Genetic Engineering: Theory & Applications
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Module VIII - Isolation and Purification of Product (Part - II)
Lecture 26 - Affinity Chromatography (Part - II)

Hello everybody. This is Dr. Vishal Trivedi from Department of Biosciences and Bioengineering IIT, Guwahati and what we were discussing? We were discussing about the affinity chromatography. So far we have discussed about the principle as well as how the two counterparts which is receptor as well as the ligands are interacting with each other and taking part and this particular type of interaction is being exploited in different types of affinity chromatography which we have discussed whether it is a bioaffinity chromatography or the pseudo-affinity chromatography.

In both of these affinity chromatography the prime requirement of running this affinity chromatography is that you have to have a receptor and then you have to have a cognate ligand.

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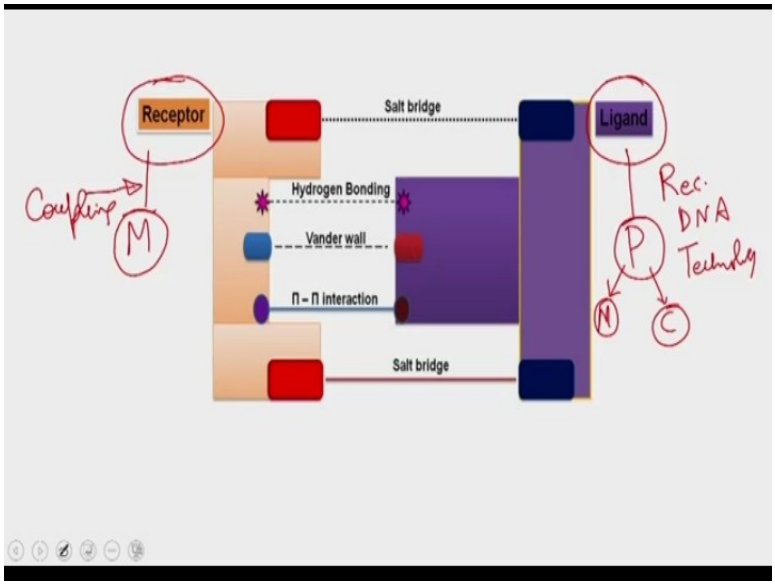

So let us see that you have a receptor. So you have the choices of either exploiting the receptor and couple it to the matrix or you can have the ligand couple it to the matrix. In that case you will express the receptor along with the protein of interest. Let us imagine that you are going to use the receptor coupled to the matrix. So which means you are going to couple the receptor to the matrix and in that case you are going to couple the ligand to the protein of your interest. Both of these events are required specialized operations. So in this case you are

going to use a recombinant DNA technology and you will couple the ligand to the protein of your interest.

It could be that you add this ligand onto the n terminus of the protein or to the c terminus of the protein. Similarly, you also have to generate the receptor. The receptor can be generated in a different way and then you have to do coupling reactions to couple this receptor to the matrix. So today we are going to discuss about how to generate the receptors and how to couple that receptor to the affinity chromatography so that you will prepare affinity column to purify the analyte of your choice and at the end of this lecture, we are also going to discuss how to operate the affinity chromatography columns.

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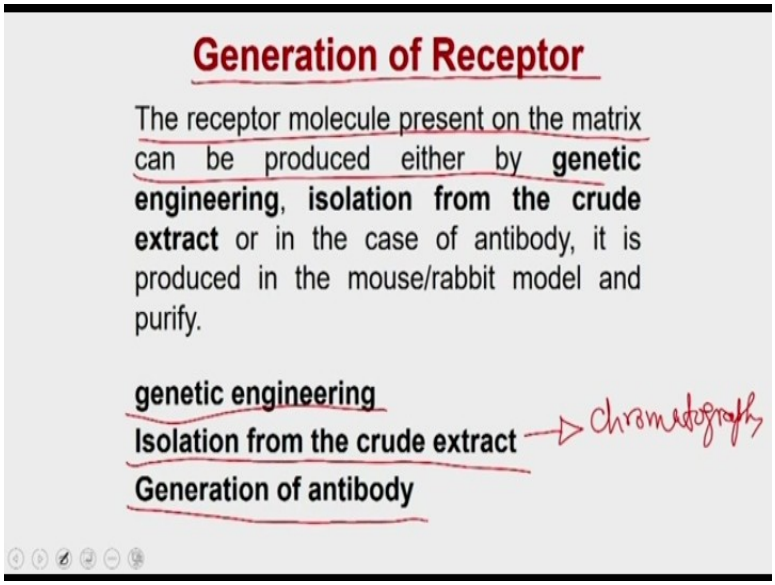

So as I just said, the prime requirement of affinity chromatography is that you should have the flexibility or you should have the ability to generate the receptor. The receptor molecule present on the matrix can be produced by 3 methods. One is called genetic engineering which means you can clone that particular receptor into a bacterial or eukaryotic expression system and then that will allow you to over excess this protein into the host and then you can purify that protein using the different chromatography technique what we have discussed and that is how you are going to have the receptor of your choice, which you are going to couple to the matrix.

The second option is that you are going to have that receptor into the crude extract and then you will use the chromatography techniques to purify. This is more relevant in those cases where you do not know about the receptor and you want to use that receptor to purify its

receptor. So in those cases, you actually know the effect of that particular factor, but you do not know what receptor is governing this particular type of phenomenon. In those cases you are going to use some SA system so that you will be able to evaluate the presence or the absence of that particular receptor into the crude mixture.

And that is how when you pass through the crude mixture through ion exchange chromatography or hydrophobic interaction chromatography or gel filtration chromatography, at the end of these chromatography techniques, you are going to get the different types of fractions and all these fractions you are going to test for the presence or the absence of this particular factor, which you are going to do by running a some kind of SA system and that actually is that SA based screening you have to perform to finally get the receptor into the purest form, which means you are going to get that particular receptor only. No impurities present.

The third option is that you are going to use the antibodies as a receptor. So in the today's lecture, since we already discuss about the genetic engineering approach we have also discussed about how you can use the different chromatography techniques to perform the purifications. Now we are going to discuss about how to generate the antibodies because antibodies can be used as a receptor for any antigen or any ligand because every ligand is can be treated as an antigen. So you can use an antibody to have the affinity for that particular ligand and that can be used instead of using a receptor.

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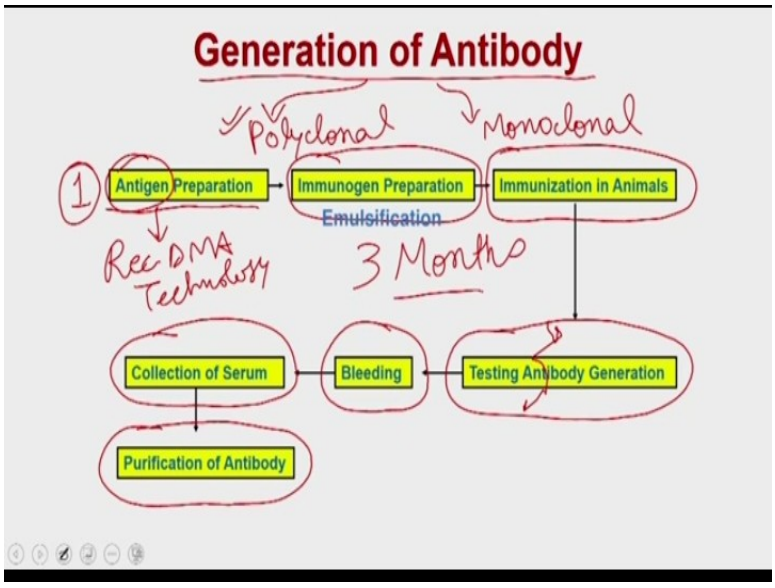

If you want to do an antibody generation, antibody generation is a multiple step process and antibodies can be generated by 2 different types. You have the antibodies which are the polyclonal and you can have the monoclonal antibodies. So we are not going to get into the details of what generation of the antibodies whether it is polyclonal or monoclonal because irrespective of whether you generate the polyclonal or monoclonal antibodies, ultimately you are going to use these antibodies to purify the ligand which is going to be the antigen in this particular case.

So what we are going to discuss, just to elaborate you or just to give you an experience how antibody is being generated in a system we are going to take an example of generating the polyclonal antibodies and the polyclonal antibodies can be generated in different types of animals, either you can use the rabbit or you can use the mice, you can use the goat and in sometimes you can also use the donkey as well. So what are the different steps?

In the step one because as I said, antibody is going to be generated against an antigen, the antigen is going to be the ligand what you are going to use to purify. So the first step is that you are going to produce or you are going to prepare the antigen. That antigen preparation also may require that you clone this particular antigen into an expression system using the recombinant DNA technology and using that technology you can actually produce the antigen in a large quantity.

Once you have produced the antigen then you are going to generate immunogen preparation which means you are going to convert an antigen into the immunogen. Once you have converted the antigen into the immunogen then you are going to use that immunogen to immunize the animals. Once you immunize the animals there is a certain steps in which first you are going to do a primary immunizations and after some time you are going to do a booster immunizations. Once your booster immunization is over then you will wait some more time and then you are going to test the presence of antibodies so that you are going to do by this that you are going to test the presence of antibodies in the particular animal and once you are sure that antibodies are being generated then you are going to bleed the animals and you can have the different sites through which you can actually withdraw the blood from that particular animal.

So once you are going to bleed the animal you are going to also prepare the serum from the blood and once you prepare the serum, you can actually purify the antibodies from that particular serum because the serum is going to have the collection of antibodies which animal

is producing for the defense against the infectious organism, which he may be encountering while you are doing the antibody generation. On the other hand it may also going to have the antibodies which you are generating against this particular antigen. So that is why the antibody generation is a multi-step process and it is a very very tedious as well as a long procedure.

On average the general antibody generation in a rabbit or a mouse approximately takes 3 months to prepare the antibody of a high titer and during these 3 months you have to keep the animal under the aseptic conditions so that you will be able to generate the antibodies against your antigen compared to the animal is going to generate the antibodies against the infectious organisms. So let us see the and discuss each and every individual steps in a more detail.

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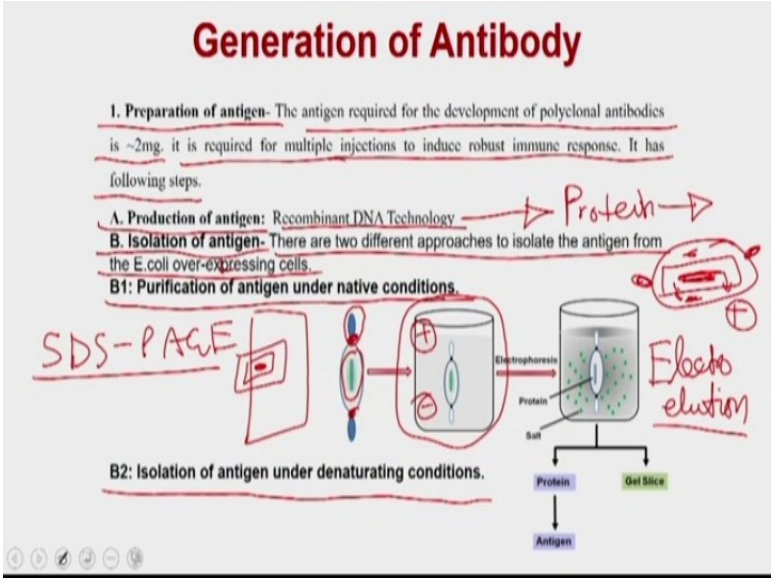

So first step is that you are going to prepare the antigen. The antigen which is required for the development of polyclonal antibodies is approximately 2 milligrams. See this depends on the the animal what you are going to use. The 2 milligram what I have given is the amount which you will need for a rabbit, but you can use the mice. Then the requirement may be lesser but consequently you are going to get the less amount of blood, so the amount of antibodies are also you are going to get. So the first question is the animal which you are going to choose and accordingly you have to prepare the antigen in the required quantity and the choice of animal which you are going to use for generating an antibody depends on many factors.

Number one, the source of antigen and the animal should be of distant relatives which means if suppose we are getting a antigen from the human then it is advisable that you use the some animal which is having the less homology to the humans.

For example, you cannot use the monkey for generating the antibodies against the human protein because in those cases this similarity between the protein which is present in the human versus the same protein which is present in the monkey may be very very close and in those cases the antibody response of the animal is going to be very less. In those cases we will use some distant animal, for example the rabbit or mice and the mice and rabbit maybe having the lesser homology compared to the monkey or chimpanzee.

The other option is what is the amount of antibody you required. For example rabbit will give you the antibodies in the range of MLs like 10 ml, 20 ml, 30ml. Whereas the mice will give you the antibodies in the range of 1 to 5 ml. Okay? That is the maximum what you get from the individual mice. Similarly, you will get large quantities of antibodies from the bigger animals like goat or donkey or like. So it depends what is the application or what is the downstream applications.

If the downstream application is only to purify some antigen then you can use the rabbit or mice but if it is downstream application is that you are going to use these antibodies for generating the some vaccine or suppose you are going to use these antibodies for passive immunizations or suppose you are going to use these antibodies for the large-scale vaccine production or diagnostic purposes, in those cases you are going to use the bigger animals like goat, donkey or in some cases we are also going to use the horse.

If you remember or if you might have this note that the most of the antibody which people develop against the cobra venom or some of the toxins that all are developed in a horse because the horse has a very high strength of withstanding that particular toxic chemical or toxic protein. The second thing is the horse is going to give you a large quantity of antibodies and most of these toxic and proteins the production of the antibody which you are going to use as a passive immunization or the protection against the cobra bites you require large quantity of these antibodies.

So it is required, the antigen is required for the multiple injection to induce the robust immune response and antigen preparation has following steps: Number one, you are going to produce the antigen so you are going to use either a recombinant DNA technology or you will

use the conventional chromatography techniques to purify the antigen of your interest. Then you are going to do isolation of antigens because end of recombinant DNA technology, it will give you the protein and that protein has to be isolated from the gel.

So there are 2 different approach what you have to use to isolate the antigen from the E.coli over-expressing cells. You can use the purification of antigen under the native conditions. In those cases you can use you can overexpress the protein in the E.coli. That will be going to overexpress and then you can use the ion exchange hydrophobic or gel filtration chromatography and that will give you the large quantity purified protein in a native conditions.

The other option is that you can do a isolation of antigen under denaturating conditions. You use these options when suppose you are overexpressing the protein in E.coli, but the protein is not present in the soluble fraction whereas the protein is present in the insoluble or the pellet fractions and in those cases you cannot use any of this conventional chromatography to purify the protein and in that case you will use the other approach that you will purify the antigen under the denaturating conditions.

So what you are going to do is you are going to run the protein onto the SDS page. So what will happen is the protein is going to run on the SDS page and on the gel you are going to have your protein of interest and once you have identified that this is my protein of interest what you are going to do, you are going to cut the gel and that gel you will put into the a dialysis bag which you are going to tie up from the both the ends and your gel is, so your protein is present inside the gel block and then what you are going to do is you are going to put this gel block into a horizontal gel operator and then you are going to do a electrophoresis.

As soon as you will do the electrophoresis what will happen is that the protein which is present in this particular block will start migrating towards the positive electrode because the protein what you have in the SDS page is going to be negatively charged. So it will start migrating towards the positive side. Because your protein is tied up in a dialysis bag the protein will migrate and eventually the protein will present into the dialysis bag because it cannot come out from the dialysis bag because the size of dialysis bag would be somewhere around 10 K da and your protein is probably going to be bigger than that.

So it will migrate, it will come out from the gel block and that is how it will going to come into your solution and you can collect the solution simply by precipitations and then you can use this particular protein for the as an antigen into the for the developing the antibodies and this whole process is called as the electro elution where you are using actually the electric field or the electrophoresis to elute the protein from the gel block.

Now once you have this protein, you can use this protein as an antigen for antibody production. Now once you have isolated or produced the antigen you have the second step is that you have to convert that antigen into the immunogen.

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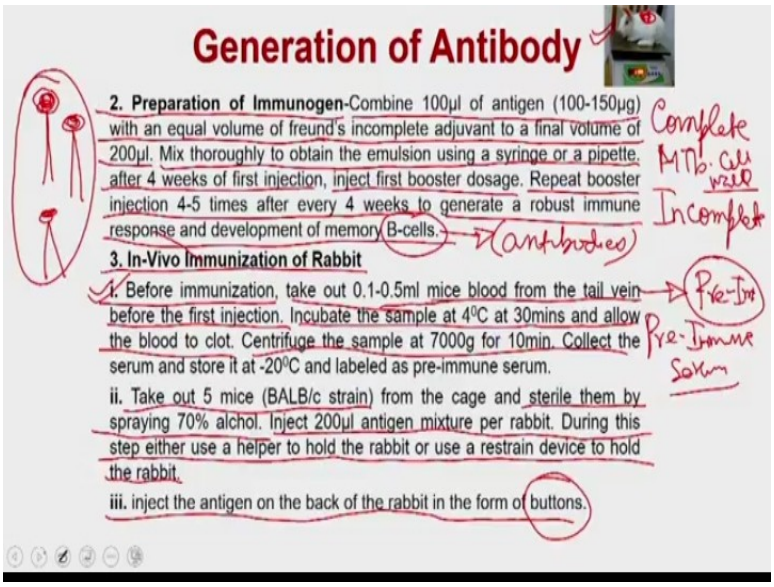

Preparation of immunogen. So for the preparation of immunogen, immunogen you are going to prepare and inject into the animal for antibody production in 2 subsequent steps. The primary injections and the secondary injections and this is almost as similar to when you are taking your babies to for vaccination. You might have noticed that we take the some of the vaccines; the doctor is giving the vaccines for on multiple occasions.

So what happen is that a single injection is not causing a robust immune response so that the cells will be present. The b-cell what you are going to produce will convert into the memory cell and that is why we will do the multiple injections and that is how some of the b-cell get converted or matured into the memory cells and that is how those memory cells will get into the spleen or memory cells get that memory gets stored into the system. And then once the new immunogen comes or the new antigen comes those memory cells start producing the antibodies.

We do the exactly the same but here the things are very different that you are going to do a primary injections and the secondary injection. The difference between a primary immunogen and a secondary immunogen is that the primary immunogen is being prepared in a complete adjuvants and the secondary injections or the booster injections are prepared in a incomplete adjuvants. So what you do is you take the 100 microliter of antigen, which is approximately going to be 100 to 150 microgram and then you add, mix it with the equal volume of freund's incomplete adjuvants or the complete adjuvant to a final volume of 200 microliter.

What is the difference between a complete adjuvant versus incomplete adjuvant is that the complete adjuvant actually contains the large quantity of MTB cell wall and because of that it actually activates the system so that the level of B-cell the level of the system is getting activated. So when you are injecting the microbacterium tuberculosis cell wall into the system you actually activate the system so that a large quantity of B-cell is present around that particular antigen and there is a small quantity of antigen which you are going to inject into the primary injections, but in a secondary injections this particular MTB cell wall is not present.

So in the secondary injections by the time the response for the micro bacterial cell wall is going to be go down and then you will inject the real antigen and that is how the response will be very very high because the amount of B cells which are being under the active state are going to produce large quantity of antibodies. Now once you mix it and make it 200 microliter you mix this thoroughly to obtain emulsion using a syringe or a pipette and then after 4 weeks of primary injections, so you will going to do a primary injection which you are going to prepare in a complete adjuvant, you will inject the first booster dose. So you repeat the booster doses 4 to 5 times every 4 weeks to generate a robust immune response and the development of memory B cells because these memory B cells are only going to give you the antibodies.

Now once you are done with this, then you have to do the immunization of the particular animal. In this case we have taken an example of rabbit. So you can see a rabbit in the picture. Before you start any experiments with any of these animals what you have to do is you have to take these animals, put it under the tidation stage where you are going to keep the animal in cage without any experiments and 1or 2 days you have to keep it in a cage so that the animal is getting habitual for the cage and it may not feel any kind of discomfort.

The second is you have to weigh the animal so that you will know the weight of this animal, so that you can decide what is the amount of antigen you can inject. Because if the animal is very big or if the animal is very small and if you inject the large quantity of this antigen, the antigen may give them the anaphylactic shocks and sometimes that happen that you are injecting an antigen and that actually is causing the death of these animals because they get a such a large immune response that they could not be able to sustain and as a shock to that particular immune response, they get, you know, they get very sick and at the end they just die because of the that particular immune response.

Now once you prepare with the immunogen, then you have to do the in-vivo immunization of the rabbit. Now before you start the immunization you take out at least 1 to 5 ml mice blood from the tailwind before your first injections because why it is required because so that you will know that what is the amount of antibodies are present into this animal and this is called as the pre-immune serum.

So before you start with the immunization you should keep the pre-immune serum because that pre-immune serum is going to be used as a comparison to see whether the antibodies are being produced in that particular animal or not. You incubate the sample at 4 degree and allow the clot to form and then centrifuge and prepare the serum. Okay? Now you take out the animal, okay? In the case of mice we use the BALB/c strain and you sterile them by the spraying 70 percent alcohol, so that does not mean that you are going to make them wet.

You are only going to sterile the area where you are going to inject the antigen so that it should not get any infection. Because suppose the animal is dirty or it has some kind of infection on its hair, that infection when you inject and cause some injury it actually that infection may get access to the body and that is how the animal may get an infection. So before you do that, you have to sterilize the area so that does not mean that you just take the animal and dip it into the alcohol which means the area where you are going to have the injection you just sterilize that area.

You might have seen many times when the doctors are going to withdraw the blood from your body they just first sterilize that particular area with the alcohol swab and then they only inject the needle into your body. Same, exactly the same what you have to do because we are going to inject this particular immunogen into the animal. So we have done the before immunization. We have the collected the pre-immune serum, then we have disinfect the

animal and then you are going to inject 200 microlitre in antigen mixture and you will inject into the rabbit and there are many places where you can inject into the rabbit.

The most preferred area is that you inject onto the backside of the rabbit. So the area where you can inject is on the backside of the rabbit so that it should not cause any discomfort. Many of the people or many times what people do is they just inject on to the foot paw. In those cases once they develop the abscess the mice or rabbit is having a very (difficult) very huge difficulty in terms of walking. Why the people inject into the food paw? Because in those cases the immune response is better, but in those cases the rabbit is having under more discomfort and that is why the I strongly recommend that we should inject the antigen on the backside of the rabbit.

So what you can do is you can remove some portion of the hairs, you sterilize and then you inject the rabbit in a small quantity. So 200 microlitre you do not have to inject at one place and you inject and makes the buttons onto its backside of this body and so during this injection process you use a helper to hold the rabbit or you can use a restraint device to hold the rabbit because the rabbit is going to have the pain because you are injecting a particular type of antigen and so that is why you need somebody to hold the rabbit. So while you holding the rabbit you should be very careful that you should not hold it very strongly, otherwise the rabbit may die or sometimes you can use the restrain devices.

The restrain devices are nothing but a small cage where you can just keep the rabbit so that rabbit would not be able to move and then you can inject the particular type of antigen. Mostly you inject the antigen on the backside of the rabbit in the form of a button, which means when you inject like suppose if you are injecting through a needle you inject and form a button like this and then you take out your needle, you inject again, make a rabbit button, then you inject again, make a back.

So suppose you have selected some area on the backside, so instead of injecting everything in one place what you do is you just spread that particular volume onto a larger area so that the antigen absorption from that particular area should be quick and that should cause the better immune response and better antibody production.

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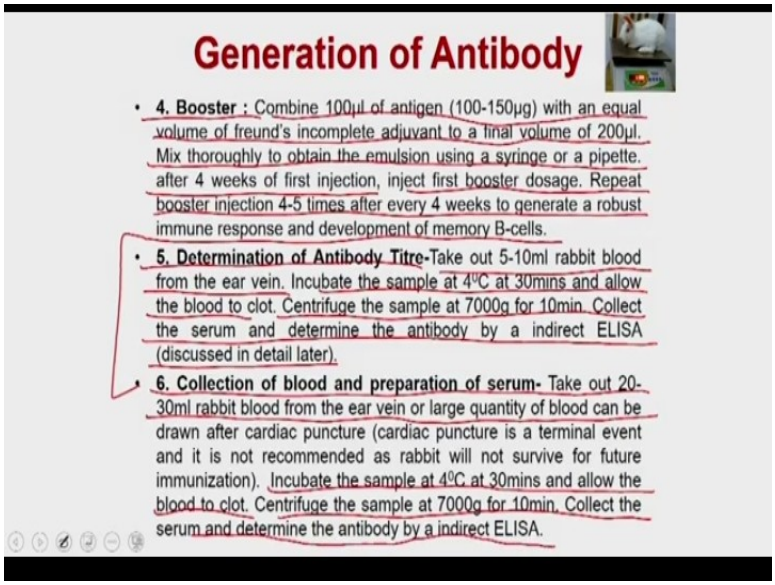

Then once you are done with the primary injection, you are going to do a booster injection. In the booster injections what you do is you combine the 100 microliter of antigen, which is 100 to 150 microgram with an equal volume of freund's incomplete adjuvant to a final volume of 200 microliter mixed thoroughly to obtain the emulsion using a syringe or pipettes. After 4 weeks of first injection inject first booster dose, repeat the booster doses 4 to 5 times and that will cause a robust immune response and development of memory B cells.

Now once you have sure that there are sufficient time is being passed, you have given the sufficient number of booster doses then you can just take out small amount of blood and you can test the presence of antibodies. So then you will determine the antibody titre. You take out a 5 to 10 ml rabbit blood from the ear vein.

So I do not that you have ever noticed a rabbit. A rabbit has a very thick vein onto the backside of the ear and that vein can be used to draw the blood from the rabbit without causing any discomfort or without causing any problem. You incubate the sample at 4 degree and allow the blood to clot, then once a blood is clot you centrifuge and you collect the serum and determine the antibody by indirect ELISA. That we can discuss or once if you do not understand then you can actually discuss with me while I will when I will be available in person or you can go through some of the YouTube videos and you will know that how to perform the indirect ELISA.

Once you are sure that the antibody is being generated then you can do the collections of blood. You take out 20 to 30 ML, even 50 ML blood from the ear vein and can be collected.

If you require a very large quantity of blood, then you can also collect the blood from the cardiac rupture but in those cases it will be a terminal treatment and the cardiac, if you do a cardiac rupture, which means you are going to rupture the heart and take out the blood, then in those cases it is going to be a terminal procedure and the animal may not be survived afterwards and it may not be available for another round of booster doses and to for antibody production.

So that is why for the larger animals like rabbit or goat or sheep, people are not doing the cardiac puncture, but for smaller animals like mice people are doing the cardiac rupture and that is how they are collecting as much as blood possible from the animal. Incubate the samples at 4 degree and allow the blood to clot, centrifuge the sample and that is how you are going to get the large quantity of serum. Again, you can determine the level of antibodies using the indirect ELISA.

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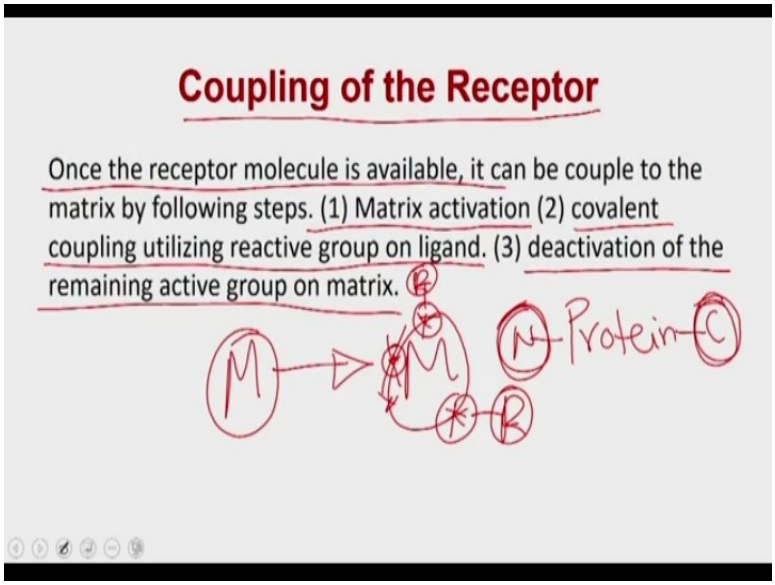

So now once the antibodies is being generated, you can couple these antibodies or couple the other receptor what you have generated through recombinant DNA technology or through the following the conventional purification system. You have to couple these particular receptors to the matrix so that the your column is ready for purifications. In these coupling reactions coupling reaction is being done in a 3 different steps.

First you are going to do a matrix activations, then you will do a covalent coupling utilizing reactive groups on the ligands and then third you are going to the deactivating the remaining active groups which are present on the matrix, which means in the step 1, you are going to

generate or you are going to activate the matrix so that the matrix is going to be having many of the active groups and in that step, you are going to add the ligand so that all these active groups are going to make the bonds with the covalent bond with the ligand or the receptor and then once you are done with that suppose you have additional activation group then you are going to destroy this activation group or you are going to deactivate these activation groups.

In most of the cases or whether you have generated a receptor through recombinant DNA technology or through conventional chromatography or suppose you have generated the antibodies, most of the time the receptors are of proteinaceous in nature. So in the protein like you have 2 different sites for the coupling which means you have 2 functional groups. One is the n-terminus amino group and on the c-terminus you have the carboxyl group, which means you have the NH4 plus as a functional group which you can use for coupling or you can have the CO minus carboxyl group which you can use.

So that is why once the matrix is activated people are activating those functional groups, which are either being directed towards the amino groups or which are directed toward the carboxyl group and these are the only 2 options exist to couple your receptor to the matrix. So, let us see, we have 3 different types of coupling reaction, which people can do to couple the ligands with the matrix.

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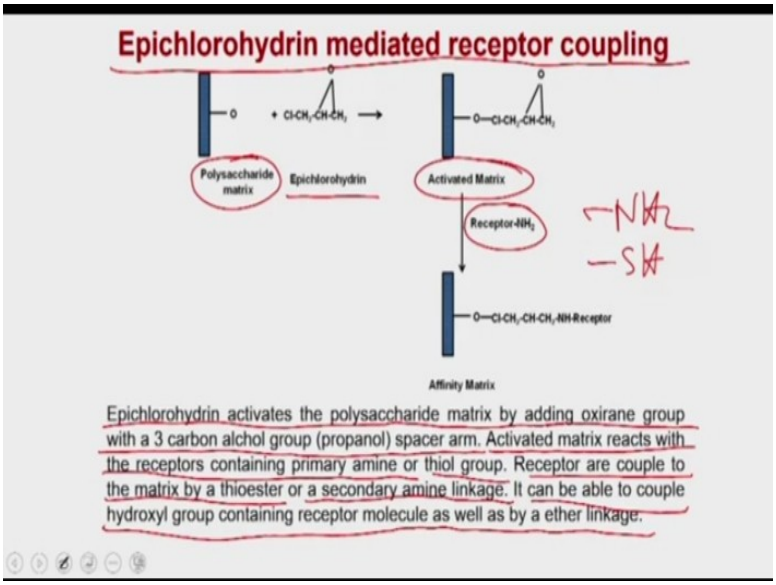

The first is epichlorohydrin mediated receptor coupling. So in the epichlorohydrin mediated receptor coupling actually is more suitable for the polysaccharide matrix and what will

happen is that epichlorohydrin activates the polysaccharide matrix by adding oxirane group with a 3 carbon alcohol group spacer arm and so that is what the activated matrix is being prepared once you incubate the polysaccharide matrix with the epichlorohydrin.

And activated matrix reacts with the receptor containing primary amine or the thiol groups. So apart from the NH2 you can also have the thiol groups which can also react with the activated matrix and once your and then at the end what will happen is the receptors are coupled to the matrix by a thioester or a secondary amine linkage, it can be able to couple hydroxyl group-containing receptor molecule as well by a ether linkage.

So when you are using the epichlorohydrin as an activating agent, you are actually activating mostly the polysaccharide matrix and that will allow the addition of the primary amine containing proteins or the thiol groups containing proteins, or you can sometimes use the hydroxyl group containing proteins as well for linkage.

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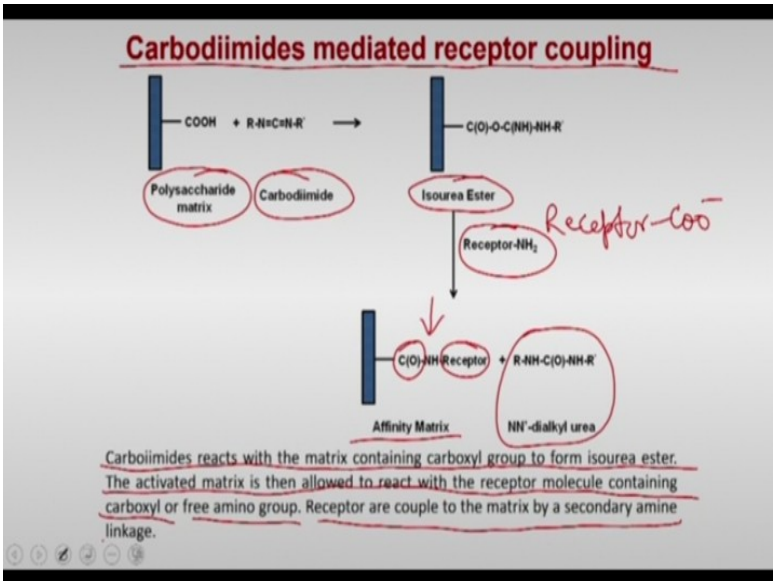

The second is the carbodiimide mediated receptor couplings. In a carbodiimide reagents are reacting with the matrix containing carboxyl group to form the isourea ester. The activated matrix is then allowed to react with the receptor molecule containing carboxyl or free amino group. So when you have the polysaccharide matrix it reacts with the carbodiimides group and as a result, it will form the isourea ester.

This isourea ester then reacts with the receptor containing primary amine or it can also react with the receptor containing carboxyl group and both are depending on and then you are actually going to have the coupling of the protein or the receptor to the matrix and that is how

your affinity matrix is ready and this is going to be the by product which is going to be released. Receptors are coupled to the matrix by a secondary amine linkage.

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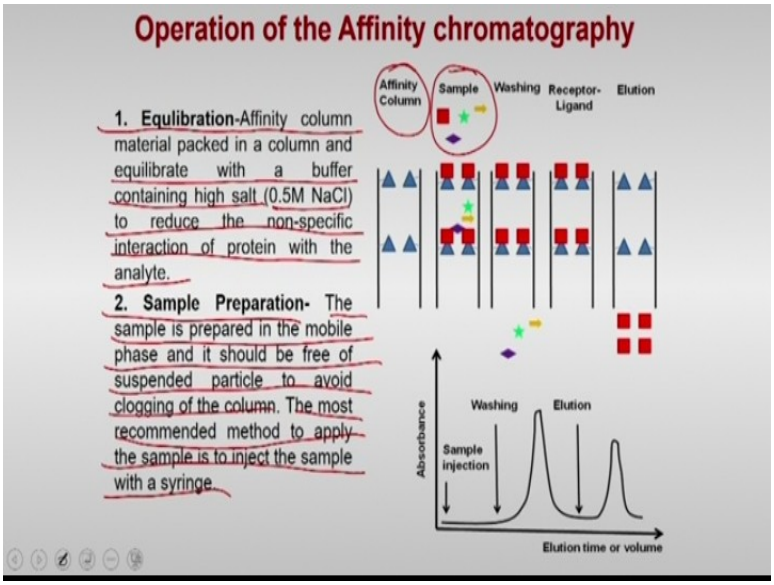

Now, let us see how to operate the affinity chromatography columns. So in affinity chromatography column operations mostly you have the 3 or 4 multiple steps. These are the general steps which we are going to discuss. In a subsequent lecture we are also going to discuss about the specific steps which you are going to do. So in generalized reactions how you are going to operate the affinity chromatography is that first step you are going to do the equilibration of the column. So once you have prepared affinity chromatography, you are going to pack the column into a tube and then you are going to equilibrate that with a buffer containing high salt.

So this is remember that the affinity is very very exclusive. So it is ligand versus receptor. So that is why if you keep a high salt the high enough so that you will disrupt the non-specific interaction but it should not be that high that it is going to disrupt the interaction between the ligand and protein or ligand and receptors. So you keep the high salt so that you will destroy the nonspecific interaction so that protein will not other protein will not react with the matrix, it will also react with only with the receptor which is present on the matrix.

So you will keep mostly 0.5 ML, 0.5 molar NaCl to reduce the nonspecific interaction of protein with the analyte. Now you prepare the sample. The sample is prepared in the mobile phase and it should be free of suspended particle to avoid clogging of the column. The most recommended method to apply the sample is to inject the sample with the syringe. So you

have prepared the affinity column. Now, you have prepared a sample. Now, you can apply the sample.

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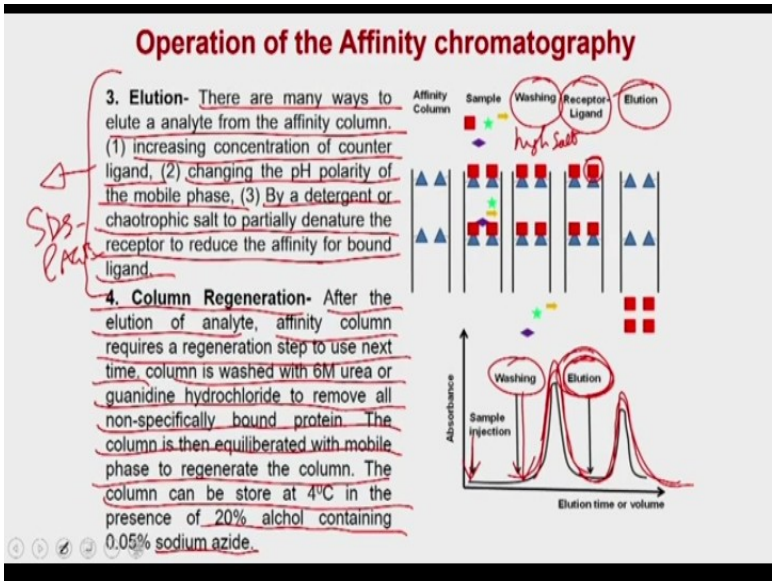

After the applying the sample you are going to have a washing step which means you can wash these columns with a very very high buffer or you can have the buffer which contains very high salt so that it should not break the interaction between the receptor and ligand but it is good enough to wash away all the protein which are being non-specifically adhered to the matrix. The third is, so once you have done that the receptor and ligand complexes are going to form and they are the only thing that will be present. Then you can do elution step. In the elution step you have the many ways to elute the analyte from the affinity column.

You can increase the concentration of the counter ligand which means the ligand which is you are using and the protein which is being overexpressed along with that particular ligand you can use that particular ligand alone in an increased quantity.

So what will happen is that ligand will compete with the ligand which is bound to the matrix through receptor and as a result because you will increase the more and more amount of ligand the receptor will prefer to bind this free ligand instead of the ligand which is bound to your protein and as a result the protein is going to be released from the sample. You can change the pH or the polarity of the mobile phase. As you remember, the receptor ligand interaction is being controlled by the electrostatic interactions, vander waal, hydrogen bonding and salt bridge interactions. All these interactions depends on the ionization of the different groups which are present on the receptor as well as the ligand.

So if you change the pH or if you change the polarity of your elution buffer, you could be able to modulate these interactions and as a result, the receptor may have the lesser affinity for the ligand and that is how it will release the ligand. At the end if any of these methods are not good enough to give you the desired, you can use a detergent or the chaotrophic salt to particularly denature the receptor to reduce the affinity of the bound ligand. One of the so if you remember we have also discussed that the three-dimensional confirmation of the receptor as well as the ligand is very very important for them to form the electrostatic, hydrophobic as well as the other kind of interactions.

So if you destroy the three-dimensional interaction or three-dimensional structure of the receptor by applying detergent or the chaotropic salt. Chaotrophic salt means either you use the urea or you use the guanidinium hydrochloride, you will going to destroy that three-dimensional structure of these receptors and as a result once the structure is collapsed for the receptor it is going to release the ligand and along with the ligand your protein of interest is also going to be eluted from the column.

So now once you have eluted the protein you can actually check the protein purification onto the SDS page and after that you have to do a column regeneration. So after the elution of analyte the affinity chromatography requires a regeneration steps to use it for the next time. Column is washed with 6 molar urea or guanidinium hydrochloride to remove the all non-specifically bound proteins. The column is then equiliberated with the mobile phase to regenerate the column. In case you are not interested to use it again next day or in the same week you can store the column in 4 degree in the presence of 20 percent alcohol containing the 0.5 percent sodium azide.

Now if you follow this particular purification using a detector system what you will see is that you have injected the samples and then you have done a washing step and after that you are going to see the elution of your protein in a different concentration of ligand and what will happen is once you do the washing you are going to see a nonspecific protein coming out from the column. After that once you are sure that all the protein, all non specific protein is being eluted then you are going to put the elution buffer and then you will see a single peak of the protein is coming out from the column.

So with this we have discussed about the ion exchange chromatography, hydrophobic interaction chromatography and the gel filtration chromatography and for the affinity chromatography we have discussed about what are the different factors which are governing

the interaction between the receptor and ligand and how you can generate the receptors and how you can couple these receptors to the matrix and now in the subsequent lecture, we are going to take up the specific examples of affinity chromatography and how you can take those particular receptor ligand combinations to perform the verification of your protein of interest.

So with this I would like to conclude our lecture here. In a subsequent lecture we are going to take up a few of the affinity chromatography riveted examples and how you can use them to purify the protein of your interest from the cells which are overexpressing your protein. Thank you.