HANDOUT

LECTURE-07

PROTEIN PURIFICATION AND PEPTIDE ISOLATION USING CHROMATOGRAPHY

Slide 1

Today, we will talk about protein purification and peptide isolation using chromatography methods. The chromatography techniques have always been prominently used for the separation of enzymes and proteins.

Protein purification by conventional chromatography is usually achieved by combining chromatography methods such as gel filtration, ion exchange and affinity chromatography.

From the complex proteome, it is challenging to purify a protein in a single chromatographic step therefore sequential pre-fractionation steps involving different modes and types of chromatographic methods are becoming necessary for proteome level analysis.

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Lecture outline-

- We will talk about gel filtration chromatography and ion exchange chromatography, affinity chromatography, these methods we will be discussed in light of protein purification.
- We will talk about few methods such as strong cation exchange and reverse phase chromatography for peptide isolation using high performance liquid chromatography methods.

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Protein purification and peptide isolation and analysis:

- This can be performed by many methods but there are few chromatography methods which are commonly used
- These include Size exclusion chromatography (SEC), Ion exchange chromatography (IEX), Immobilized metal ion chromatography (IMAC), Normal phase chromatography (NPC), Reverse phase-HPLC (RP-HPLC), Hydrophobic

interaction chromatography (HIC) and Hydrophilic interaction chromatography (HILIC).

- These are only few chromatographic methods but there are many more which are also used for different type of applications.

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So if your aim is to purify proteins, there are various techniques which can separate proteins and they rely on different type of principles such as

- differential solubility of the proteins
- size of proteins
- charge on the given protein
- affinity for various ligands

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Definition of chromatography.

- The International Union of Pure and Applied Chemistry (IUPAC) defines chromatography as a physical method of separation in which components to be separated are distributed between two phases one of which is stationary and other is mobile phase moves in a definite direction.
- A mobile phase is described as a fluid which passes through a stationary phase in a defined manner.
- It may be a liquid, a gas or it could be a supercritical fluid. The stationary phase may be a solid or a liquid. If it is a liquid, it may be distributed on a solid which may or may not contribute to the separation process.

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- Chromatographic methods involve separation of proteins over a bed of appropriate material, these materials are usually packed in a given column and known as matrix or resin.
- These matrix and resins are usually beads with or without attached chemical groups.
- The binding and interaction of proteins with the column matrix is an important feature of chromatography.

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The chromatographic techniques involves four major components an inlet for sample introduction, the mobile phase, stationary phase and a detector.

- Each type of chromatography requires very educated and informed choice of matrix considering the bead shape, size and porosity. In additional to the functional group type the charge distribution and density as well as elusion condition such as pH, the ionic strength and gradient shape.

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- As previously stated,,t chromatography involves the selection of right matrix.
- The matrix is very important for binding of proteins and they can determine the efficiency of separation which we want to achieve from these types of chromatographic methods.

So let's move on to more specific chromatographic techniques-

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Gel Filtration Chromatography

- The gel filtration chromatography separates proteins on the basis of difference in size.
- It is used to separate a protein of interest from a protein of mixture that are of larger or smaller size. If the proteins are having similar sizes then gel filtration or size exclusion chromatography is not an appropriate choice for doing the protein purification.
- So this method is also used for various proteomic applications when there is even need to remove contaminants such as low molecular weight contaminants and low molecular weight detergents.

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- Gel filtration chromatography is also known as size exclusion chromatography it means the molecules are separated according to the given size.
- The small molecules such as salt those will be retained longer by gel the filtration systems and large molecules such as proteins will elute first because they cannot enter inside these beads and they can be used to separate protein based on the size.

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- The gel filtration chromatography column is composed of porous beads which are made from polyacrylamide, dextran or agarose. Now these columns are packed with the hydrated porous gel matrix.

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- The protein sample which contains mixture of un-purified proteins of different size is then loaded on these columns.

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- Now when protein sample is applied on to the column, the small proteins pass through thepores of the beads while the large proteins are excluded therefore this technique is also known as size exclusion chromatography.
- The bead of different pore sizes or increasing retention time by adjusting the increasing column length or decreasing the flow rate can be adjusted to achieve higher resolution of proteins.

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- After these step, the fraction are collected and analyzed for protein content so you can expect that larger proteins will elute first and then small molecules will be eluted later.

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- So gel filtration chromatography is useful for removal of contaminants, desalting steps as well as buffer exchange.
- However its drawbacks are its low capacity, broad distribution of its pore size and small sample volume to be analyzed. So let me describe gel filtration chromatography in following animation-

Definition of components

- Size exclusion gel matrix- the matrix filling the gel filtration column consists of highly hydrated polymeric material commonly dextran, agarose and polyacrylamide. The protein mixture is a mixture of unpurified proteins of different sizes which is applied on top of the column.
- Mobile phase – the proteins are moved out of the column by using a suitable mobile phase that carries the protein out for elution. For gel filtration a salt solution of appropriate strength is commonly used so that it will not have any effect on properties of proteins being purified.
- Solvents or buffer systems are often used in other types of chromatography.
- A fluent sample fractions, the solution leaving the column is collected in suitably sized fractions, initial fraction will contain only the mobile phase while later fractions will have purified proteins.

- Let me give an analogy of gel filtration with sieve. This process is very similar to separation of small particulate matter from food grains using a sieve. The larger grains remain behind the sieve while the smaller sand or stone particles pass through them and are removed.

In keeping with this the gel filtration chromatography is also known molecular sieve. Now let me show you how this process works.

Animation 1

- So first the matrix which is suitable for the required protein separation, this packed gel matrix is loaded with the protein sample contain a mixture of unpurified proteins of different sizes.
- The column is then eluted with a salt solution of an appropriate concentration. The large proteins that cannot enter into the pores of the gel move down through the interstitial spaces at a faster rate and are eluted first.
- The smaller proteins move and out thereby taking longer time to be removed from the column. The fractions of appropriate size should be collected and analyzed for their protein content.
- The largest proteins eluting out first will be present in the initial fractions while the smaller proteins which elute out later will be present in later fractions.
- Once all these fractions are collected, then it can be analyzed for their protein content by using a UV-visible spectrophotometer. Now analyse the protein content by using UV-visible spectrophotometer at 280 nm.
- Once all the absorbance values are recorded a graph of eluant volume versus protein concentration can be plotted.
- The highest molecular weight is in the beginning and lowest molecular weight at the end.

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<u>Ion exchange chromatography.</u>

- This is one of the versatile chromatographic methods which rely on differences between number of charges and distribution of charge groups in defined pH and solvent conditions.

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- In ion exchange chromatography, proteins are separated based on charge difference the proteins with overall negative charge will interact with positive charges

or vice versa. So, varying the amount of positively and negatively charged amino acids and pH influences net charge on proteins.

- So let's say if the pI of the protein is known, an anion exchange medium with pH above pI of the target protein or a cation exchange medium with pH below pI of the target protein should be selected.

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- So in this slide some of the common ion exchange matrixes are shown like Carboxymethyl (CM) and Diethylaminoethyl (DEAE). So when a desired protein is positively charged, the cation exchange chromatography should be used. When a desired protein is negatively charged the anion exchange chromatography method should be used.

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- So in ion exchange chromatography, the column is packed with a resin, either a it's cation or anion exchanger depending upon the charge of the protein that need to be bound to the column and purified.

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- So proteins are adsorbed to the ion exchange column and then it can be desorbed by increasing the salt or altering the pH of the buffer which can change the on protein. So various anionic buffers such as acetate and phosphate are used for cation exchange and cationic buffers such as tris chloride or ethanolamine are used for anion exchange.

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- Now the buffer solution exchange so that the net pH of the protein of interest can modified and it no longer binds to ion exchange resin therefore the bound protein can eluted out as shown in this slide.

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- So if you have negatively charged protein which gets eluted first will be present in initial fractions while the positively charged protein that bound to the column will be eluted in the later fractions or it will vice versa.

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Definition of concepts

The Charged stationary phase

- The column stationary phase consists of a positively or negatively charged polymeric matrix which will bind the molecules of opposite charge.
- Commonly used ion exchangers are included negatively charged carboxy-methyl cellulose or CM cellulose which is a cationic exchanger and positively charged DEAE cellulose which is an anion exchanger.
- The protein mixture- unpurified mixture of proteins which consist of proteins of different net charges is loaded on to the column.
- The proteins having charges opposite to that of stationary matrix will bind to it while remaining proteins will be eluted.

Mobile phase-

- The proteins are eluted of the column by using suitable mobile phase and then samples are collected by using different sample fractions.
- The solution leaving the column can be collected in suitably sized fractions for further analysis. After giving you the description of components,

Animation 2

- The column is packed with a suitable cation or anion exchange resin depending upon the charge of the protein that needs to be bound to the column and purified. The anion exchange column is then loaded with the impure proteins mixture consisting of various positively and negatively charged proteins.
- The column is eluted with a buffer solution of suitable pH such that negatively charged molecules are removed from the column while the positively charged molecules remain bound to the anion exchange resin.
- The buffer solution is then changed such that the net pH of the protein of interest is modified and no longer binds the ion exchange resin therefore the bound protein also gets eluted out of the column in this manner.
- The fractions of appropriate size must be collected and analyzed for their protein content. The negatively charged protein which gets eluted first will be present in the initial fractions while positively charged proteins which bound to the column are eluted in later fractions.

- Once all the fractions are collected then the protein content can be analyzed by using a spectrophotometer. So analyze these fractions for their protein contents using a UV-visible spectrophotometer at 280 nm.
- A graph of volume of eluant versus protein concentration can be plotted. In this particular example negatively charged large molecules coming first and then positively charged molecules are coming later.

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Affinity chromatography

- It is a desirable method for protein purification to the homogeneity.
- Due to the selectivity of immune recognition it is possible to purify a protein in a single step under the favorable conditions and affinity chromatography is one of the methods for protein purification.

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- Affinity chromatography is based on affinity of proteins to its ligands and other protein molecules. Metal chelation is widely used in purification of recombinant proteins.
- Various substrates, products, cofactors, antibodies and metals can show the affinity for the given protein and this is used to purify a protein based on its affinity. The matrix beads are chemically coupled to ligand.

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- So in affinity chromatography, the column is packed with a resin which is covalently coupled to the ligand specific to the protein of interest.

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- The protein mixture is passed over the derivatized affinity column the protein of interest binds through a specific interaction while all other proteins which do not interact will not bind.

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- Now the column is washed with a suitable mobile phase to remove the unbound protein and protein of interest which has higher affinity for ligand remains bound to the derivatized column matrix and is not removed during the washing step. Now these proteins interest can be desorbed by applying excess ligand in the solution.

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- So due to the specific interaction the affinity chromatography achieves very high degree of protein purification. It is not limited by sample volume which is the case of gel filtration chromatography and because of its superiority in achieving pure protein it is usually considered as a final step the protein purification.

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- There are various examples where affinity chromatography can be used especially the antigen – antibody pairs they are commonly used for bio-affinity pairs.
- The matrix containing protein A which is used for IgG purification so in this case the protein A recognizes the Fc region of IgG and this interaction is being used for protein purification using affinity chromatography.
- Another strategy is concavalin A protein binds to the glucose molecule so by adding a concentrated solution of glucose the glucose can displace the column where these molecules are attached on the binding site of concavalin A but these are only few examples there are many other examples where different type of fusion partners and ligands are used for affinity chromatography method as shown in the slide.
- Protein A binds with IgG and the proteins can be eluted out by lowering the pH, ABP binds with HSA, elution is again with the low pH, Histidine tag binds with Ni-NTA columns the metal chelators and imidazole or low pH conditions can be used for elution. GST (glutathione S-transferase) binds with glutathione and reduced glutathione is used for elution. The maltose binding protein (MBP) binds with amylase and it can be eluted with maltose. Then FLAG protein can bind to M1/M2 Ab and EDTA or low pH can be used for eluting the proteins.
- There are various other examples but these are common strategies being employed for protein purification.

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- So we just discussed various strategies, by adding a tag or by applying some affinity interaction, the protein can be purified.
- The genetic engineering methods have made it possible to make fusion proteins which shows strong affinity between fused protein and ligand. So for example I have shown in the slide the affinity of the Histidine tag with Ni-NTA column. Now because of interaction of Histidine with Ni, the proteins which contain Histidine tag will bind to these Ni-NTA resins.. Now after washing with mild Imidazole such as 20-50 mM unbound residues will come out but by increasing the concentration such as 100-500 mM Imidazole the proteins can be eluted out.

- The effectiveness of protein purification should be further assessed on SDS-PAGE gel where one need to see whether one pure band is shown or even some contaminating band are also seen. So one case is shown on the left side where contaminating bands are seen whereas in the other case only a pure band can be seen.

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So let's discuss affinity chromatography methods step by step in following animation-

Definition of few components-

The derivatized stationary phase-

The stationary phase resin in affinity chromatography consists of a covalently bound ligand that will specifically bind the protein of interest by interacting it.

The protein mixture-

It is unpurified protein mixture which consist of proteins having different proteins and interaction specificity of ligand bound to the column matrix.

Mobile phase-

Following the sample loading, the unbound proteins are washed out of the column using suitable mobile phase. The ligand solution- the solution is passed through the column to elute the bound protein of interest. Since it contains the same ligands that are bound to the column matrix it is capable of eluting the proteins by interacting with it.

The effluent sample fraction-

The solution leaving the column is collected in suitable sized fractions for further analysis. The unbound proteins are eluted from the column first, followed by the bound fractions which are removed after washing with the ligand solution.

Animation 3

- The column is first packed with a suitable resin that has been covalently coupled to the ligand, specific for the protein of interest.
- The derivatized affinity column is then loaded with the protein mixture containing various proteins having various properties and interaction specificity.
- The column is washed with a suitable mobile phase to remove all the unbound proteins.

- The protein of interest which has higher affinity for the ligand remains bound to the column matrix and is not removed during the washing.
- The samples collected during washing can be analyzed assays and discarded if not required.
- After the column has been washed thoroughly the protein of interest is eluted by passing a ligand solution which binds to the matrix bound protein and removes it from the column.
- The fractions are then analyzed for its protein content by UV-visible spectrophotometer at 280 nm. A graph of eluant volume versus protein concentration is plotted.

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High performance liquid chromatography (HPLC)-

- HPLC is a separation technique that separates molecules based on their different adsorption and desorption between stationary phase matrix in column and mobile phase. Superior separation and resolution of the components can be achieved by HPLC.

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Liquid chromatography

- Liquid chromatography separates mixture components on basis of differences in affinity for stationary and mobile phase. It can remove the undesired impurities therefore for various type of mass spectroscopy based methods.
- Iit increases the reproducibility and robustness for analysis of peptides and proteins. It also concentrates the diluted samples and therefore it increases the sensitivity and detection of low level proteins when you are applying the different type of proteomic technologies.
- It is mostly used for separating the peptide mixtures.
- One of the diagrams is shown here represents a typical liquid chromatography setup which consist of solvent bottles, degasifiers, dual or quaternary pump, the sample injector, column and detectors.
- Different solvents can be placed in solvent bottles depending upon the purification requirements.

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Reverse phase chromatography-

- The reverse phase chromatography is based upon the hydrophobic binding interaction between the peptides or the proteins in the mobile phase and immobilized hydrophobic ligands in the stationary phase.
- The reverse phase consists of long aliphatic carbon chain which is highly hydrophobic in nature. Molecules are bound on the column by means of hydrophobic interactions and are eluted out when the solvent polarity is modified.
- The reversed phase chromatography is mostly used with electro-spin ionization (ESI) in mass spectrometry due to its compatibility of its acidic aqueous and polar mobile with ESI. It is also used for desalting the peptides before injecting for ESI.

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Strong cation exchange (SCX) chromatography-

- SCX consist of stationary phase matrix which is made up of negatively charged sulfonic acid groups which bind the oppositely charged peptide molecules.
- These molecules can be eluted out using a positively charged mobile phase which binds the analyte molecules more firmly.

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Multi-dimensional protein identification technology (MudPIT)

- So MudPIT method combines cation exchange and reverse-phase HPLC chromatographic separation of tryptic peptides for the proteome based analysis.

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- So we have already discussed to separate methods SCX and RP.
- So SCX is based on the electrostatic attraction between the negatively charged sulfonic acid and positively charged peptides and the elution can be caused by addition of positively charged mobile phase.
- The RPC is shown on the right side is based on the hydrophobic interactions between the analyte and stationary phase.
- The elution can be brought about by modifying the mobile phase polarity. The SCX can be used offline and then each fraction can be analyzed reverse phase HPLC followed by mass spectrometry.
- Alternatively, both RP and SCX resins can be packed into single column and by introducing buffers in the series, the multidimensional separation can be achieved.

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Choosing correct methodology for analysis of the complex proteome

- Now when application is to separate complex proteome and analyze using mass spectrometry then one has to select what type of chromatography method is applicable for their sample type.
- Now multi-dimensional approaches are usually coupled prior to the mass spectrometry analysis.
- And as we have discussed there are certain chromatographic methods which are commonly available and one has to make an educated choice of selecting what type of chromatography methods can be used for first dimension and what can be used for second dimension separation.
- So when size exclusion chromatography has been used followed by the reverse phase, to check the compatibility of first and second dimension separation based on the size in the SEC and the charge various type of combination methods have been tried out?
- So let me first give you abbreviations here and then what properties they separate proteins and peptides based on and then we can talk about how these combinations can be applied.
- So we have talked about size exclusion chromatography which separates proteins based on the size or the molecular weight. Reversed phase based on the hydrophobicity and then we have capillary electrophoresis which separates based on the charge. IMAC is affinity based interaction, reverse phase again hydrophobicity. SC is based on the charge. So these are different methods which we have discussed. Now what are we looking at how the combinations of these can be applied for proteomic investigation. So when SEC followed by RP-HPLC was tried the poor resolution of peptides in SEC occurs.
- The RP-HPLC followed by capillary electrophoresis or SEC followed by capillary electrophoresis is limited with the loading capacity.
- Now third combination of using affinity chromatography as first separation followed by RP-HPLC has been used. But what is most popular is SCX in first dimension followed by the RP-HPLC in the second dimension. It has been used for various proteomic analysis.

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So in summary, in the last few years there has been an increased effort to develop the technologies which are capable of analyzing protein expression at the proteome scale.

Proteins can be fractionated into different groups having similar physical and chemical properties by a variety of different analytical methods.

Some of the chromatographic methods such as gel filtration, ion exchange and affinity chromatography were discussed. The SCX and RP-HPLC are fundamental tools for the isolation and analysis of peptides. Nano-liquid chromatography has gained popularity for the proteomic studies due to its high sample throughput and analytical sensitivity.

So in summary we have talked about principle of different type of chromatography commonly being applied for proteins and peptides.