Analytical Technologies in Biotechnology
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Module - 3
Chromatographic methods
Lecture - 6
Affinity chromatography

In previous lectures we have discussed two specific techniques, chromatographic techniques. One ion exchange chromatography, and gel filtration chromatography. One was based on the charge an analyte carries, and another was based on the size or shape of the molecule.

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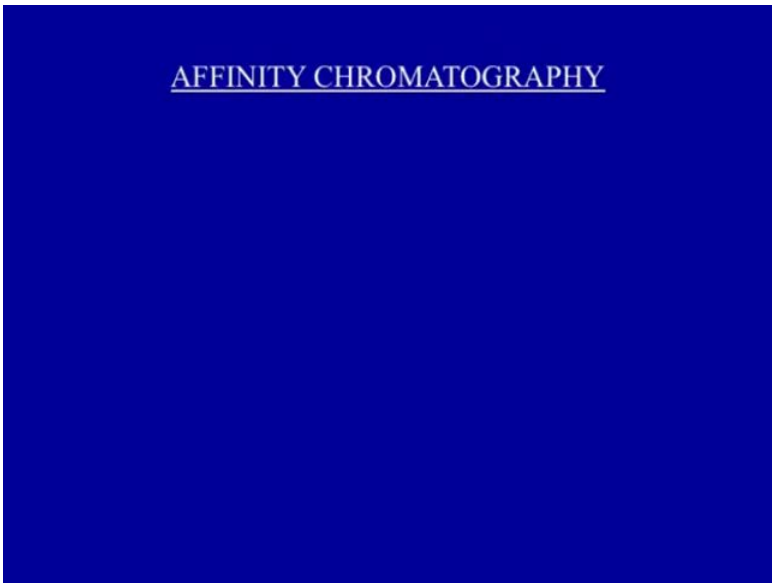

Today we are going to discuss about another chromatographic technique, which is affinity chromatography, which has very high selectivity. Now, lot of molecules or substances they interact with each other quite strongly, and this is the basis of affinity chromatography, that is one molecule has high affinity for another molecule and will bind to it reversibly, but strongly. So, affinity chromatography separates proteins or other analytes on the basis of a reversible interaction between a protein or say group of proteins and a specific ligand coupled to chromatographic matrix.

Now, this combinations there could be a lot of different kind of combinations, like say protein inhibitor combination, it could be receptor hormone combination, it could be evident biotin combination or it could be simply antibody antigen combination. This technique is ideal for a capture or intermediate step in a purification protocol and can be used whenever a suitable ligand is available for the protein of interest to be purified. With a very high selectivity and hence high resolution, high capacity for the proteins of interest could be obtained.

So, purification labels in the order of several thousand fold with high recovery of active material are achievable in this technique. Target protein can be collected purified and also could be concentrated, mostly it is like in a concentrated form as in ion exchange chromatography, but it is much more selective than ion exchange chromatography. So, it is a high capacity with high selectivity and has much higher resolution. Now, biologic interactions between ligand and target molecule can be a result of electrostatic or hydrophobic interaction, it could be wonder world forces or hydrogen bonding.

Now, to elude the target molecule from affinity medium the interaction can be reversed either specifically using a competitive legend or non-specifically by changing the ph or ionic strength or polarity. So, if I have to explain in a very simple way, let me explain this technique on your screen. Now, so what is happening is first thing is that you have to choose a pair that is a ligand that needs to be immobilized for a particular protein of interest to be purified and this ligand then needs to be put on a matrix that is support material.

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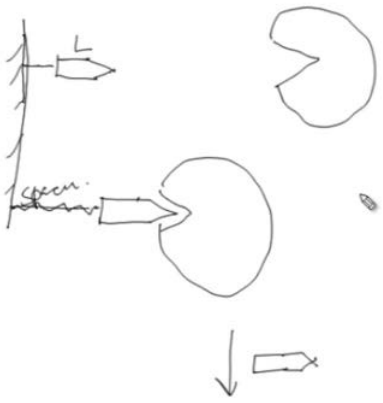

So, what you have is supposing this is your support material and which could be anything like as we discussed agros, or dextrin or polychromite depending on what you choose to use. There will be a particular ligand which will be immobilized, say this is your ligand which is immobilized, this is your ligand. Now, this ligand could be immobilized either directly on to the matrix or it could be mobilized through a say spacer arm, spacer arm could be 6 to 8 carbons or others as we discussed. Then the ligand could be attached to the spacer arm, a spacer arm is attached to the matrix.

Now, this kind of increases the accessibility of the protein to be purified to the ligand. Sometimes the larger molecules the bigger molecules, if the ligand is too close to the matrix and because of the matrix like physical conformations protein cannot access. So, in either case what is happening is there is a protein or this is analyte which has a complimentary shape here. This is just a schematic to make you understand. Now, when the protein molecule to be purified is loaded on to the column and this ligand which is immobilized, like charges are immobilized on stationary phase, this ligand is also immobilized and one beat might carry many ligands. Also the porosity and other factors will affect the flow and binding.

So, as this protein molecule and many of the protein molecules will come they will be binding to this particular ligand and due to their biological interactions they will bind. Now, here affinity has to be good enough in the sense that it binds specifically and non

specific binding could be avoided and should be reversible also, so that you can elute the sample. So, once it is bound, then second step would be to wash the contaminants and then elute this protein. Now, elution can be done by either I can take a free ligand here in higher concentration.

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Now, when you have free ligand in higher concentration, what will happen? This free ligand in higher concentration will replace the bound ligand to the column. So, what you will get in, you will get elusion pattern where the free ligand has replaced the bound ligand and this whole thing could be then passed in the elusion or elute from the column. So, this is how you will be able to purify the sample very specifically and it is high capacity and high selective, you can also elute it by changing the p h and other conditions. So, this is in a very simple way this is the basis of affinity chromatography. Let us explain this, let us get into the details of this technique.

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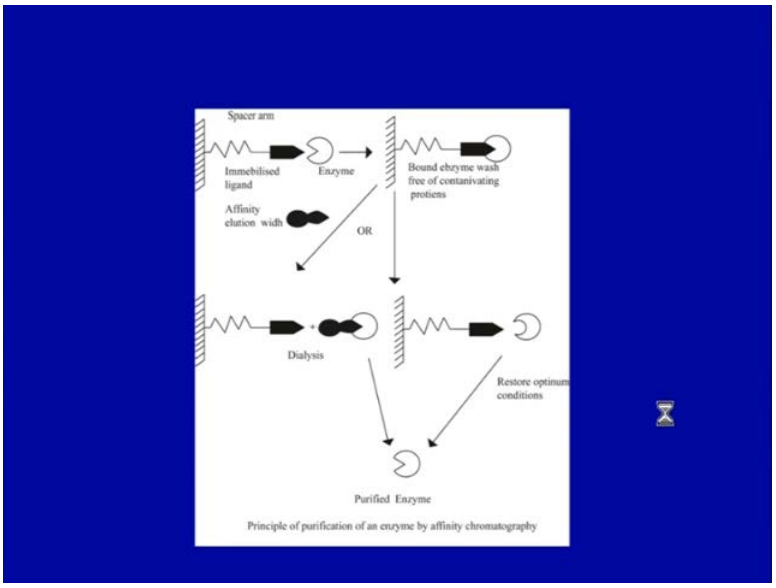

Now, here if you see what I have explained this depicts the same thing, you have ligand which is the spacer arm, this comes and binds here, this could be eluted with another free ligand or p h conditions could be taken and then you restore and then again purified enzyme is obtained and this can be used as many times as you want by cleaning it up. Let us discuss different things about this chromatographic technique, there are certain terms which are important, one is matrix which we have discussed earlier, but in terms of affinity chromatography.

So, matrix for ligand attachment is very important, so matrix should be chemically and physically inert and should be able to attach to the ligand. Then spacer arm, spacer arm like I have shown you it is used to improve the binding between the ligand and target molecule by overcoming any facts of a steric hindrances of the matrix. Ligand is molecule that is binds reversibly to a specific target molecule or group of target molecules. Then binding, the buffer conditions are optimized to ensure that the target molecule interact effectively with the ligand and are retained by the affinity medium, as all other molecules will be washed through the column, so binding another important term here.

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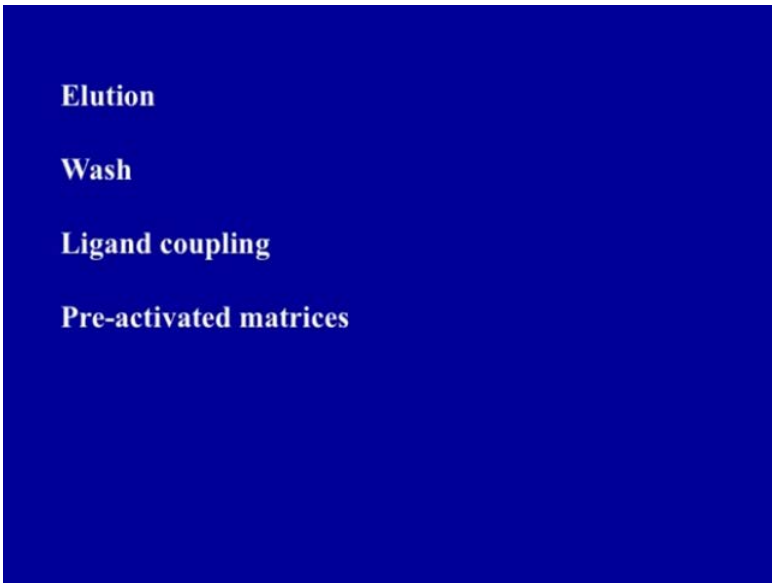

Then elution, in elution in affinity chromatography buffer conditions are changed to reverse the interaction, that is weaken the interaction between the target molecule and the ligand and like I said it could be done specifically and non specifically. Then you wash for the column for non specific binding, so that unbound substances which are not really need to be binding there will be taken off. Ligand coupling is another important term it is a covalent attachment of ligand to a suitable pre activated matrices to create affinity medium.

So, this could be done, commercially these resins are available and even this could be done purchase a pre activated matrices and you can couple a particular ligand. Pre activated matrices like I said it is a matrix which have been chemically modified to facilitate the coupling of the specific type of ligand and you can purchase and it could be could attach any particular type of ligand. Now, the operation of affinity chromatography involves lot of different steps, if you go through, if you have to do affinity chromatography, then you have to ah go through certain steps.

One is you have to choose an appropriate ligand, so you have to know what you are going to purify or which group of analytes proteins you are going to purify. So, the choice of appropriate ligand is most important, then the immobilization of the ligand on to a support matrix. So, that you can really do that particular, purify the particular analyte, then contacting the protein mixture of interest with the matrix that is another

important part, then removal of nonspecifically bound proteins and finally, elution of protein of interest in a purified form.

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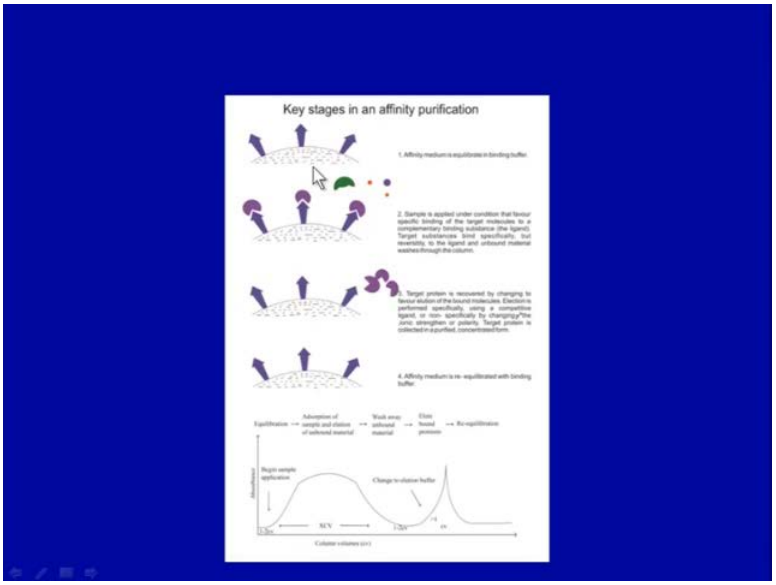

If I see this particular figure, if you can see here, this is very simple like what you have done is first thing is you have ligand, this is kind of a mobilized and you have to equilibrate in a particular buffer. These protein molecules are coming and binding in here as you can see non-specific binders will not be allowed then what is done? You will be eluted by, target protein will be eluted or recovered by changing the conditions and finally you can regenerate it. Now, you can see here this graph, this is for non-bound compounds non specific comes out and a peak could be eluted, as very sharp peak in a concentrated form.

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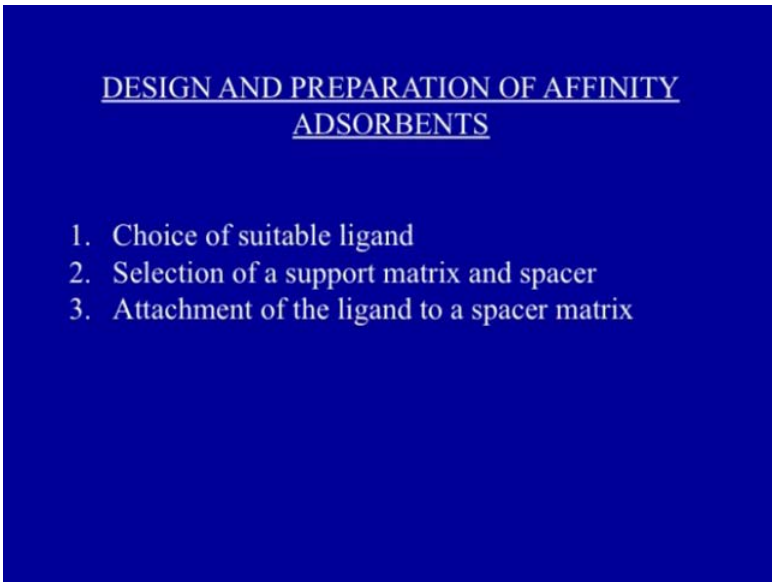

So, if you consider design and preparation of affinity chromatography matrix, like I said it requires choosing certain particular things, affinity adsorbent. For a purification of a particular protein and it involves three major steps or three major factors here. One is choice of suitable ligand, selection of a support matrix and a spacer, then attachment of the ligand to a spacer matrix. So, let us discuss each of these things here in detail.

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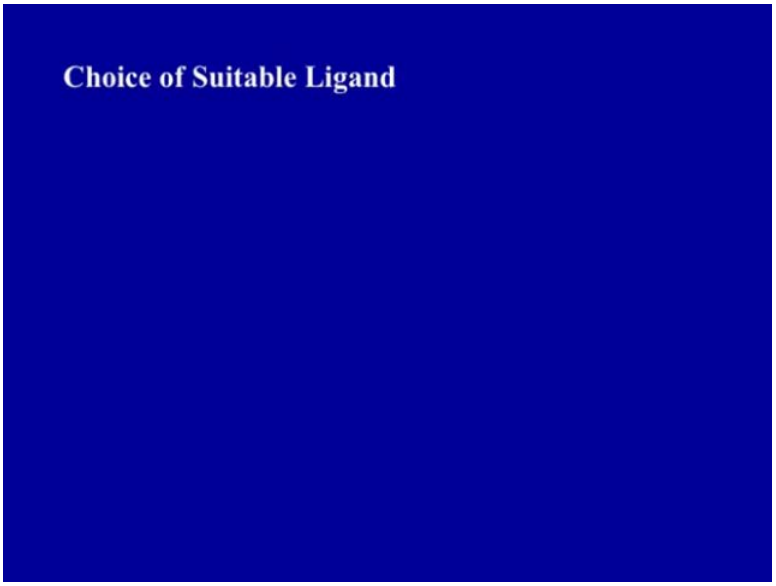

Now, first thing is choice of suitable ligand, so what kind of ligand one needs to choose for successful affinity purification? Now, so this requires for a successful affinity purification you require a bio specific ligand that is, that particular ligand could be

covalently attached to a chromatography matrix. It should be attached in a way that it does not lose its binding capacity. So, the couple ligand must retain its particular specific binding affinity for the target molecule and after washing away unbound materials, the binding between the ligand and the target molecule must reversible.

So, reversibility is a very important factor, otherwise we will not be able to elute the sample, so and purify the sample in active form. Any component can be used as a ligand to purify its respective binding partner, there are lot of different biological interactions and they are typical biological interactions used in affinity, chromatography. For example, there could be enzymes substrate interaction or substrate analog could be taken, there could be antibody, antigen interaction, there could be lactic polysaccharide interactions, nucleic acid, nucleic acid like say enzyme interaction, hormone receptor interaction. So, there are whole lots of interactions which are present here, like I said evident iodine could be there. So, lot of interactions could be there and these are natural biological interactions that are occurring all the time and could be utilized for affinity chromatography.

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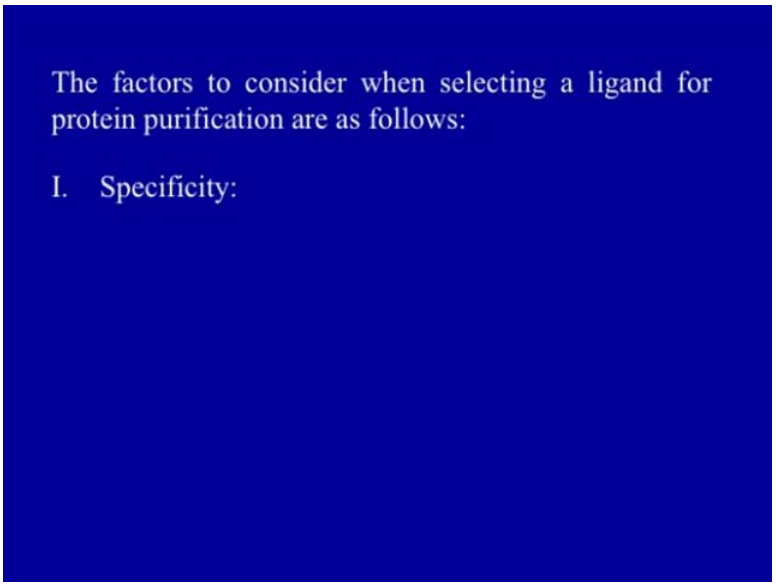

Now, the factors to consider when selecting a ligand for protein purification are like specificity one. Now, specificity is very important, the ligand should recognize only the protein to be purified and it should be highly specific. Now, there could be two different types of ligand, one is mono specific that is they will recognize only one type of

substances or compounds or proteins we can say, these are called mono specific compounds. Like for example, mono specific anti body, they could be a possibility that is a ligand is known to recognize a group of compounds or group of proteins, then it will be called group specific in nature.

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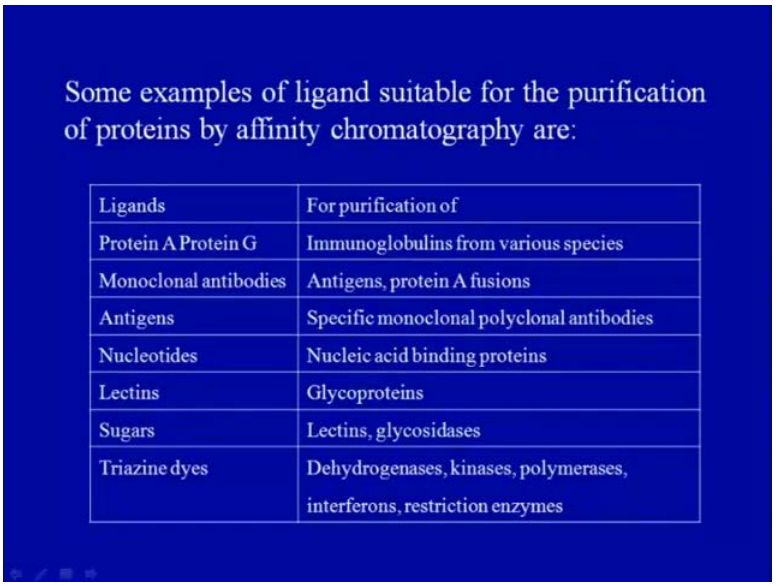

Some typical examples of ligand suitable for the purification of proteins by affinity chromatography are you can see on your screen. Like protein A or protein G which could be utilized for immunoglobulins, monoclonal antibodies for different kinds of antigens and then nucleotides for nucleic acid binding proteins, lectins for glycoproteins, sugars for lectins and glycosidases and likewise you can use lot of different combinations. So, one more specificity which is very important, the second is reversibility that is very important that ligand should form a reversible complex with the protein to be purified.

So, the complex should be resistance to the composition of the free stream and washing buffers and the complex should be easily dissociable without requiring denaturing conditions, it should be, affinity should be high enough, but not so that it becomes irreversible. Then stability of the ligand is very important, it should be stable to the conditions to be used for immobilization as well as the conditions for elution and other things. Like say resistance to proteolysis, resistance to denaturization by eluents or cleaning agents, that is very important for a ligand to be immobilized.

Now, size of the ligand if we consider, then the ligand should be large enough such that it contains several groups to interact with the protein, resulting in sufficient stereo selectivity and affinity. Then a ligand should contain a functional group which can be used for immobilization without affecting the protein binding characteristics. Remember if it is connected in a way that its binding site is kind of inhibited or it is towards the matrix then it is of no use then actually. So, it should have a functional group which can bind to the matrix without affecting its binding characteristics.

Smaller ligand might not be accessible to protein molecules because of the matrix back bone interference; so many times spacer arms could be utilized. Very large ligand is likely to be susceptible to denaturation and degradation, so it can also cause increased non-specific binding through other parts of molecules. So, these things need to be taken care when you are choosing the size of the ligand.

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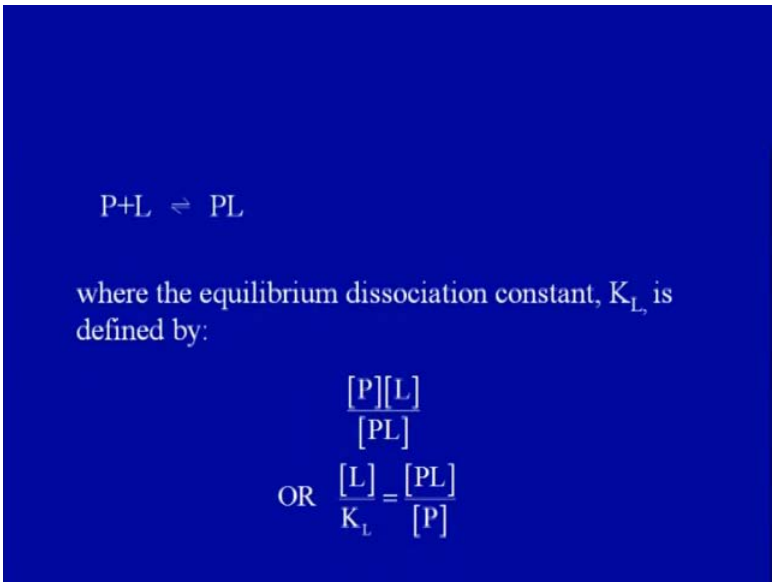

Now, affinity like I said that the interaction of a protein, which is P and ligand L can be described in this that is P plus L equilibrates with PL and the dissociation constant could be given by this equation here. If you consider that a substance that is which is substantial absorption of the protein from solution, it should have like values of dissociation constant must be about two adders of magnitude less than the concentration of the immobilized ligand. Now, many affinity techniques they operate quite well between the dissociation constant of 10 is to minus 4 to 10 is to power minus 8 molar, so

that is how you select a particular ligand. So, this was about ligand, the next important thing is support matrix and the spacer.

Now, when you are selecting the matrix there are few things needs to be taken care of, a ligand is mobilized to a solid support or a matrix by one or more covalent bonds, so the effectiveness of the immobilized ligand in purification will depend on the structure of the matrix. Following criteria as we are going to discuss are very important in selection of the matrix, one is that matrix should have a high degree of porosity.

So, that right fluorides and the accessibility could be achieved. Large proteins like earlier also discussed will have problems if there is not enough porosity and they needs to have unhindered access to the ligand immobilized on the interior portion of the latest. So, that is another important part where cross linking is taking place high porosity is very important factor.

Then the matrix should be chemically stable under the conditions used for activation and coupling, as well as they are used during for operation and regeneration. So, those conditions they will be lot of different conditions and matrix needs to be stable, then matrix should be physically rigid in order to allow good flow properties. This is very important for any chromatography that mechanical properties of matrix where it should be quite rigid, so that right flow could be obtained or you can say even flow throughout the running could be obtained.

Then matrix should stand reasonable range of p h and temperature change. Matrix should be easily activated for coupling and in case of affinity chromatography, coupling of ligand at high density and should be inert to non-specific binding of proteins. Matrix property should be substantially altered on functionalization. So, if a matrix is there it should not be substantially altered when you are doing all this processing, chemical processing matrix. Matrix should be uniform in structure, particularly when functionalized so that the ligand molecules can be homogenously distributed, that is very important.

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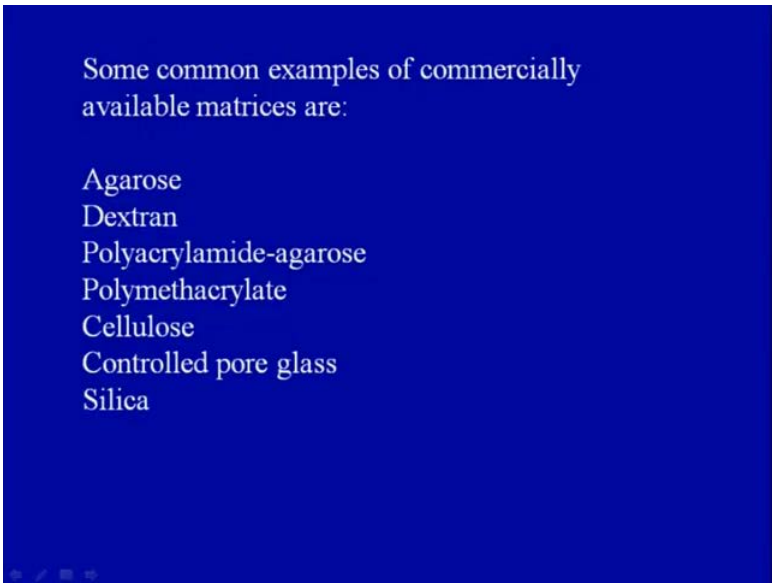

Now, there are lot of different kinds of matrices are used, commonly used matrices include agarose, dextran, then polymethacrylate, polyacrylamide, cellulose, controlled pore glass could be utilized silica and so on. So, there are whole lots of matrices are available, now once this matrix is chosen it has to be reactivated and the ligand has to be put in.

Now, many times you one needs to put spacer arm, so when you have to choose a spacer arm for connecting ligand and matrix there are certain things to be, like if you see in general. Spacer molecule is used to distance the ligands from the matrix and so when small size of the ligand excludes it from free access to protein molecule in the solvent. Ligand is mobilized through a near enough to the protein binding surface to interfere with the protein binding.

So, what is happening here is because of say ligand is smaller and it is very close to the matrix, the protein which is larger to be purified is not able to access the ligand and in that case you need to include a spacer arm. Now, length of the spacer is very crucial must be determined very carefully and the number of methyline groups, most often successfully has been used is from 6 to 10. So, the chemical nature of the spacer is critical to the success of separation.

Now, some spacers or spacer arms are hydrophobic, but most commonly consisting of methyline groups, but there could be others which are hydrophilic in nature and may

contain carbonyl or amido groups. Spacer should be resistant to bind protein that is non-specific binding should not occur and it should be stable chemically. Like for example, it could have a chemical nature say hydrophobicity or hydropholicity or charge, so it should be resistant to bind protein itself like non-specific binding should not occur.

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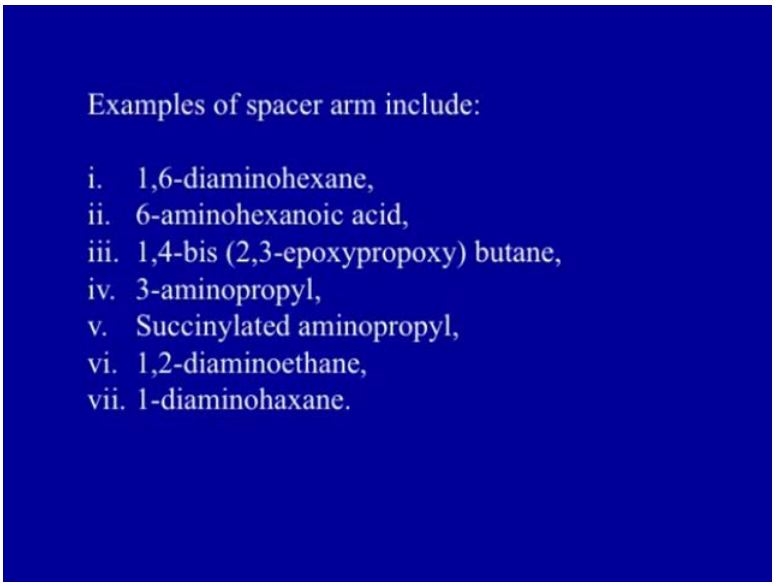

There are lot of different examples of spacer arms they could be like ah 1,6 diaminohexane is used, 6 aminohexanoic acid is used, 3 aminopropyl, succinylated aminopropyl and so on, this is list of spacer arms is available. Now, attachment of a ligand to a spacer matrix when it needs to be done, then large number of methods are developed for coupling ligand to matrix material, the most common procedure is to link the coupling agent to the matrix material and then add the ligand. So, what we see is pre activation of the matrix. So, the reaction conditions and the relative proportions of the regents will determine the number of molecules that can be attached to each matrix particle.

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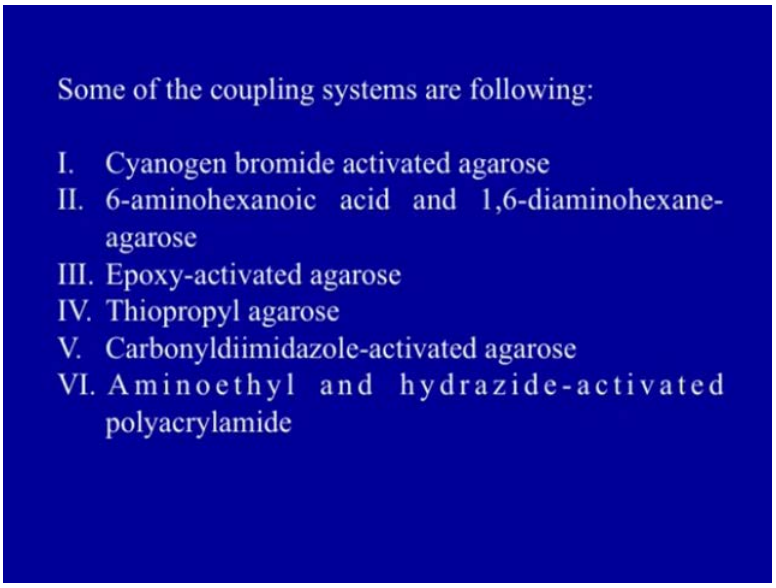

Now, there are certain coupling agents which are available, which will activate the matrix so that the ligand could be put in. Like say cyanogens bromide activated agarose. Cyanogens bromide is very commonly used coupling agent, it reacts strongly with amino group, it is extremely useful in coupling enzymes, co-enzymes, inhibitors, antigens antibodies and so on.

There could be others like 6 aminohexanoic acid and 1,6 diaminohexane-agarose could be utilized. In case of small ligand where hysteric interference occurs because ligand is too near to the matrix surface, it is a 6 carbon atom spacer is inserted between the matrix and the ligand here by using agarose to which 6 aminohexanoic acid or 1, 6 diaminohexanoic are coupled.

So, you can have a coupling agent with a spacer arm, so this could be utilized in this case. There could be lot others like epoxy activated agarose and it is the agarose to which 1 4 is to 3 epoxy propoxy butane is coupled, which contains free oxiding groups. So, this group allows linkage of sugars carbohydrates or any other ligand containing a hydroxyl amino or thio groups. Then there could be thiopropyl agrose and there are other materials which are available in here, they could be carbonyldiimidazole-activated agarose could be utilized in place of c n b r coupling.

This is like utilized that like many times c n b r coupling or cinogen bromide coupling, the n nucleoifile reacts with the cinate ister of c n b r resulting in isourea linkage and that

carries a potential charge and can act as an ion exchanger and thus affinity chromatography will be kind of specificity is decreased, so the chromatography run might not be proper. So, to avoid this problem carbonyldiimidazole could be utilized rather than c n b r, then there are aminoethyl or hydrazide activated polyacrylamides which could be utilized for coupling.

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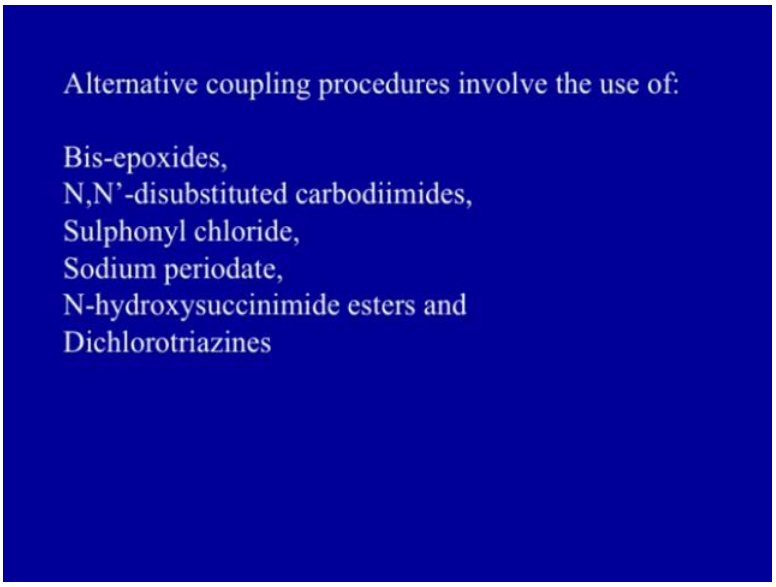

Lot of alternate coupling procedures is available which could be utilized here.

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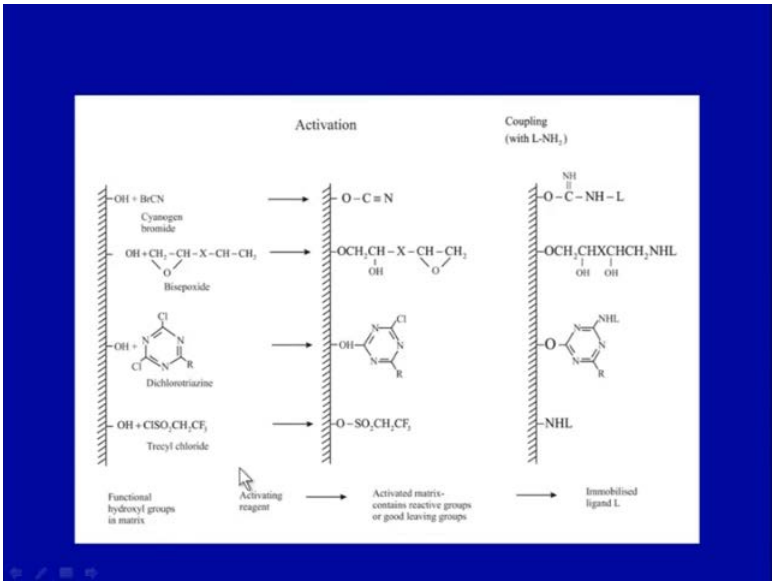

If you see here on your screen, this is a very simple way of showing activation and coupling. So, first thing is activation and then couple the ligand, so if you see in the case say cynanogen bromide, it acts and it is activated in here. Then to this group it is very easy to couple the ligand in here through C N group. Likewise, there are other examples here which could be, there are other functional groups and they could be activated and finally the ligand could be immobilized. So, these are methods that could be utilized for immobilization of different ligands through activation and coupling.

So, lot of chemistry is involved here which we have not gone too much of detail, but that is important this chemistry can be done in your lab also. But many times you might not be able to do it very uniformely when you repeat it or many ligands may not be attached because of many problems, unless you do it very carefully, otherwise commercially available matrices with couple ligands could be always purchased. So, the already activated and coupled, you have pre activated matrices that could be utilized, if you have to couple a particular ligand.

Now, estimation of ligand concentration can be done, it is very essential to determine the success of ligand immobilization, when you have done this. Like I said that sometimes not so successful in lab conditions, but you have to determine the success of ligand immobilization and it could be done by different methods, like difference analysis. So, what you can do is by measuring the amount of ligand added to the coupling mixture and that could be recovered after washing procedures.

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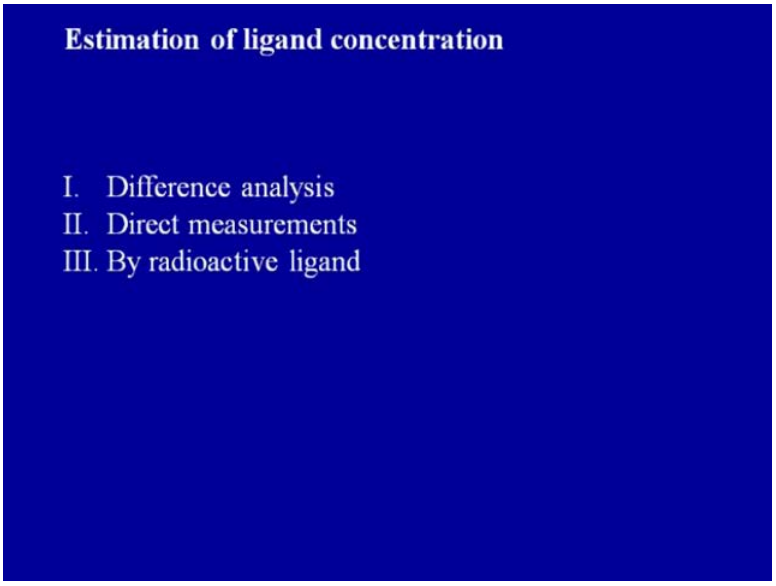

So, difference analysis could be one method, there could be direct measurements where absorbents of derivatives and underivatized matrix can be compared. Derivitized gels can be asset for proteins using lauren methods, direct element analysis can be done the immobilized ligand is having unique group. Say for example, phosphate there could be direct analysis; this could also be done by radio-active ligand.

Now, radio-active ligand is incorporated into the coupling reaction, then it could be analysed for total immobilized ligand. So, there could be different methods of to analyse the ligand binding here. So, affinity chromatography like I said is very highly selective, high capacity, gives high resolution and it could be utilized for separation of many different kinds of analyte.

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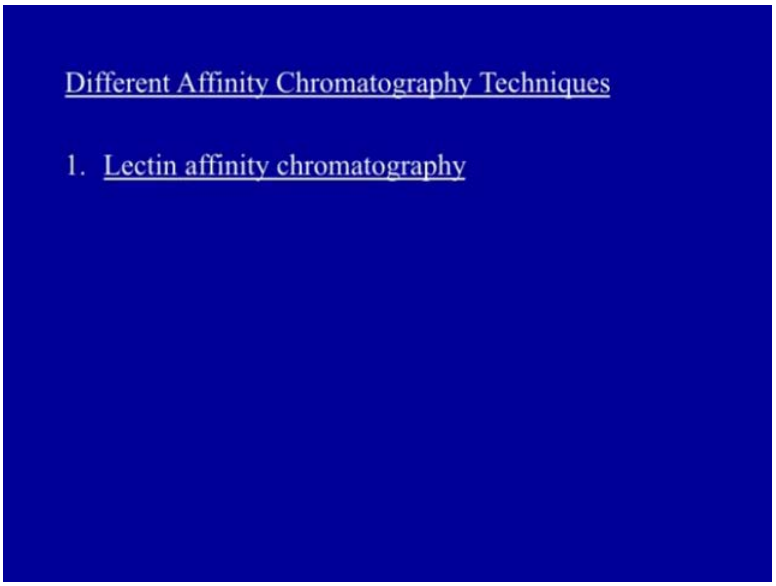

There are lot of different kinds of chromatography techniques which are available in affinity chromatography, like different combinations and different types of like basic we have discussed here. But let us see what are the different combinations or different types of affinity chromatography. So, what here the ligands that are used in affinity chromatography are either we said group specific or mono specific. On the basis of these ligands lot of different combinations of affinity chromatography could be there and for example, lectin affinity chromatography.

Now, lectins are a group of proteins produced by animals and plants and other organisms and they have the ability to bind carbohydrates specifically and hence glyco proteins. So,

they have a polymeric structure most being tetra make and there sub units may be either identical in which case they recognize a single specific saccharide or of two types in which they can recognize two different saccharide molecules. They are highly valuable in the purification of glyco proteins particularly membrane receptor proteins and once the glyco protein has been bound to the immobilized lectin, elution can be achieved by a number of different ways.

Like we have discussed earlier like for example, by affinity elution by using simple monosaccharide for which the lectin has the affinity. So, you can have a free monosaccharide which can be used and through competition, by use of borate buffer which forms a complex with glyco proteins it could be utilized for elution. You can change p h that could be one method for elution or by addition of a reagent, like ethylene glycol to reduce the ligand hydrophobic interaction. Lectin affinity chromatography can be carried out in the presence of little bit of high salt concentration, because it does not rely on ionic interactions and it is also used to separate mixtures of cells by taking advantage of the saccharide component on outer membranes.

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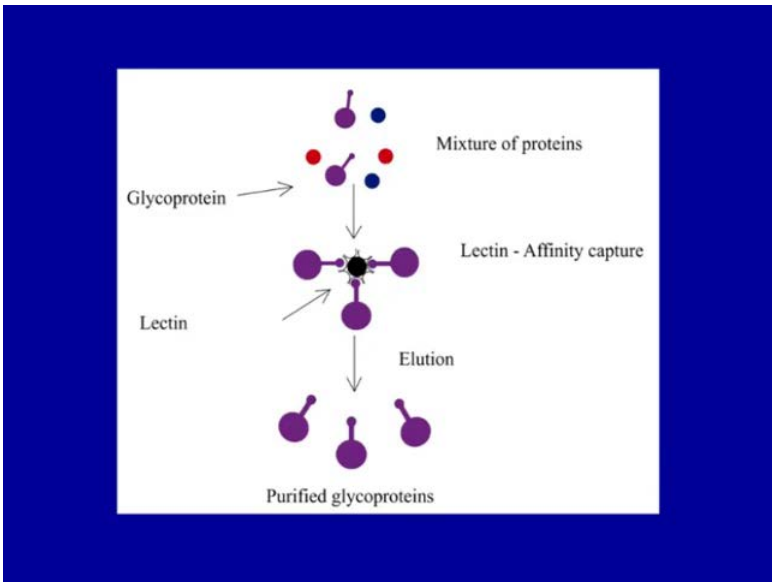

So, if you see here it is a very simple depiction that you have two kinds of, one is glyco proteins another simple proteins, this is lectin. So, glycoproteins will bind others will not bind in the mixture, then you can elute it by having the monosaccharide or different methods you can use for elution. So, this is very simple depiction of this particular

chromatography technique. Then there could be immunoaffinity chromatography, immunoaffinity when we say necessarily it is antibody antigen interaction.

So, the antibodies are used as the immobilized ligand in the isolation and purification of various proteins including membrane proteins of viral origin. Monochloro antibodies may be linked to agarose matrices by the say c n b r coupling, and then protein binding to the immobilized to the antibodies is achieved in neutral buffer solutions containing moderate salts concentration. There could be a lot of different ways to elute it like use of high salt concentration use of urea or curadium hydrochloride is isotropic agents, lowering the p h and likewise.

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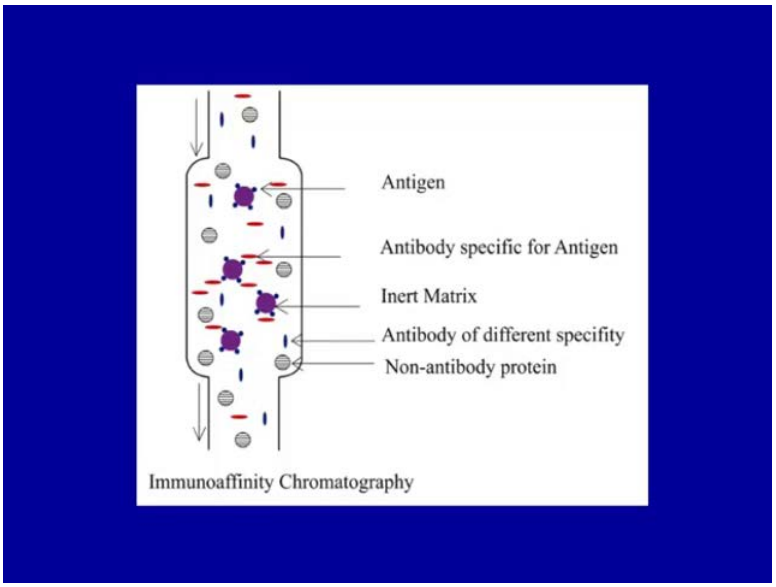

So, if you see immunoaffinity chromatography as you can see on your screen, if you see here there is an antigen immobilized on to the matrix material, the protein, the antibody the specific antibody will bind to it. And then antibody of different specificity could be especially brought in then it could be eluted or by p h or other changes it could be eluted. So, that is the immunoaffinity chromatography is another important form of affinity chromatography.

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There could be metal chelate chromatography, in this technique an immobilized metal line which could be copper, zinc or mercury or cadmium and so on or transition metals is used to bind proteins selectively. Binding involves reaction with the immidazole group of hystedine residues or cistine residues or endole groups of tetrophan. The immobilization of protein involves formation of a coordinate bond that allows protein attachment and retention during the elution or non-binding contaminated material.

The elution can be achieved again by similar methods, like lowering the p h, using some complxing agents such as e d t, etcetera. Nickel column is quite widely used to purify recomenate proteins having hystedine tag or polished tag. Zinc columns could be utilized for isolation of say human interferon, there are other things like dye ligand chromatography where certain dyes which binds with certain molecules. The exact mechanism is not known it is not specific, but they could be very good way to purify the certain proteins, like say d n a binding protein and other things.

One say black ground blue that is one method of achieving purification, there also you can have elution or through salt gradient or affinity elution could be done, which is very important, it is a cheap, there are very good methods of purification and very specific. Then there could be covalent chromatography, in covalent chromatography technique it could be used it is used to separate thiol containing proteins by exploiting their interaction with an immobilized ligand containing a disulphide group. Now, the most

commonly used ligand is disulphide two paridol group, on the reaction with the thiol containing protein in parallel two thiol is released.

The process can be monitored spectrofort amatically at 343 nano meter, thereby allowing the adsorption of the protein to be followed. When the protein is attached covalently to the matrix non-thiol containing proteins are eluted and unreactive thio-paradol groups are removed by use of 4 mille molar dye or dithiothreitol, d t t we can say or mud captor phenol could be utilized. Protein can be then be released with replacement of thiol containing compound which could be d t t or reduced dulto thyoines or systeens.

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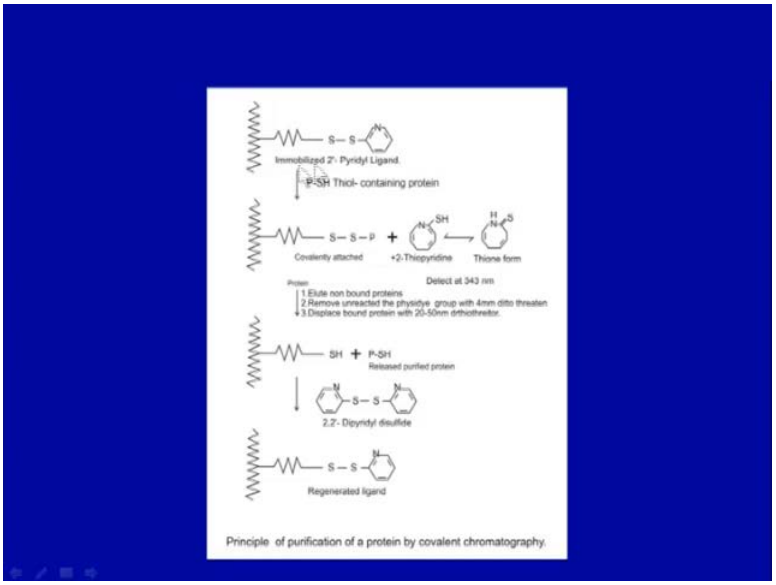

So, this could be utilized in this way, here particular figure shows particular thing that you have immobilized two prime paradol ligand, a proteins binds and then releases the thion form and then with another group it could be eluted. It is a very good technique for purification or a lot of different asset containing compounds. Apart from the above techniques lot of different metric ligand systems could be developed for purification.

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Like for example, protein a agarose could be utilized, it is derived from the cell wall of the bacterium, staphylococcusorius, it has a high affinity for the absy region of human hemoglobin G.

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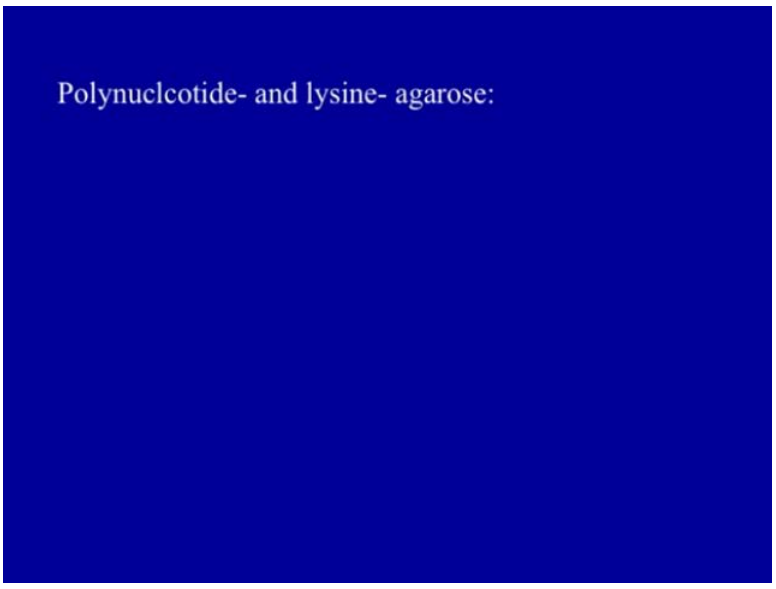

There could be poly nucleotide and lysine-agarose could be utilized where nucleic acid binding proteins can be purified.

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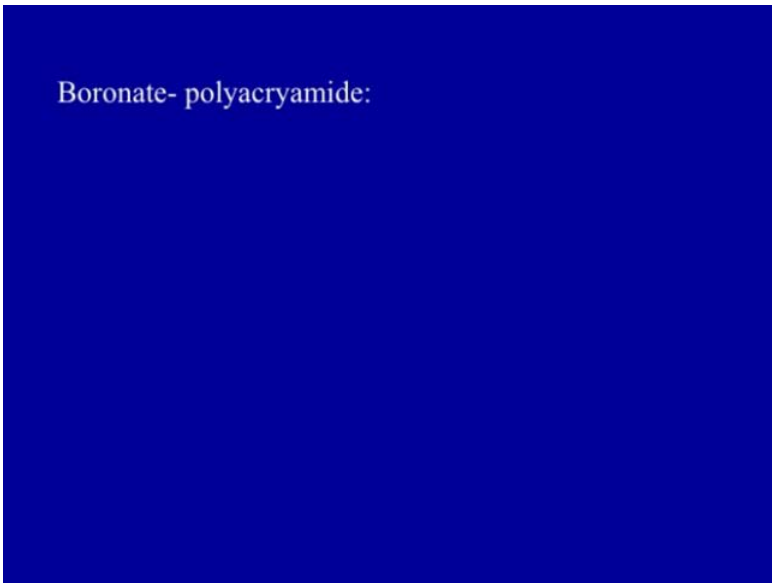

There could be boronate-polyacryamide which has high affinity for binding low molecular weight compounds with cyst-thiol groups. Like say like ribonucleiotides, sugars or other many coenzymes which can be utilized for purification.

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Heparin agarose system could be developed which prevents blood clotting and used to purifying bone colagenes, hepatitis b, serfice antigens, plasma antithromin three and several other like say hydrogen receptors and other molecules here.

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So, there could be lot of different other combination there is one acriflavine agarose, it is intercalated reversibly between the base spheres of d n a and also binds weakly to the for related hetrocycles, etceteras, for example, nucleotides. So, the binding is electrostatic in between the positive charge on the accreding ring and negatively charged phosphate group of nucleotide. Then it could be used to separate nucleotides, algonucleotides and etcetera. So, there as you see there are whole lots of different kinds of combinations or different types of affinity chromatographic methods could be developed.

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So, what we have seen is affinity chromatography can be used for lot of different applications for like say purify and concentrate a substance from mixture and depending on the specificity, reduce the amount of substance in a mixture. Then you can discern what biological components point to a particular substance that could be also determined. Then you can purify concentrate certain enzyme solutions and other kinds of analytes. So, affinity chromatography with different kinds of combination, different kinds of affinity chromatography methods could be very useful technique and highly selective, high capacity, one step purification technique which could be utilized.

So, this completes the section on affinity chromatography system. The one technique which is left to be discussed and we are going to discuss in the next lecture that it is gas liquid chromatography technique, which is a very useful technique of for certain substances which can be volitalized. The gas liquid chromatography has been used widely earlier by chemist's biochemists and for different by different applications; h p l c has kind of replaced it, but not completely. So, we are going to discuss in the next lecture about the gas liquid chromatography.

Thank you.