side chains are being buried

Secondary structure formation insulates the polar protein backbone from the nonpolar protein interior

Hydrogen bonds, disulfide bonds and salt bridges begin to form and stabilize structure

van der Waals interactions bring protein substructures into stable contact



The hydrophobic side chains are being buried. Secondary structure formation insulates the polar protein backbone from the nonpolar protein interior. Hydrogen bond disulfide bonds and salt bridges begin to form and stabilize the structure, van der Waals interactions bring proteins substructures into stable contact.

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What's most important in folding?

Nonspecific interactions (hydrophobic effect, van der Waals) are required to bring the protein together into a globular conformation



Steric interactions (restraints on the backbone) limit the ways in which the globular conformation can form

Chemically specific interactions (hydrogen bonds, ionic interactions, dipolar interactions) determine the fine detail of the protein structure

What are the most important in folding? Nonspecific interactions, hydrophobic effect, van der Waals are required to bring the protein together into a globular conformation. Steric interaction restraints on the backbone limit how the globular conformation can form; if you remember, this is where we are getting our Ramachandran plot. Chemically specific interactions like hydrogen

bonds, ionic interactions, solid breeze dipole interaction determined the fine detail of the protein structure.

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Enzymes that speed folding:

- Protein disulphide isomerase** Facilitates formation of correct disulphide bridges
- Peptidyl proline isomerase** Catalyzes cis-trans isomerisation of peptide bonds involving proline
- Molecular chaperones** Help folding, especially of large proteins, by preventing interaction with other proteins



Some enzymes speed up the folding. Protein disulfide isomerase facilitates the formation of correct disulfide bridges. Peptidyl proline isomerase catalyzes cis-trans isomerization of peptide bonds involving proline. Molecular Chaperones helped to fold, especially large proteins, by preventing interaction with other proteins.

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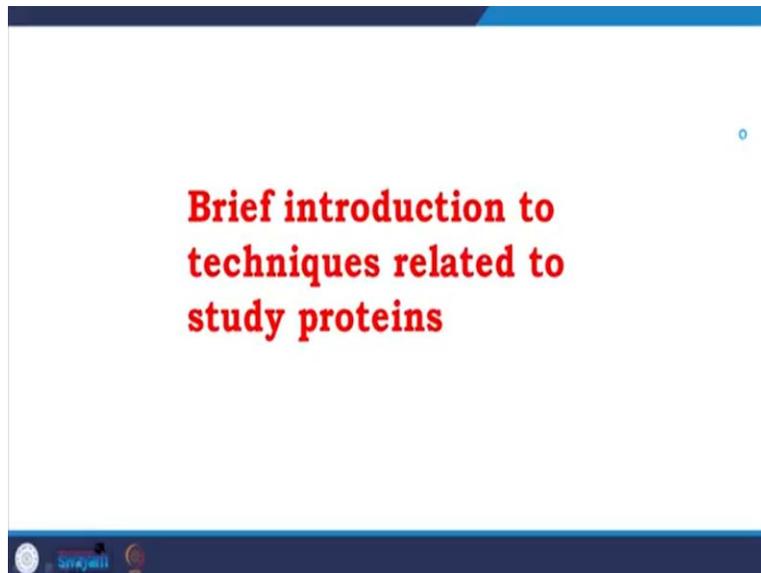
Thermophilic organism → high temp → hot spring → bacteria → Gene pool → stable → stable
bacteria → normal temp → mesophilic

What is the molecular basis of the observation that proteins of thermophiles are more stable than their homologues in mesophiles?



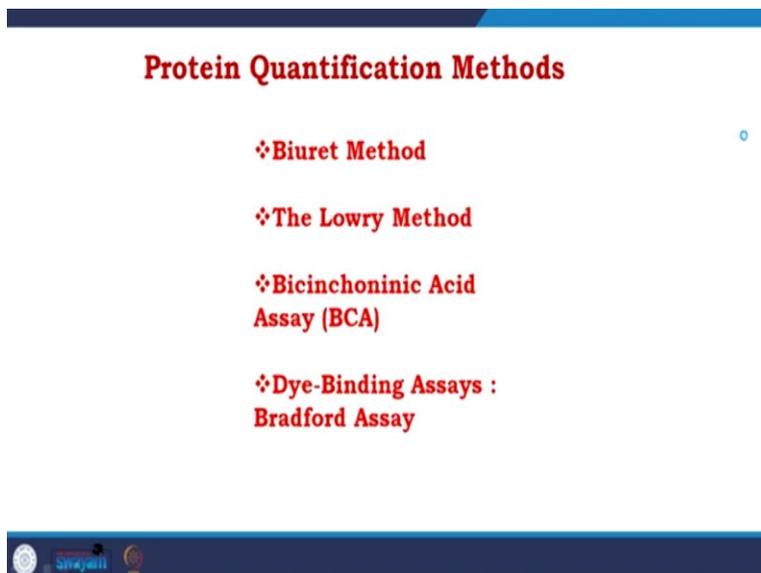
What is the molecular basis of the observation that proteins of thermophiles are more stable than homologs in mesophiles? Thermophiles are organisms that live in high temperatures. For example, go to a hot spring, and you collect bacteria from there. You will find that a protein, a gene product, I would say coming from that bacteria if you study, will show more stability than the same protein in a bacteria that is staying in normal temperature called mesophile.

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In the second part of today's lecture, I will give you a very brief introduction to the techniques related to studying proteins; I will start with a very simple protein quantification method.

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There are different methods, but I will talk about Biuret method, Lowry method, Bicinchoninic acid or BCA Assay, and Dye-Binding Assays. I will take an example of Bradford Assay.

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Quantification of Protein:

Biuret Method:

A test for detecting the presence of peptide bonds.

Under alkaline conditions, Cu^{2+} forms a violet-colored complex.

Protein concentration is directly proportional to the intensity of color, absorbed at 540 nm.

The Biuret reagent contains sodium hydroxide, hydrated copper (II) sulfate and potassium sodium tartrate (to stabilize the complexes).

Reduction of copper results in a color change, which can be read at 550 nm. The linear range is typically 0.5-20.0 mg of protein.



Blue: negative Violet: positive

The Biuret method is a test for detecting the presence of peptide bonds. Under alkaline conditions, copper forms a violet-colored complex. Protein Concentration is directly proportional to the intensity of color absorbed at 540 nm. To stabilize the complex, the Biuret reagent contains sodium hydroxide, hydrated copper II sulfate, and potassium sodium tartrate. Reduction of copper results in a color change. The linear range is typically 0.5 to 20 milligrams of protein

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Quantification of Protein: Lowry Method

The development of the Lowry method (named after Oliver Lowry) introduced a more sensitive assay for determining protein concentration.

A modification of the Biuret test, the Lowry method relies on the reaction of copper with proteins, but the sample is also incubated with the **Folin-Ciocalteu reagent**.

Reduction of the Folin-Ciocalteu reagent under alkaline conditions results in an intense blue color that absorbs at 750 nm

The Lowry method is best used with protein concentrations of 0.01-1.0 mg/mL.

Tyrosine, tryptophan, and cysteine of proteins reduce molybdenum acid and phosphotungstic acid of a phenol reagent, turning the solution blue.

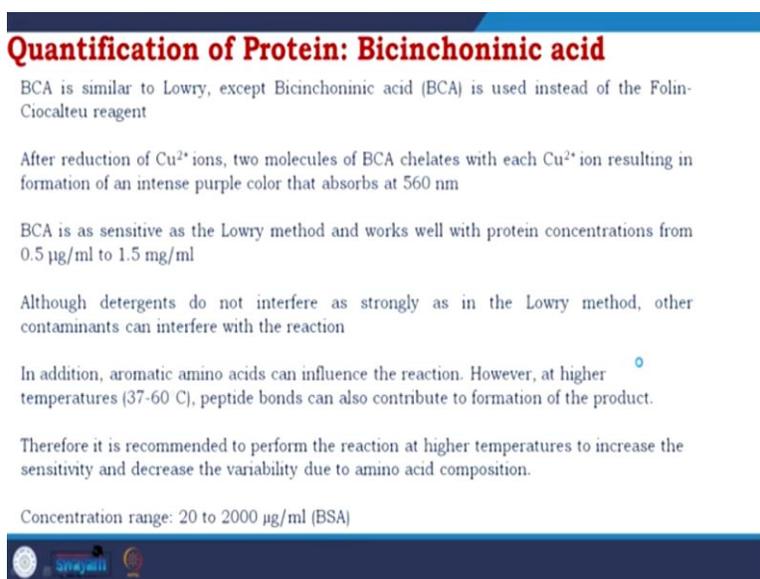
Concentration Range: 5 to 200 $\mu\text{g/mL}$ (BSA)



Blue: negative Violet: positive

It is named after Oliver Lowry, who introduced a more sensitive assay. It is a modification of Biuret tests. The Lowry method relies on the reaction of copper with protein the same, but the sample is also incubated with a reagent called Folin-Ciocalteu reagent. The reduction of the reagent under alkaline conditions results in an intense blue color that absorb at 750 nm. The Lowry method is best used with a Protein Concentration of 0.01 to 1 mg per ml. Tyrosine, Tryptophan, and Cysteine of protein reduce molybdenum acid, and phosphotungstic acid have a phenol reagent, turning the solution blue. The concentration range is 5 to 200 micrograms per ml. So, this is estimated with the protein bovine serum albumin.

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Quantification of Protein: Bicinchoninic acid

BCA is similar to Lowry, except Bicinchoninic acid (BCA) is used instead of the Folin-Ciocalteu reagent

After reduction of Cu^{2+} ions, two molecules of BCA chelates with each Cu^{2+} ion resulting in formation of an intense purple color that absorbs at 560 nm

BCA is as sensitive as the Lowry method and works well with protein concentrations from 0.5 $\mu\text{g}/\text{ml}$ to 1.5 mg/ml

Although detergents do not interfere as strongly as in the Lowry method, other contaminants can interfere with the reaction

In addition, aromatic amino acids can influence the reaction. However, at higher temperatures (37-60 C), peptide bonds can also contribute to formation of the product.

Therefore it is recommended to perform the reaction at higher temperatures to increase the sensitivity and decrease the variability due to amino acid composition.

Concentration range: 20 to 2000 $\mu\text{g}/\text{ml}$ (BSA)

Bicinchoninic acid is again similar to Lowry, except the BCA replaces the Folin-Ciocalteu reagent. After the reduction of copper ions, 2 molecules of BCA chelates with each copper iron, forming an intense purple color that absorbs at 516 nm. This test is as sensitive as the Lowry method and works well with a Protein Concentration from 0.5 micrograms per ml to 1.5 milligrams per ml.

Although detergents do not interfere as strongly as the Lowry method, other contaminants can interfere. In addition, aromatic amino acids can influence the reaction. However, at higher temperatures, peptide bonds can also contribute to the formation of the product. Therefore, for these problems, performing the reaction at higher temperatures is recommended to increase the sensitivity and decrease the variability due to amino acid composition—concentration range from 20 to 2000 micrograms per ml.

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Quantification of Protein: Dye-Binding Assays

Dye binding assays rely on an absorbance shift that occurs when a dye binds to proteins.

Several different dyes can be used:
Coomassie Brilliant Blue G-250, bromocresol green, pyrogallol red.

The most commonly used dye-binding assay is the **Bradford Assay**



Dye-Binding Assays rely on an absorbance shift that occurs when a dye binds to a protein. Several different dyes could be used Coomassie Brilliant Blue G-250, Bromocresol green, Pyrogallol red, but the most commonly used dye-binding assays are Bradford Assays.

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Quantification of Protein: Bradford Assays

The Bradford assay, is an easy, sensitive and accurate method for protein quantification.

Binding of Coomassie Brilliant Blue G-250 to proteins, causes a shift of the dye from red (465 nm) to blue (595 nm) under acidic conditions.

It is compatible with more common reagents, although detergents can cause interference

Proteins with a concentration of 20-2000 $\mu\text{g/ml}$ can be measured using the Bradford assay.



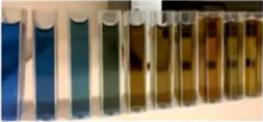
The Bradford Assay is an easy sensitive, and accurate method for protein quantification. The binding of Coomassie brilliant blue G-250 to proteins causes a shift of the dye from Red 465 to blue 595, nanometer under acidic conditions. It is compatible with more common reagents, although detergent can cause interference. Protein with a concentration of 20 to 2000 micrograms per ml can be measured using Bradford Assay.

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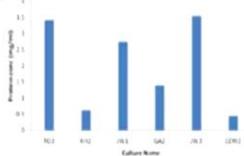
Experimental Procedure:

Take 200 μ l of the Reagent \longrightarrow Add 800 μ l of the Reagent \longrightarrow Make 1 ml of the solution

Intensity measured at 595 nm \longleftarrow Change of colour is observed \longleftarrow Add 2 μ l of the Protein



Increased Protein Concn.

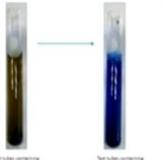


Compare Protein production from different culture

So, how is it used to measure concentration? Take 200 microliters of the reagent, and add 800 microliter of water. So, you have 1 ml of the solution and add 2 microliters of protein, a change of color is observed, and intensity is measured at 595. You see that when you keep changing the higher protein concentration, you gradually start getting a more intense blue color.

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Quantification of Protein: Bradford Assays

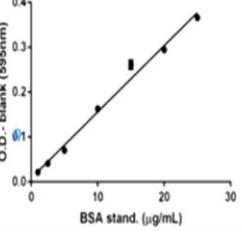


Test tube containing Bradford reagent alone (Color of the solution)

Test tube containing Bradford reagent with protein added (Color of the solution)

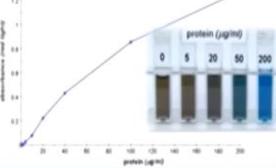


Coomassie Brilliant Blue G-250 Dye
 $C_{24}H_{12}N_6Na_2O_6S_2$
MW 654.62



O.D. - blank (595nm)

BSA stand. (μ g/mL)



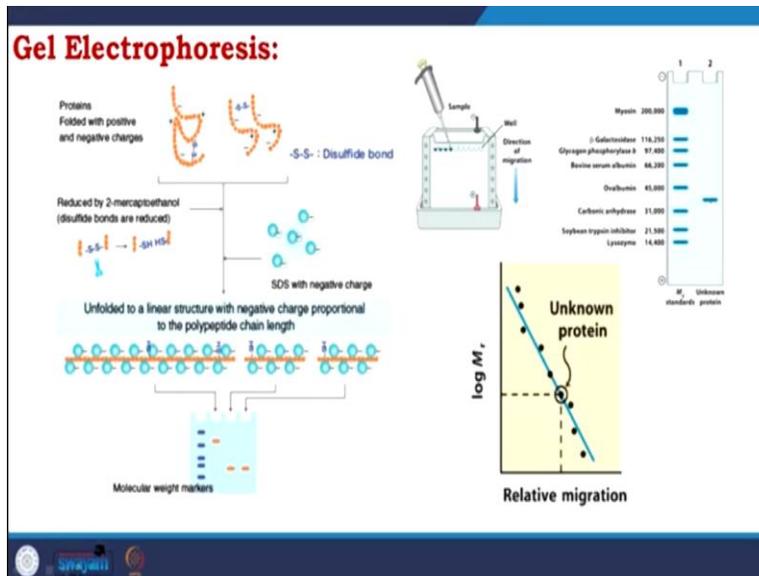
Absorbance (595nm)

protein (μ g/ml)

protein (μ g/ml): 0, 5, 20, 50, 200

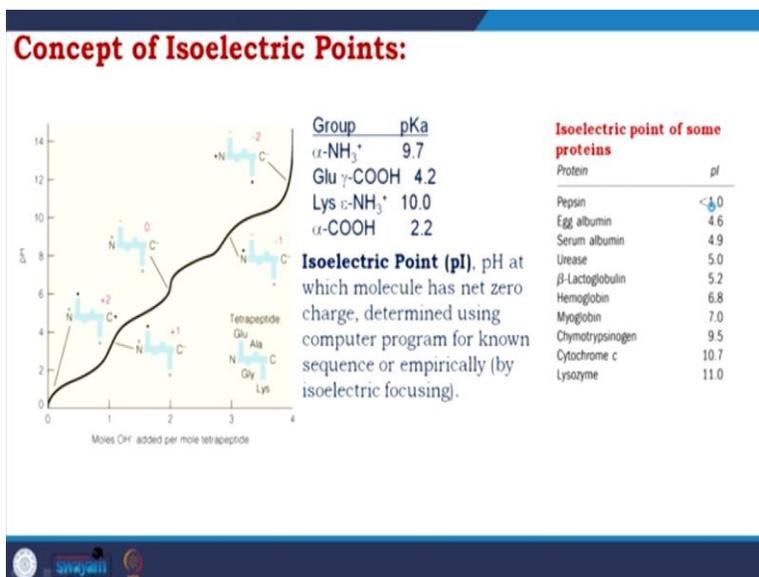
You could also do real quantification. So, for that, you have to use the BSA standard. So, using those reagents, you could quantify protein.

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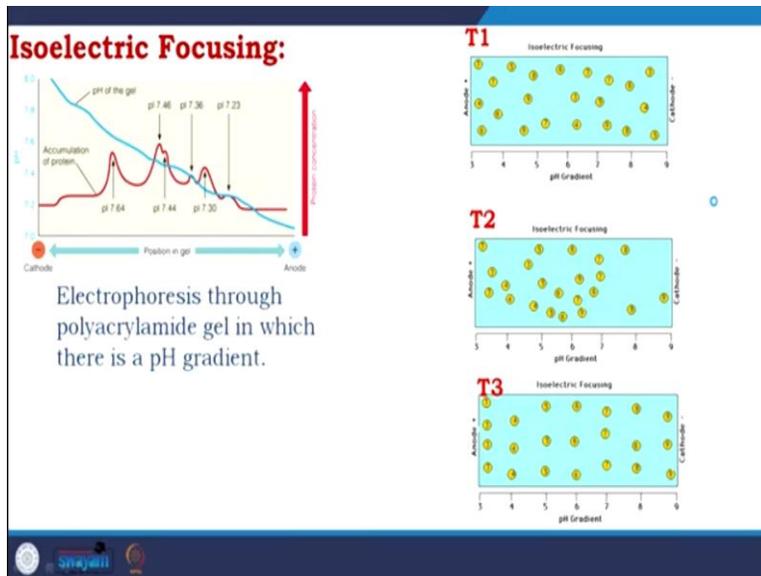


Another very interesting technique is gel electrophoresis. You have the protein. The protein might have a disulfide bond and a positive or negatively charged surface. To reduce the disulfide bond, add 2-mercaptoethanol and boil. Also, you add sodium dodecyl sulfate, which is called SDS gel electrophoresis. More importantly, SDS is now the negatively charged ion, so the entire protein is bound with SDS. So, all of them are negatively charged. So, when you run the gel, you stain it and see the bands, and nowadays, there are advanced markers that will give bands even without staining. Also, from the relative migration, you could get the mass of the unknown protein more accurately.

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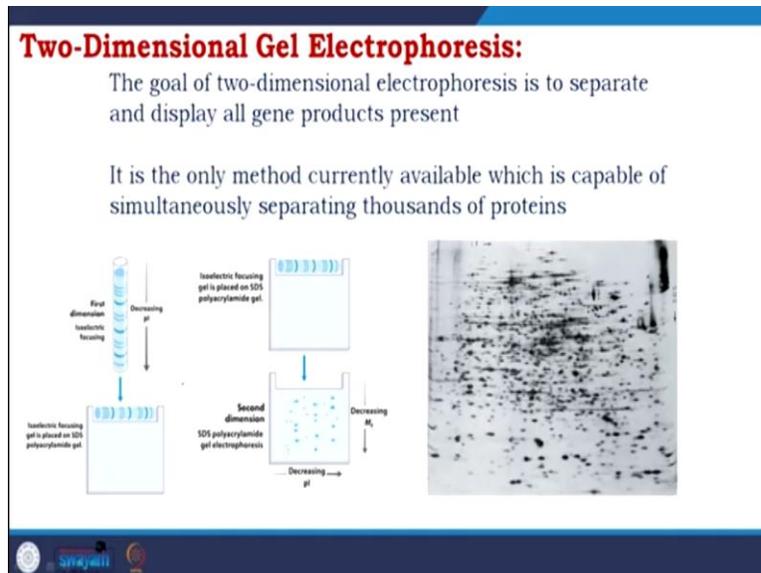


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According to that pI, you could perform isoelectric focusing electrophoresis through polyacrylamide gel with a pH gradient. So, you start in time 1; they are random, then gradually separated, and ultimately, they are separated from anode to cathode because of the Isoelectric Focusing the difference in the pH gradient along with the different pIs.

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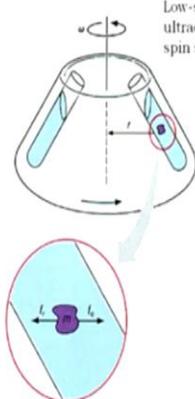


Now, you combine these 2 techniques, Isoelectric focusing and gel electrophoresis, called 2-dimensional gel electrophoresis. 2-dimensional electrophoresis aims to separate and display all gene products present. Let us say in a cell you are experimenting with, it is the only method currently available that can simultaneously separate 1000s of protein. That is the beauty.

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Centrifugation:

Low-speed, high-speed, or ultracentrifugation different spin speeds and g forces



Centrifugation Methods:

- Differential (Pelleting) - simple method for pelleting large particles using fixed-angle rotor (pellet at bottom of tube vs. supernatant solution above)
- Zonal ultracentrifugation (e.g., sucrose gradient) - swinging-bucket rotor
- Equilibrium-density gradient ultracentrifugation (e.g., CsCl) - swinging-bucket or fixed-angle rotor

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Centrifugation is a very important technique. Low, high, or ultra-centrifugation different spin speeds and G forces. So, it needs different work to have a cell grow. You want to get a pallet. So, you have the lowest one if you have protein to isolate, so, your higher speed and then ultracentrifugation, you have to differentiate between cell organelles or something.

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Ultracentrifugation:

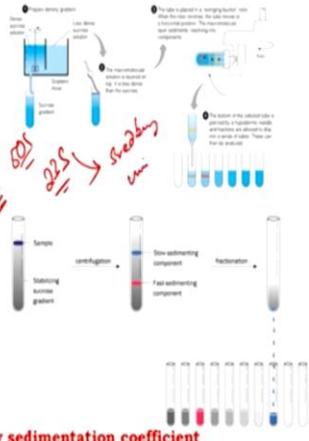
Sucrose density gradient ultra centrifugation is a powerful technique for fractionating macromolecules like DNA, RNA, and proteins

For this purpose, a sample containing a mixture of different size macromolecules is layered on the surface of a **gradient** whose **density** increases linearly from top to bottom

Density gradient centrifugation as developed by Meselson and Stahl employed a process called **equilibrium sedimentation** or isopycnic **centrifugation**

Equilibrium sedimentation is the process by which particles in a solution reach a point where they reach their isopycnic position and stop moving

Separates by sedimentation coefficient (determined by size and shape of solutes)



Sucrose density gradient ultracentrifugation is a powerful technique for fractionating macromolecules like DNA, RNA, and protein organelles. For this purpose, a sample containing a

mixture of different-sized macro molecules is layered on the surface of a gradient whose density increases linearly from top to bottom.

Meselson and Stahl developed this in their famous experiment, which employed an equilibrium sedimentation process, or Isopycnic centrifugation. Equilibrium sedimentation is a process by which particles in a solution reach a point where they reach their Isopycnic position and stop moving.

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Cleavage of Polypeptides for Analysis:

	Reagent (biological source)*	Cleavage points [†]
Strong acid (<i>e.g.</i> , 6 M HCl) - not sequence specific	Trypsin (bovine pancreas)	Lys, Arg (C)
	Submaxillary protease (mouse submaxillary gland)	Arg (C)
Sequence-specific proteolytic enzymes (proteases)	Chymotrypsin (bovine pancreas)	Phe, Tyr, Trp (C)
	Staphylococcus aureus V8 protease (bacterium <i>S. aureus</i>)	Asp, Glu (C)
	Asp-N protease (bacterium <i>Pseudomonas fragi</i>)	Asp, Glu (N)
	Pepsin (porcine stomach)	Phe, Tyr, Trp (N)
Sequence-specific chemical cleavage (<i>e.g.</i> , cyanogen bromide cleavage at methionine residues)	Endoproteinase Lys C (bacterium <i>Lysinibacterium</i> <i>enzymogenes</i>)	Lys (C)
	Cyanogen bromide	Met (C)

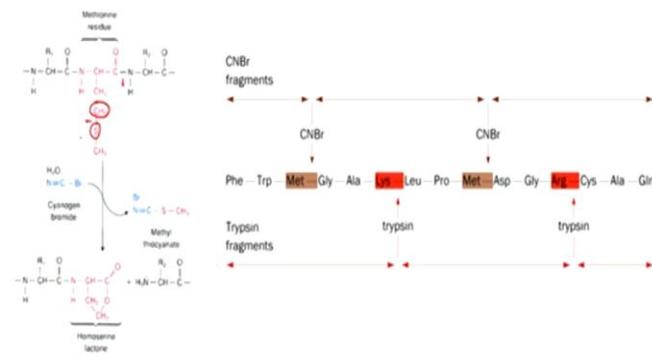
Protease enzymes are most commonly used

*All reagents except cyanogen bromide are proteases. All are available from commercial sources.

So, how a protein or polypeptide would be cleaved for further analysis? You could use strong acid like 6 molar HCl, but you would not get any specificity there. You use sequence-specific proteolytic enzymes, which are called proteases. You could use sequence-specific chemical cleavage and cyanogen bromide cleavage at methionine residues. Among them, the protease enzymes are most commonly used and are diverse. So if you look, trypsin cleavage points are basic amino acids, lysine, and arginine. Then you get Submaxillary protease which cuts in arginine specifically, Chymotrypsin, phenylalanine, tryptophan, tyrosine, Staphylococcus aureus V8 protease aspartate, and glue the acidic ones. Asp-N-protease, aspartate and glutamate, pepsin phenylalanine, tryptophan tyrosine, endoproteinase Lysin.

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Cyanogen Bromide Cleavage at Methionine Residues:



So, how cyanogen bromide keeps the Methionine residue, you will see that it attacks and keeps the residue between the methyl group and the sulfur, forming a homoserine lactone. It could mainly work on Methionine and be used in combination with trypsin.

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Edman Degradation:

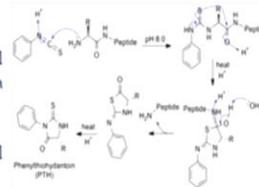
Edman degradation, developed by Pehr Edman, is a method of sequencing amino acids in a peptide

In this method, the amino terminal residue is labeled and cleaved from the peptide without disrupting the peptide bonds between other amino acid residues

Phenyl isothiocyanate is reacted with an uncharged N-terminal amino group, under mildly alkaline conditions, to form a cyclical *phenylthiocarbamoyl* derivative

Then, under acidic conditions, this derivative of the terminal amino acid is cleaved as a thiazolinone derivative

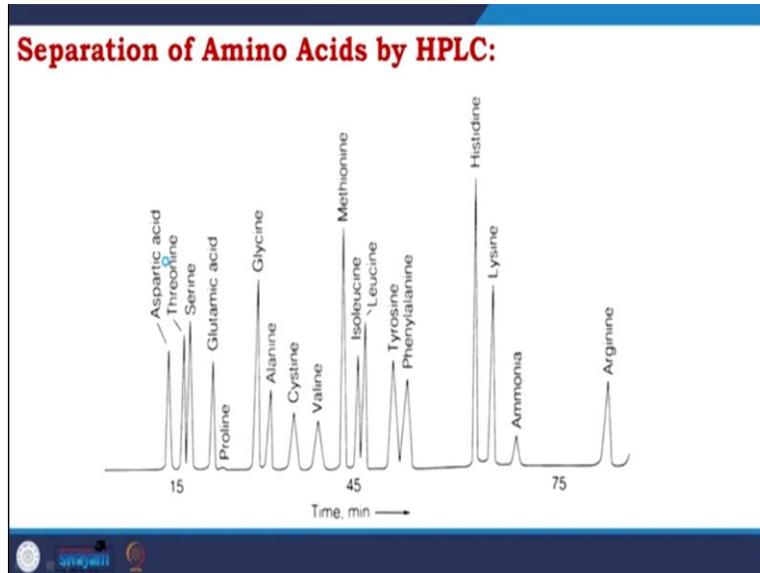
The thiazolinone amino acid is then selectively extracted into an organic solvent and treated with acid to form the more stable phenylthiohydantoin (PTH)-amino acid derivative that can be identified by using chromatography or electrophoresis



Edman degradation is a very famous method. Phil Edman developed it as a peptide sequencing method of amino acids. In this method, the amino-terminal residue is labeled and cleaved from the peptide without disrupting the peptide bonds between other amino acid residues. Phenyl isothiocyanate reacts with an uncharged N-terminal amino group under mildly alkaline condition to form a cyclical phenylthiocarbamoyl derivative and does not affect the other peptides.

Then under acidic conditions, this derivative of the terminal amino acid is cleaved as a thiazolidine derivative. And ultimately, this isothiazolinone amino acid is selectively extracted into an organic solvent and treated with acid to form a more stable PTH or phenylthiohydantoin. This can be identified by using chromatography or electrophoresis. So, in this way, you could keep identifying the N-terminus amino acid.

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When you cleave them, you can separate them using high-performance liquid chromatography, a very popular method of identifying small molecules.

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Mass Spectrometry:

Peptide mass fingerprinting:

Proteolytic digestion of protein

Then determination m/z of peptides by MS (e.g., MALDI-TOF or ESI-TOF)

Search "fingerprint" against database

Success of identification depends on quality and completeness of database for specific proteome

Tandem MS (MS/MS - e.g., nanoLC-ESI-MS/MS):

Proteolytic digestion of protein

Separation and determination of m/z of each fragment (MS-1)

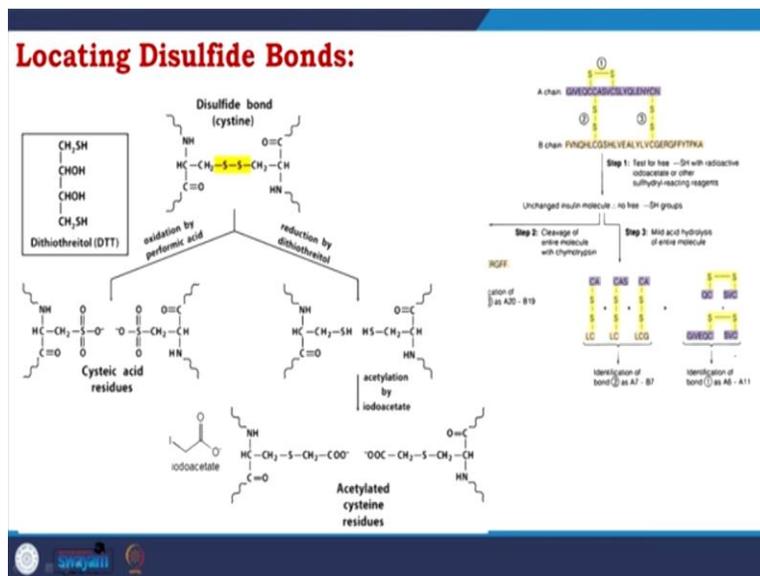
Then determination of collision-induced dissociation fragment spectrum for each peptide (MS-2)

Gives context/sequence-dependent information, so more of a *de novo* sequencing method

Mass Spectrometry is a very popular method. Generally, 2 types of mass spectrometry method are used here, and I am talking about peptide mass fingerprinting. You have to do the proteolytic digestion of protein for any process. You have to do that first, and then you determine the m/z of peptides by mass spectrometry either by MALDI-TOF or ESI-TOF electrospray. Then you search for fingerprints from the database.

And the success of this method depends on the quality and completeness of the database of the specific proteome where you are working. Another method is known as Tandem mass spectrometry. Here the same thing you do is the proteolytic digestion of protein, but then you do a separation, you could say. So, you could do the proteolytic digestion of protein instead of going there to do m/z . You do separation and then a determination of m/z . This is called the first step and then the determination of each peptide's collision-induced dissociation fragment spectrum separately.

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How you look at the disulfide bond if you have a disulfide bond present, you either do the reduction by adding dithiothreitol do the oxidation by performic acid. In the case of performic acid, you get cystic acid residues. In the case of dithiothreitol, disulfide bond would be reduced, an individual S-H group would be formed, and then you put iodoacetate, it would do escalation, and now you get asked related cysteine residues.

So, in the protein, you identify the escalated cysteine residues and know where the disulfide bonds are.

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Procedure	Result	Conclusion
hydrolyze: separate amino acids	A 5 H 2 R 1 C 2 I 3 S 2 D 4 K 2 T 1 E 2 L 2 Y 1 F 1 M 2 Y 2 G 3 P 1	Polypeptide has 38 amino acid residues. Trypsin will cleave three times (at one R (Arg) and two K (Lys)) to give four fragments. Cyanogen bromide will cleave at two M (Met) to give three fragments.
react with FMOB: hydrolyze: separate amino acids	2,4-Dinitrophenylaminate detected	E (Glu) is amino-terminal residue.
reduce disulfide bonds (if present)		
digest with trypsin	<ol style="list-style-type: none"> GASMALIK EGAATHDFEIDPR DCVHSD YLIACGPMTRK 	<ol style="list-style-type: none"> placed at amino terminus because it begins with E (Glu). placed at carboxyl terminus because it does not end with R (Arg) or K (Lys).
digest with cyanogen bromide	<ol style="list-style-type: none"> EGAATHDFEIDPFGASMA TKDCVHSD ALIKYLACGPM 	<ol style="list-style-type: none"> overlaps with <ol style="list-style-type: none"> and <ol style="list-style-type: none"> allowing them to be ordered.
establish sequence	Amino terminus: EGAATHDFEIDPFGASMAALKYLACGPMTRKCYHSD Carboxyl terminus	

Reduction of di-sulphide bond using DTT

Apply Trypsin which would cut into arg and lys

Apply CNBr, which would cut into Methionine

Apply MS to sequence peptides

And what you do is the reduction of disulfide bond using dithiothreitol. You apply trypsin, the protease which would cut into arginine and lysine. You apply CNBr, which would cut into Methionine, and then you do Edman degradation, know the N-terminus ones. You apply mass spectrometry to get a fingerprint of the sequence.

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Proteome Reflects Gene Expression Repertoire:

Sample → 2D electrophoresis → Pure protein → Proteolytic digestion → Proteolytic fragments

Proteolytic fragments → MALDI-TOF → Mass spectrum → Database searching

Proteolytic fragments → Capillary Electrophoresis HPLC → LC/MS/MS → Amino acid sequencing

Database searching

Mass spectrum

Capillary Electrophoresis HPLC

LC/MS/MS

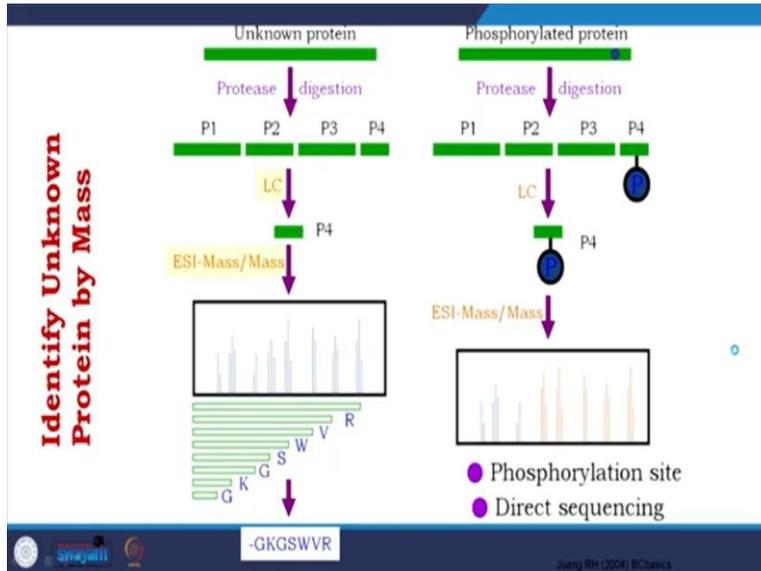
Amino acid sequencing

N [Colorful sequence]

You have the sample, and you do the 2d electrophoresis, you get the pure protein, you do the proteolytic digestion and get the proteolytic fragments that proteolytic fragments go through the

MALDI-TOF you get the mass spectrum, or you purify with capillary electrophoresis. Then you go for LC-MS-MS for amino acid sequencing, whereas with the mass spectrum or fragment, you go for database searching.

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But then you have an unknown protein, you do protease digestion again, you get the fragments p1, p2, p3, p4 with them, you go for MALDI-TOF these fragments are there, you get m / z values for each of them, and you know their mass. Now, you search the database, get the candidate protein, make the digestion simulation, calculate the molecular weight, compare the peptides, and identify the unknown proteins.