

# NPTEL VIDEO COURSE – PROTEOMICS

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### LECTURE-06

#### PROTEIN PURIFICATION AND PEPTIDE ISOLATION USING CHROMATOGRAPHY

#### TRANSCRIPT

Welcome to the proteomics course. Today, we will talk about protein purification and peptide isolation using chromatography methods. The chromatography techniques have always been considered prominent 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 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.

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. And then we will talk about few methods such as strong cation exchange and reverse phase chromatography for peptide isolation using high performance liquid chromatography methods.

Protein purification and peptide isolation and analysis- it can be performed by many methods but there are certain chromatography methods which are commonly used, such as 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

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 a given protein
- affinity for various ligands

So let's talk about what is chromatography.

So 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

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resin. So these matrix and resins they are usually beads with or without attached chemical groups. Now the binding and interaction of proteins with the column matrix is an important feature of chromatography.

The chromatographic technique 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, bead shape, size and porosity. In addition, functional group types the charge distribution and density as well as elution condition such as pH, the ionic strength and gradient shape.

So we have just talked that chromatography involves the selection of right matrix, these matrix are very important for binding and interaction of proteins and they can determine the kind of achievement, which we want to make from these types of chromatographic methods.

So let's move on to more specific chromatographic techniques such as gel filtration Chromatography.

The gel filtration chromatography it separates proteins on the basis of difference in size. It is used to separate a protein of interest from a protein mixture that are 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 need to remove even contaminants such as low molecular weight contaminants and low molecular weight detergents.

So 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 system and large molecules such as proteins will elute first because they cannot enter inside the pores of these beads and they can be used to separate protein based on the size.

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.

The protein sample that contains a mixture of unpurified 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 the pores 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 increased column length or decreasing the flow rate can be adjusted to achieve higher resolution of proteins.

After these steps, the fractions 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.

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

Let's first give the definition of few 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.

Sample fractions, the solution leaving the column are 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 as molecular sieve.

Now let me show you how this process works. So first the matrix which is suitable for the required protein separation, this packed gel matrix is loaded with the protein sample containing a mixture of unpurified proteins of different sizes. The column is then eluted

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with a salt solution of 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 in and out of pores 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 the later fractions. Once all these fractions are collected, then it can be analyzed for their protein content by using a UV-visible spectrophotometer. Now analyze 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 towards the end.

Now let's move on to next chromatographic method, which is ion exchange chromatography. This is one of the versatile chromatographic separation method, which rely on differences between number of charges and distribution of charge groups in defined pH and solvent conditions.

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 by varying the amount of positive and negative charge amino acids and even pH influences net charge on proteins.

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.

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

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 charge 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 the anion exchange.

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

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 be vice versa.

So let me describe how ion exchange chromatography works step by step in following animation.

The charge stationary phase; the column stationary phase is consisting of a positively or negatively charged polymeric matrix, which will bind 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 protein mixture, 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 out 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 brief description of components, let me show you the process in animation-

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.

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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.

So let's now talk about another very important method, which is 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.

So affinity chromatography is based on affinity of proteins to its ligands or other protein molecules. The metal chelation is widely used in purification of recombinant proteins. Various substrates, products, cofactors, antibodies or metals, these 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 these ligands.

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

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

So due to the specific interaction the affinity chromatography achieves very high degree of protein purification. It is not limited by the 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.

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 the IgG purification so in this case the protein A recognises 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

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different type of fusion partners and ligands are used for affinity chromatography method as shown in the slide. The 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.

So we just discussed various strategies, by adding a tag or by applying some affinity interaction the proteins can be purified. The genetic engineering methods have made it possible to make fusion proteins which shows strong affinity between the 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 tag with Ni, the proteins which contain Histidine tag will bind to these resins of Ni-NTA. Now after washing with the 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.

So let's discuss affinity chromatography methods step by step in following animation. Let me give you 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. Depending on protein of interest this could be either water or sometimes salt solution.



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The ligand solution- the solution is passed through the column to elute the bound protein of interest. Since it contains the same ligand that is 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.

Now let's see the process in following animation.

The column is first packed with a suitable resin that has been covalently coupled to the ligand, specific to the protein of interest. The derivatized affinity column is then loaded with the protein mixture containing various proteins having different 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 derivatized 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 their protein content by UV-visible spectrophotometer at 280 nm. A graph of eluant volume versus protein concentration can then be plotted.

Let's now talk about high performance liquid chromatography (HPLC). HPLC is separation technique that separates molecules based on their differential adsorption and desorption between stationary phase matrix in column and mobile phase. Better separation and resolution of the components can be achieved by HPLC.

So what is liquid chromatography?

The liquid chromatography separates mixture components on basis of differences in affinity of stationary and mobile phase. It can remove the undesired impurities therefore for various type of mass spectroscopy based applications it increases the reproducibility and robustness for analysis of peptides and proteins. It also concentrates the diluted samples and therefore it increases the sensitivity, detection of low-level proteins when you are applying the different type of proteomic technologies. It is mostly used for separating the peptide mixture one of the diagrams is shown here which is a typical liquid chromatography setup which consist of solvent bottles, degasifiers, dual or



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quaternary pump, the sample injector, column and detectors. Different solvents can be placed in solvent bottles depending upon the purification requirements.

Let's first talk about reversed phase (RP) chromatography. The reversed 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.

In reversed phase, the stationary phase consist of long aliphatic carbon chains, 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-spray 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.

Let's now talk about 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.

So what is 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.

So we have already discussed two 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 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 by reverse phase HPLC followed by mass spectrometry.

Alternatively, both RP and SCX resins can be packed into a single column and by introducing buffers in the series, the multidimensional separation can be achieved.

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

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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 so 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 the 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, SCX 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 investigations. 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 separation in first dimension followed by the RP-HPLC in the second dimension that has resulted in the best separation of peptides and it has been used for various proteomic analysis.

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. 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. The nano-liquid chromatography which makes use of C18 capillary columns has gain popularity for the proteomic studies due to their ability to achieve finer separation.

So in summary we have talked about principle of different type of chromatography methods commonly being applied for proteins and peptides and we looked at some of these chromatographic methods in more details. Thank you.