Enzyme Science and Technology
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Module - V
Enzyme Production (Part 3: Purification)
Lecture - 27
Chromatography (Part-III)

Hello everyone, this is Dr. Vishal Trivedi from Department of Biosciences and Bioengineering, IIT Guwahati. And, what we were discussing? We were discussing about the different properties of the enzyme in the course Enzyme Science and Technology. In this particular module, we are discussing about how you can be able to purify the protein from the you know cells which are over expressing the particular clone right.

And, what we said is that protein is providing you the different types of options, you can be able to use the charge hydrophobicity or the surface area as a criteria to purify the protein. And in the previous lecture, we have also discussed about the basic principle of the affinity chromatography and how you can be able to use that for purification of the proteins.

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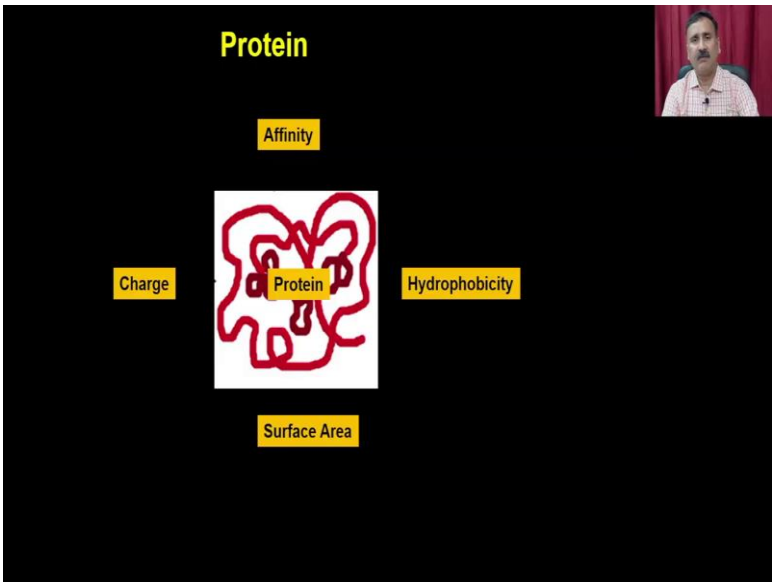

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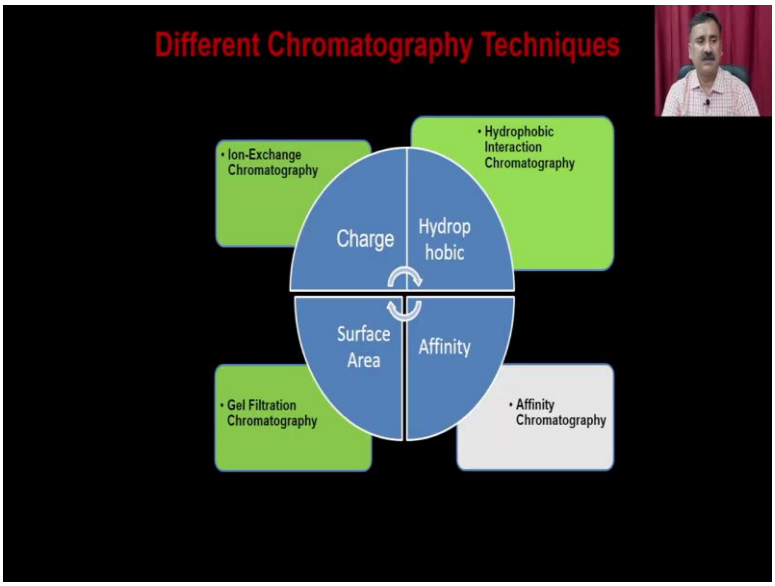

If you recall what we have discussed, we have discussed about that the affinity chromatography relies on the interaction between the receptor and the ligand.

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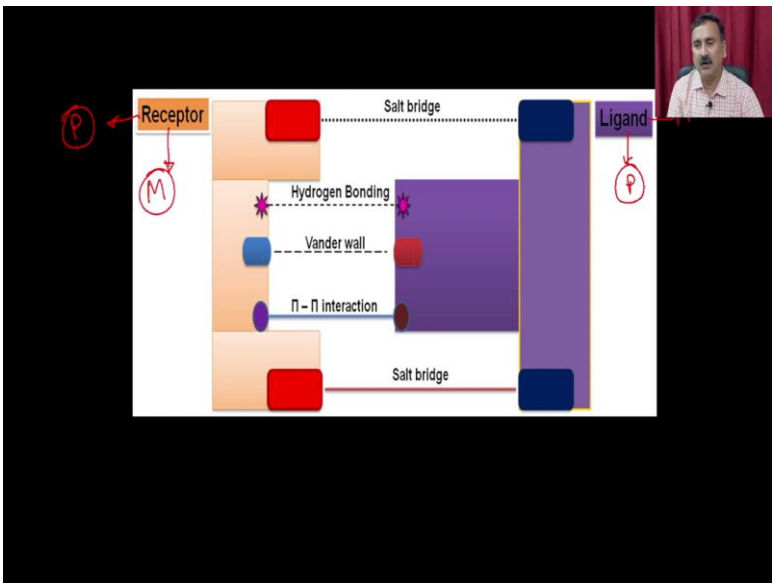

And you have the option of either putting the receptor along with the protein or the ligand with the protein and that is how you can be able to use them for the purification of the particular protein. So, in a typical affinity chromatography, what you have is you have the receptor and the ligand which are you know exclusively been interacting with

each other. And you have the option that you can actually be connect the receptor to the matrix, right or you can actually be able to connect the receptor to the protein.

So, either of those cases the ligand has to be placed on the counterpart. For example, if you are putting a receptor onto the matrix, then the ligand has to be placed onto the protein of your interest with the help of the recombinant DNA technology. If the you are putting the receptor onto the protein, then you are have to put the ligand onto the matrix. So, we have couple of examples which we are going to discuss in this particular lecture.

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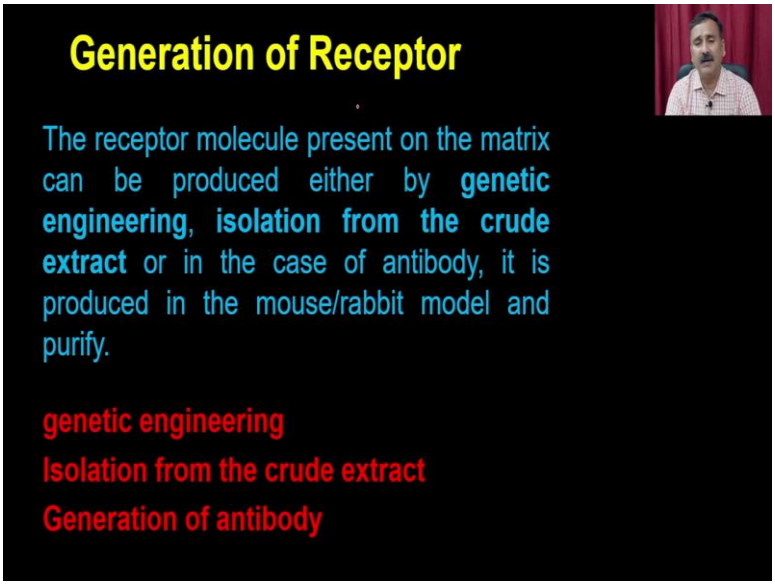

So, first thing what we require to perform the affinity chromatography is that you should have the receptor or the ligand. The receptor molecule present on the matrix can be produced either by the genetic engineering or the isolation from the crude extract or in the case of antibody, it is produced in the mouse or rabbit models.

So, you have the three options. You can actually be able to purify the receptor either with the genetic engineering or you can isolate the receptor from a crude extract or you can actually be able to generate the antibodies. How you are going to generate the antibodies?

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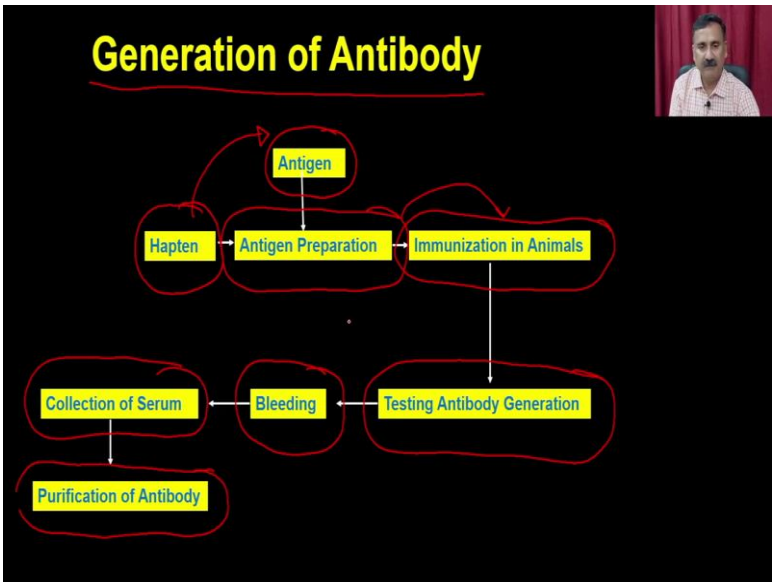

So, for in generation of the antibodies what you have to do is you have to first prepare the antigens. If you are you know dealing with a protein which or if you are dealing with a molecule which is very small and it is coming under the category of hapten, then you have to convert the hapten into an antigen.

After that you have to prepare the antigen for the injections and after that you have to do the immunization to the animal, you have to inject the animals right. And, then once the animal are actually going to produce, the antibody you have to test the vessels of antibodies in the animal.

And, once you see that the antibody is in a very high quantity, then you can be able to extract out the blood and that blood is going to contain the antibodies. And, from the blood you are actually going to collect the serum and the serum is going to be and then you can be able to purify the antibodies from the serum. So, let us see and discuss some of these steps individually.

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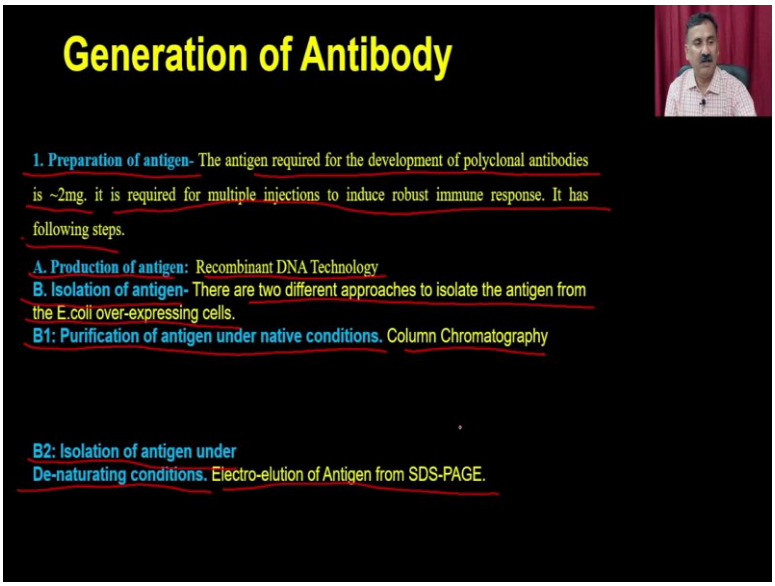

So, the preparation of the antigen. So, the antigen required for the development of polyclonal antibody is approximately 2 milligrams. It is required for the multiple injection to induce the boost immune response. So, it has a following steps. First you are going to produce the antigens.

So, you can actually be able to use the recombinant DNA technology to clone the antigen of your interest and then you can be able to over-express that in a large quantities. Then you can do the isolation of the antigen. There are two approaches to isolate the antigen from the E. coli over-expressing cells.

Purification of antigen under the native conditions. So, you can use the column chromatography. You can use the ion exchange chromatography or any other affinity chromatography to purify the antigen or you can actually be use the antigen under the de-naturating conditions. So, you can do the electro elution of antigens from the SDS-PAGE.

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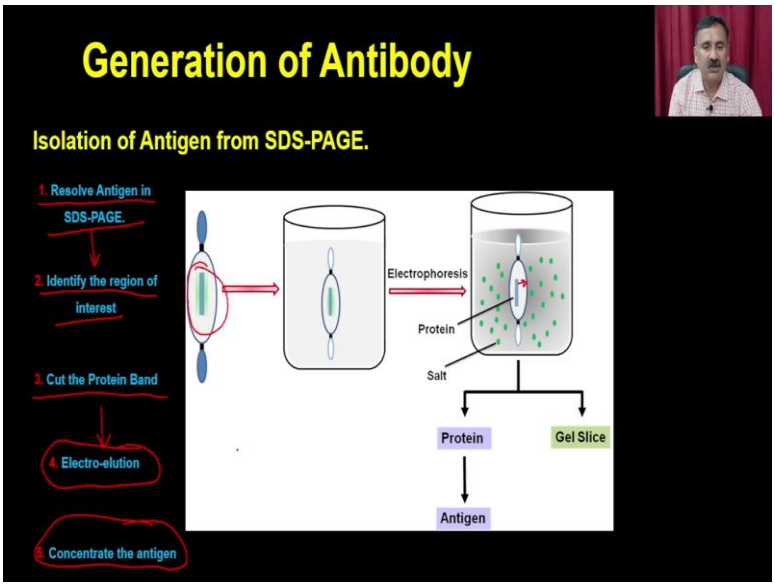

These are the steps what you have to take to isolate the antigen from the SDS-PAGE. So, first what you are going to do is you are going to run the antigen on the SDS-PAGE followed by you have to identify the region of interest site. So, you are going to identify the region of interest.

Then, you are going to cut the protein bands and you are putting into a dialysis bag and this dialysis bag you are going to keep into a horizontal apparatus. And, then you are going to run the you are going to perform the electrophoresis. And, as a result what will happen is that the protein band is going to be electro-eluted from this from this eclamide block and it will come into the dialysis bag.

And, then you can concentrate the antigen and you when you use the antigen for the injections into the animal. But you cannot inject the antigen as such, you have to prepare the antigen for the injections.

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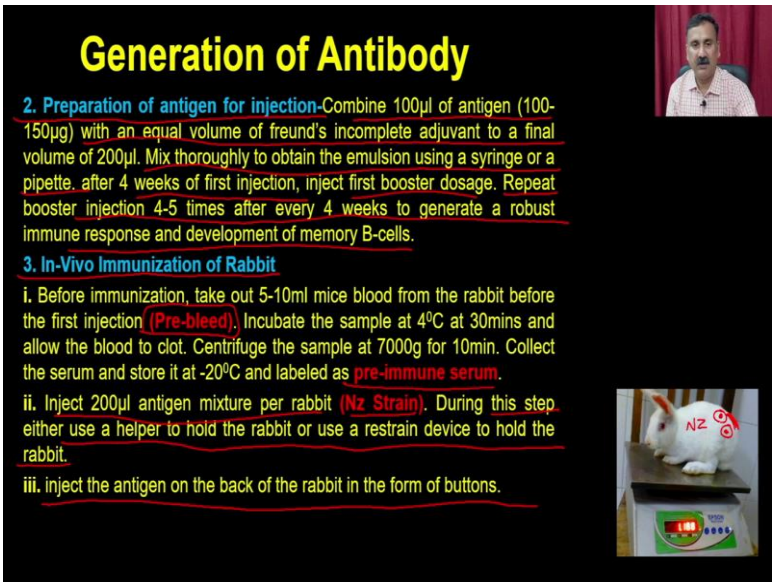

So, preparation of the antigen for the injection, you have to combine 100 microlitre of antigen which is approximately around 100 to 150 microgram with an equal amount of fluids freund's incomplete adjuvant to a final volume of 200 microlitre. You mix thoroughly to obtain the emulsion using a syringe or a pipette, after 4 weeks of injection, inject the second booster dose. Repeat the booster injections 4 to 5 times every 4 weeks to generate a robust immune response and development of the memory b cells.

Then, you once an antigen is ready then you can do the in vivo immunization on the rabbit. So, before immunization, you can just check out some amount of blood so, that it is actually going to serve as a control and it is called as pre bleed or the pre immune serum. And, then you can inject the 200 microlitre antigen into the rabbit which is going to be the Nz strain.

So, this is the rabbit, what you have this is the Nz strain rabbit. And, during this step either use a helper to hold a rabbit or use a restrained device to hold a rabbit. Inject the antigen onto the back of in the rabbit in the form of the button. So, you are actually going to inject the rabbit somewhere here in the form of the buttons. And, these buttons are going to release the antigen in a very very slow mode.

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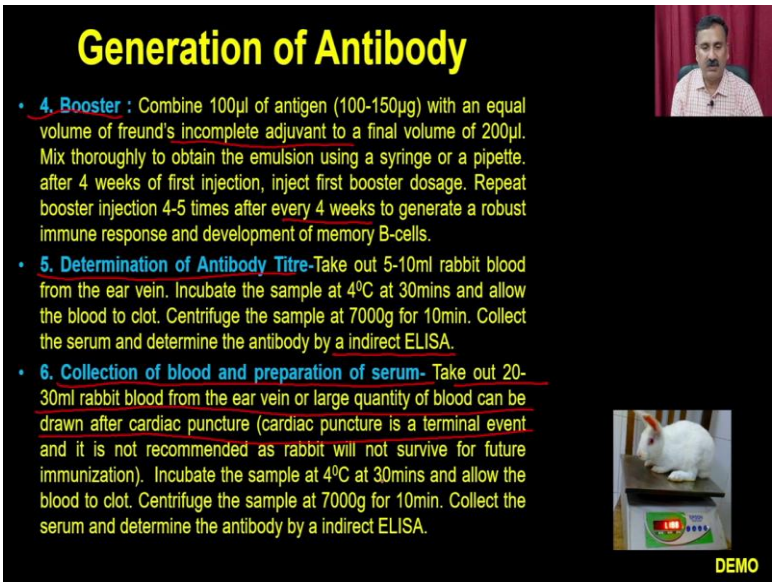

Then you are going to do the booster. So, exactly the same way you are going to prepare the antigen, except that its here you are going to use the incomplete adjuvants and that is why you are going to put the boosters. Once you do the booster, you have to wait for the 4 weeks and that is how it is actually going to have the a robust immune response and it is actually going to produce large quantity of antibodies.

Then, you can determine the antibody titres. So, you can use the indirect ELISA to determine the antibody titres. And, then once you see that the antibody amount is very high, you can collect the blood and prepare the serum. So, take out the 20 to 30 ml of blood from the ear vein or large quantity of blood can be drawn after the cardiac puncture.

And, you can actually be able to collect the blood and then you can actually be able to prepare the you know the serum and you can actually be determine the concentration of the antibody by the indirect ELISA.

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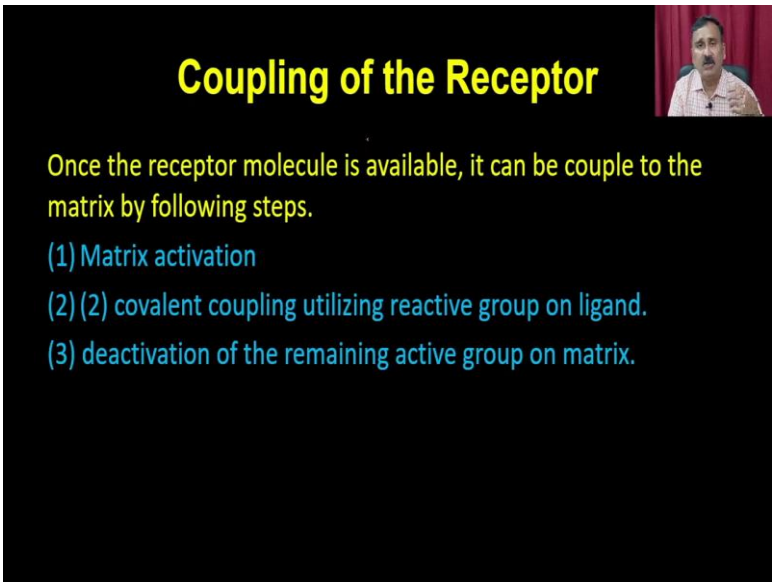

Then, how you are going to couple the receptor to the matrix right? So, once the receptor is been available, it can be coupled to the matrix by the following steps. In the step 1, you are going to do the matrix activation. In the step 2, you are going to do the covalent coupling and in the step 3, you are going to do deactivate the remaining active groups onto the matrix.

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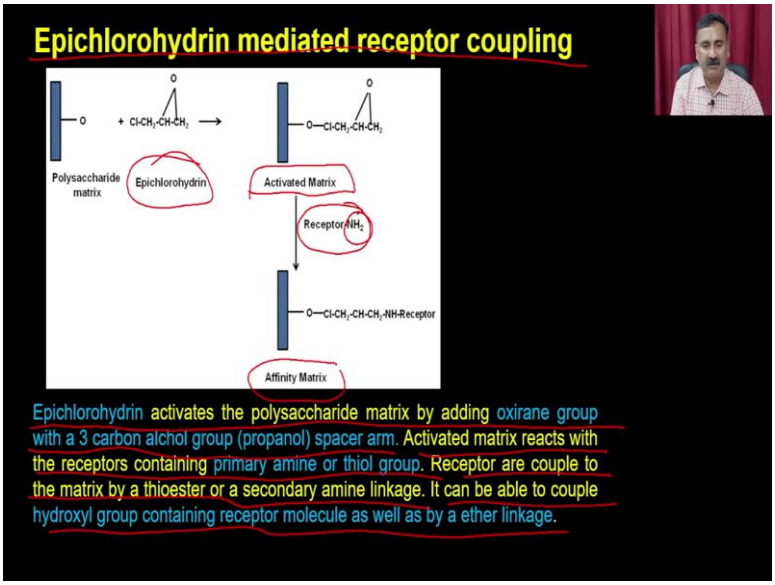

So, you can have the multiple options, you can use the epichlorohydrin mediated receptor coupling. So, in the epichlorohydrin activates the polysaccharide matrix by

adding the oxirane groups with 3 carbon alcohol groups is spacer arm. The activated matrix reacts with the receptor containing primary amine or thiol groups. Receptors are coupled to the matrix via thioester or secondary amine linkage.

It can be able to couple the hydroxyl group containing receptor molecule as well as by the ether group. So, in a polysaccharide matrix when you added the epichlorohydrin, it is going to form the activated matrix. And, on this activated matrix when you are going to add the receptor which is going to have the free amino group, it is going to you know get tagged and that is how you are going to have the affinity matrix.

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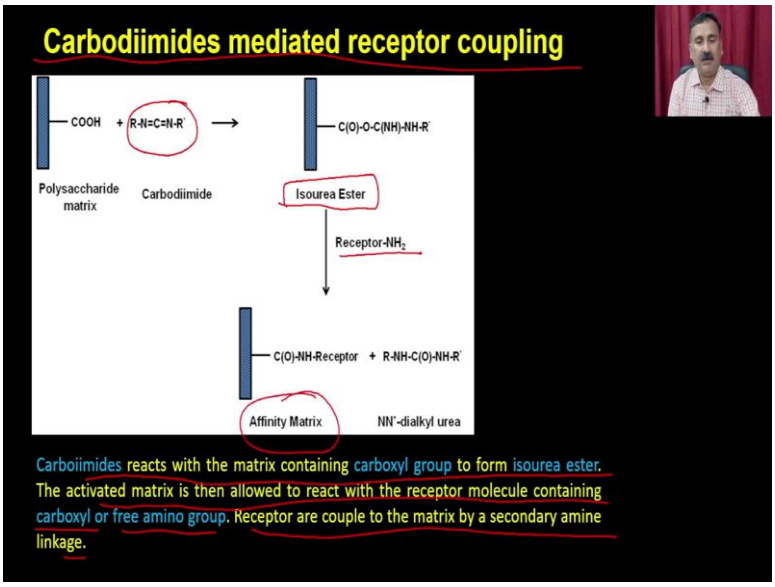

Then, you can also use the carbodiimides mediated receptor coupling. So, in the carbodiimides receptor coupling, carbodiimides reacts with the receptor containing carboxyl group to form the isourea ester. And, the activated matrix and then allow to react with the receptor molecule containing the carboxyl or the free amino group. The receptors are coupled to the matrix by a secondary amine linkage.

So, this is what its happened right carbodiimide when you react with the carboxyl group, it is going to form the isourea esters. And, when it reacts with the receptor, it is actually going to form the affinity matrix.

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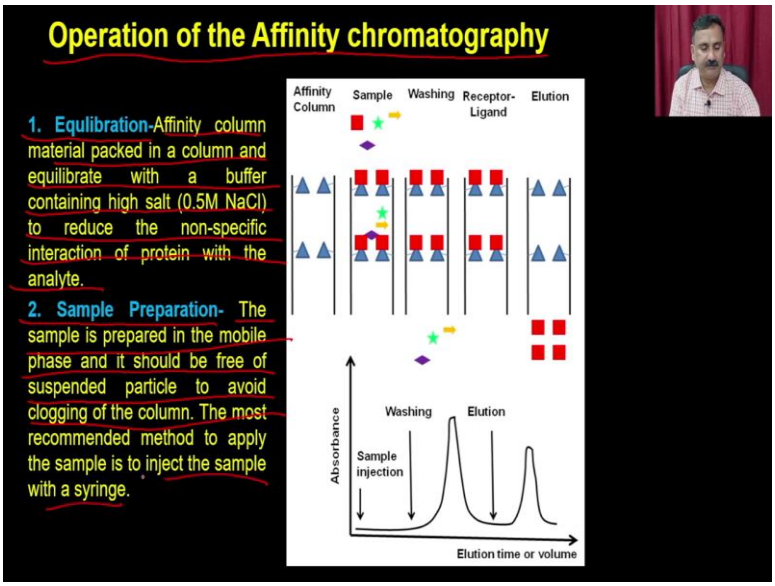

Once you have the affinity matrix, you can actually be able to do the affinity chromatography. So, in affinity chromatography what you have is you have the equilibrium. So, affinity chromatography material is a packed in and equilibrate with a buffer containing high salt to reduce the non-specific interaction of the protein with the analyte and you can have the sample preparation.

The sample is prepared on the mobile phase and it should be the free or suspended particle to remove the clogging of the column and so on. And, then you inject a sample with a syringe.

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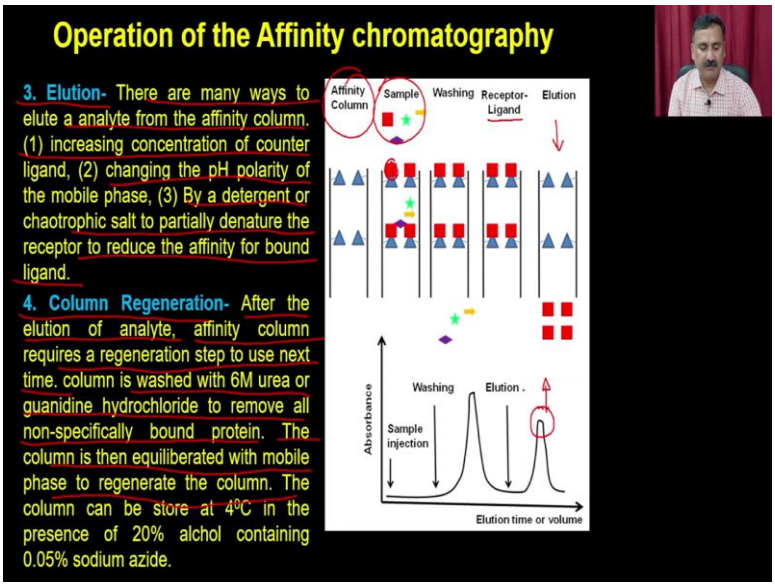

In the elution, the elution there are many ways to elute a analyte from the affinity chromatography. You can increase the consideration of the counter hands or you can change the pH of polarity of the mobile phase or by a detergent or the chaotrophic salt to partially denature receptor to reduce affinity for a bound ligand. And, once you are done with the elution, you have to also do the column regenerations.

So, after the elution of a analyte, you can affinity chromatography require a regeneration step to use it for the second time, column is washed with the 6 molar urea or guanidine hydrochloride to remove the all non-specifically bound proteins. The column is then equilibrated with a mobile phase to generate the regenerate the column. The column can be stored at 4 degree in the presence of 20 percent alcohol.

So, this is what you have the affinity column where you have the you know antibody or the receptor tagged. So, when you add the samples, the receptor is going to have the affinity only for the 1 molecule right, one of the ligand. So, it will go and bind to this ligand right and the rest of the molecule are going to be washed away in the washing step.

And, then you are going to have the receptor ligand onto the column and then you are going to do the elution. So, you are going to have the different options of the elution and that is how you are going to monitor this. So, what you see is that the molecules are getting eluted in the elution step.

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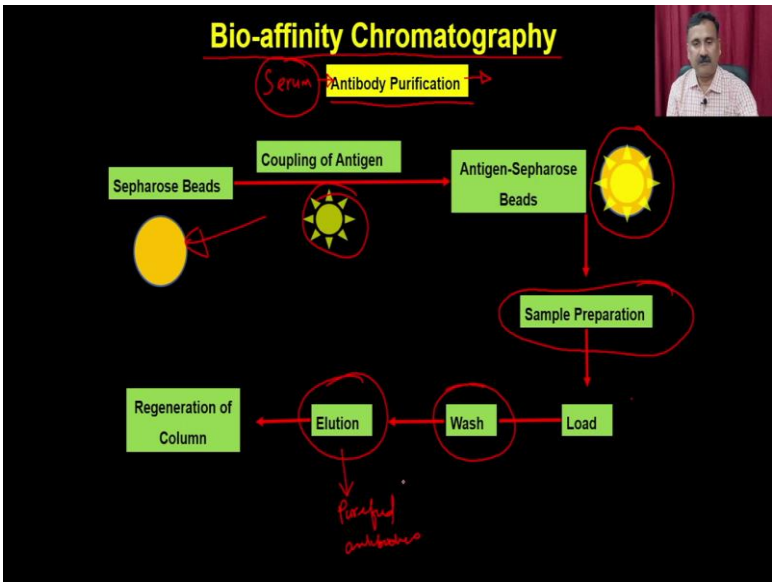

So, bio- affinity chromatography is a step where you are actually going to do the affinity chromatography its ok. So, in a if you want to do a bio- affinity chromatography, you also going to have the you should you know, you can use that for the even for the antibody purification as well. I am sure you we have seen how we can be able to produce the antibody in the serum. So, if you see all those procedures are very very complicated.

So, to just to explain you those procedure, how you can be able to produce the antibody and the antibody the crude antibody is going to be present in the serum, we have prepared a small demo clip. And, that demo clip is actually going to explain you the all the steps what we have just discussed; how you can be able to prepare the antigen, how you can inject that into the animals and so on.

I am Amogh Anant Sahasrabuddhe, I work in CSIR-CDRI, Lucknow. And, in today's demo we will be discussing different steps involved in generation of antibodies in rabbits.

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So, further first step, we require several things like freund's complete adjuvant, here it is from sigma. We need a micro emulsifying needle which has two openings connected with a bind needle. We need antigen which is purified and filtered.

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So, they are no contaminations, it is crystallized solution of antigen. Then, we take out some of the freund's complete adjuvant in Eppendorf and then mix them together. Since, one since the this adjuvant is oil based, it does not mix easily with the watery system like the antigen is in previous. So, therefore, we mix them, rigorously, vigorously and forcefully.

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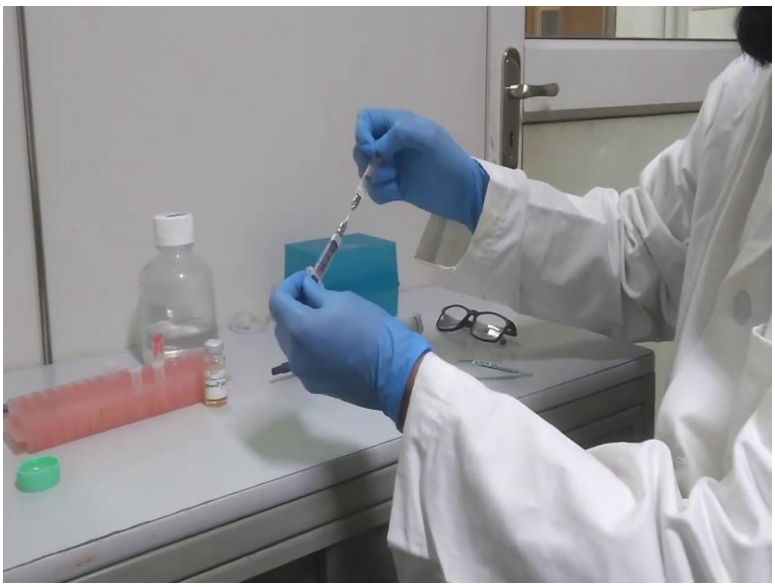

For that purpose, it is these two we mix these emulsion and the we mix these PVS, I mean PVS containing antigen and the adjuvant, oil based adjuvant. After mixing we can

we take out in a needle, using a needle we take out in a syringe like this and then we fix the micro emulsifying needles into it. Attach another syringe into it like this.

So, once you once you have filled your antigen and the adjuvant in this needle, you push it here and then you keep pushing from one side, keep pulling from another side, keep pushing from one side and keep pulling from another side. So, this process forcefully pushes your material, I mean the oil and the antigen through this fine needle and with that in that process the emulsion is formed.

Emulsion can be called as watering oil or oiling water, because both are in the same concentration, same volumes. So, you can call them anyway. So, it is the emulsion by this method the emulsion is formed. So, for ready reference, we have already prepared very much. This emulsion looks like white, initially it was two phase and then slowly it has turned into single phase.

Now, you can push this emulsion from one needle to another side and from another this syringe to another syringe. So, this process creates very good emulsion which does not separate out later on when you are ready to ready to inject.

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So, how do you check them? So, for checking purposes, we drop one of this emulsion drop a drop of emulsion on the water surface like this. If emulsion is not formed perfectly, this will spread out otherwise it will not spread. So, this is a check that your

emulsion is formed correctly. So, once you find that this drop is not spreading, your emulsion is actually ready for injection.

So, this was the process by which you prepare the emulsion for injection purpose. So, this is the first step of preparing the emulsion. So, now let us understand why we prepare the emulsion. We have checked that emulsion is formed. Now, the purpose of making the emulsion because you have antigen and antigen through antigen you can raise antibodies, but after emulsifying them, you actually make the antigen releases slowly.

So, it is a sustainably is kind of preparation so, that the antigen is exposed to system in a systematic manner. So, that the more and more memory cells can be is generated and that is the sole purpose of having emulsion. Otherwise, if you inject antigen as such in PVS or in other water system.

It will spread out in the body and it will be cleared up by the immune system readily, and no memory cells will be generated. So, these are this is the purpose main purpose of preparing the emulsion.

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So, now we have prepared the emulsion, we have come to animal house. This is our rabbit which will be immunized and before immunization we have to take pre-immune breed. So, that we can compare better the serum and the antiserum. So, we will now to start how we immunize it.

So, now we are preparing to immense. Here first and important thing is in all this animal processes is we have to avoid the pain to the animal. So, for that purpose we strain we have to strain the animal because we have to inject. So, we strain the animal in a way that it has less and less pain and the movement is also very less.

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So, we will inject this into the emulsion into the thigh, we have to catch hold of both the legs and we have to sterilize the area using alcohol. We have to look at the thigh muscles, they should be cleaner, cleanly visible, skin should be cleanly visible. There are two kind of injection that we give, one is intradermal and another one is subcutaneous.

So, today we will be doing subcutaneous injection. This is our emulsion that we have prepared by micro emulsifying needle, we have seen earlier. This is the area where we would like to inject. We have to take out all the airs from syringe and the needle. We have taken out, clean the area again and then we apply some anti-septic powder, here it is betadine powder. So, that the infection cannot develop later on.

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Just sprinkle some of it at the area of injection. Then slowly release, leave the animal relaxed and it is immunized. So, this is a button form which is made, this is the region with the emulsions line.

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So, when we have to bleed, we have to strain the animal because we cannot, you in general you do not you know it is not advisable to anesthetize the animal. So, we use this strainer, this wooden strainer where we keep this rabbit here. Keep his neck here inside and the head outside. Then, we strain his neck using this.

Then, we strain the rabbit from back side using these kind of cassettes. According to the size of the rabbit we have these slots given and after using this, we strain the animal from the top like this.

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Like this and we lock the neck region and the animal is ready, with this head outside and the ear outside from here to bleed. But, the drawback of this strainer is that if the rabbit is smaller or very, (Refer Time: 22:58) depending upon its behaviour, if it is panic. Sometimes it rotates itself and the backbone is broken.

Its rabbits backbone is very, very sensitive and it breaks. So, if it breaks, it you have to sacrifice the animal unnecessarily. So, we normally we do not use this though it is used for a rabbit bleeding. And, we normally got (Refer Time: 23:25) rather method, is to strain the animal.

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So, rather method is to strain the animal in a towel or a this kind of cloth. So, the advantage of having this cloth to strain is it where the animal has its claw inside, outside of this cloth and then it cannot move. So, then we have to restrict the movement when he is similar in a strainer and you know on this cloth. So, we use this (Refer Time: 24:05)

Now, we will strain the animal on this cloth. We keep the animal relaxed on this cloth and then we strain it. Make sure that the ear are outside and the animal is strained properly so, as to reduce its movement and now this ready to bleed.

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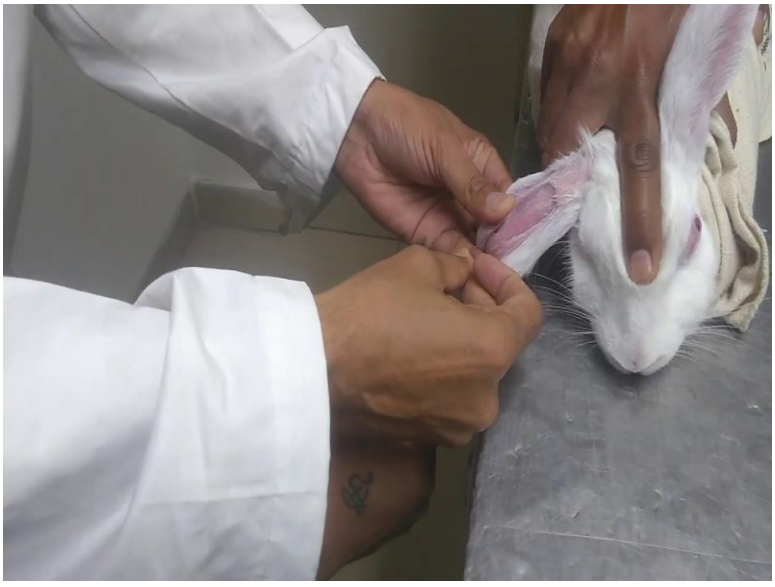

You will bleed the animal from his mid ear vein, ear vein and you rub it so, that it gets heated up and the circulation is faster. The vein is also expand and more and more flow into there. So, this is the method which is normally we apply. When the vein is properly visible, since the mid ear vein from which vein bleed, then you slightly sterilize it using alcohol and using a 20 gauge needed which is wide enough to give sufficient bleed. We will prick to the vein and collect the bleed.

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Then we to stop it, we will just with less and less pain we can collect the bleed like this.

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Now, we have to make sure that no further bleeding occurs. Then, we wipe out whatever bleed is outside using sterile water, we wipe out all the blood here and there if any spillage and keep, then wipe out all over, using water. So, that the vein becomes cool and get (Refer Time: 27:29) Then, we will just check, still it is bleeding. So, keep it pushed until the bleeding stops.

So, now, now I think the blood has stopped coming out and then now we apply some antibiotic. Here, in this case it is Betadine powder so, that there is no further infections or inflammation in rabbit. And, this also ensures that there is this is ear (Refer Time: 28:30) And, if there is any inflammation, it will have some pain so, it will it will avoid the kind of (Refer Time: 28:37)

So, we have the isolated approximately 10 to 12 ml of blood. This will give us approximately half of the volume of the blood serum this and this will be (Refer Time: 28:54) the blood will be (Refer Time: 28:55) at 37 degree centigrade for 1 hour and then it will be kept at (Refer Time: 28:59)

So, that the clot is shrunkened properly and the serum is maximally taken out. And, then we will assort the serum, add some preservative like sodium (Refer Time: 29:10) and keep it if minus 20 or minus 18 as per requirement. And, then we also test simultaneously the title of it and the specific (Refer Time: 29:24) using (Refer Time: 29:25) test and rest in the (Refer Time: 29:27)

Now, you can see this whole of the animal and thus this animal is I mean really relaxed. It has I think it has rigorous pain or a negligible pain and this is actually very important for handling (Refer Time: 29:43) that in whatever procedure you go through with animal, animal should be ensured not to have a pain.

Maybe you can if the procedure is painful to understand, its a painful procedure; you exercise it. Since, this procedure is not painful, it has not been (Refer Time: 30:01) So, that this is very important step to ensure that there (Refer Time: 30:05) would be (Refer Time: 30:06) should be relaxed.

So, in whole of the process, I think you have you have got to know all the steps of and where development in rabbit. And, we had been prepared medicine, we injected the emulsions, we isolated the blood after giving chain booster doses. And, and (Refer Time:

30:30) the process is (Refer Time: 30:32) I think you have understood most of this processes and you liked it. Thank you.

Once you collected the serum in that particular step, it is actually going to have the crude antibody, it means going to have the antibody and all other proteins. So, you can use the bio-affinity chromatography and you can be able to use that for affinity chromatography. And, for antibody purification what you can do is you have to first couple the antigens onto the beads right.

And, that is why you are going to have the antigen bound sepharose beads and then you can be able to load this particular serum as a sample. And, that is how you can actually you have the washing step, then you can have the elution step and that is how it is actually going to give you the purified antibodies.

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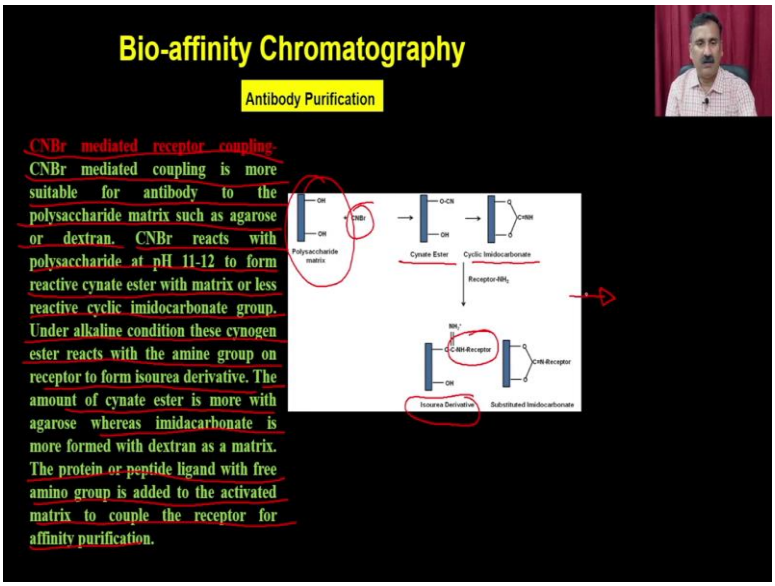

So, antibody how you can actually be able to prepare the column right. So, what you can do is you can use the CNBr mediated receptor coupling. So, CNBr mediated coupling is more suitable for the antibody to the polysaccharide matrix such as agarose or dextran. CNBr reacts with the polysaccharide at pH 11 to 12 form to form the reactive cynate ester with a matrix or less reactive cyclic imidocarbonate group.

Under alkaline conditions these cyanogen esters react with the amine groups on the receptor to form the isourea derivatives. The amount of cynate is more in the agarose

whereas, this and that. The protein or the peptide ligand with the free amino group is added to the activated matrix to couple the receptor for the affinity purifications.

So, this is what you have, you have a polysaccharide matrix. When it reacts with CNBr, it is actually going to form the cynate esters and as well as the cyclic imidocarbonate, And, that is going to react with receptors to give you the isourea derivatives and the receptor is going to coupled onto the matrix, And, this coupled matrix can be used for the purification of antibody or it can be used even for the purification of the antigen as well.

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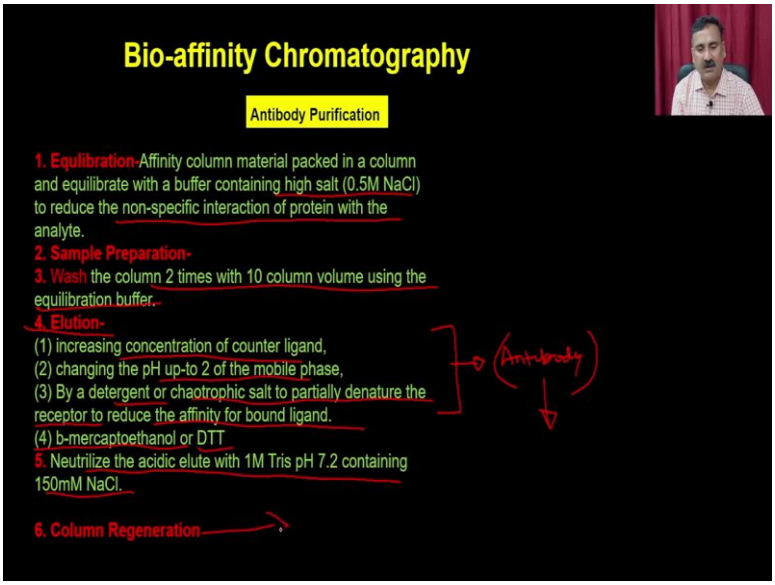

So, in the antibody purifications, you can just packed a column with a receptor onto this and you can equilibrate the affinity column with a high salt, high salt containing buffer. So, that it can reduce the non-specific interactions. Then, you can wash the column with 10 column volume using the equilibration buffer and then you can do the elutions.

So, you can use the counter ions or you can use the pH of 2 or you can use the detergent or chaotrophic salt to partially denature the receptor to reduce the affinity for the bound ligand or you can use also the beta mercaptoethanol or DTT. And, all of these are going to release the antibodies from the matrix and that you can actually be able to collect and purify.

Once this has been done, you can actually neutralize the acidic elute with the help of the 1 molar trace containing pH 7.2 containing 150 millimolar. And, once you are done with the purification, you have to do the column regenerations.

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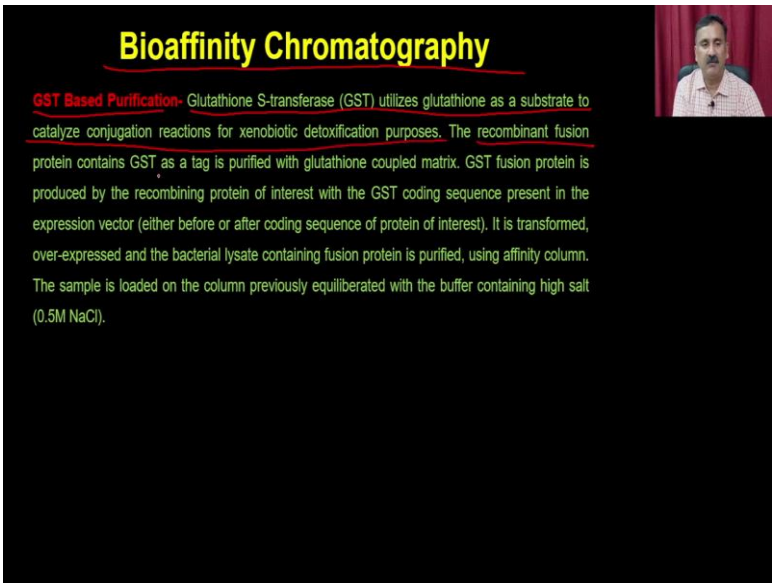

Then, we also have the another examples of the bio affinity chromatography where you can use the GST based beat based purifications. So, glutathione GST utilizes glutathione as a substrate to catalyse the conjugation reaction for xenobiotic purpose. A recombinant fusion protein containing GST as a tag is being purified with the help of the glutathione coupled matrix.

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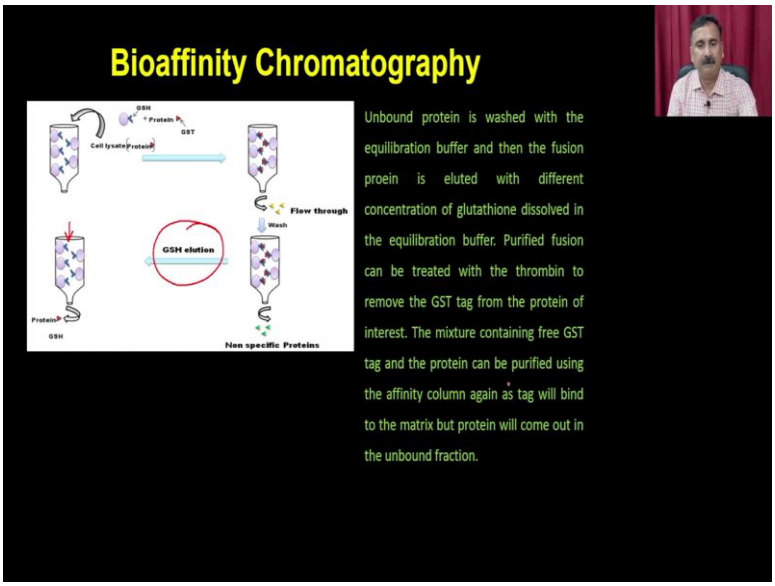

How you are going to do that is that you are going to prepare a column which is having the GST right. So, that is how the protein is actually going to contain the GST tag and the column is going to have the GSH as a ligand. So, when you purify when you load the lysate, it is actually go and bind because there will be an interaction of the GST and GSH. And, then you are going to have the washing step and ultimately you are going to use the GSH for elution.

So, GSH is going to competete for the protein and that is how the GSH will bind to this to the GST which is already been tagged with the protein and that is how the protein is going to come out. After this step you have the option either to remove the tag. So, that also you can be do you can be able to do with the help of the different types of proteases.

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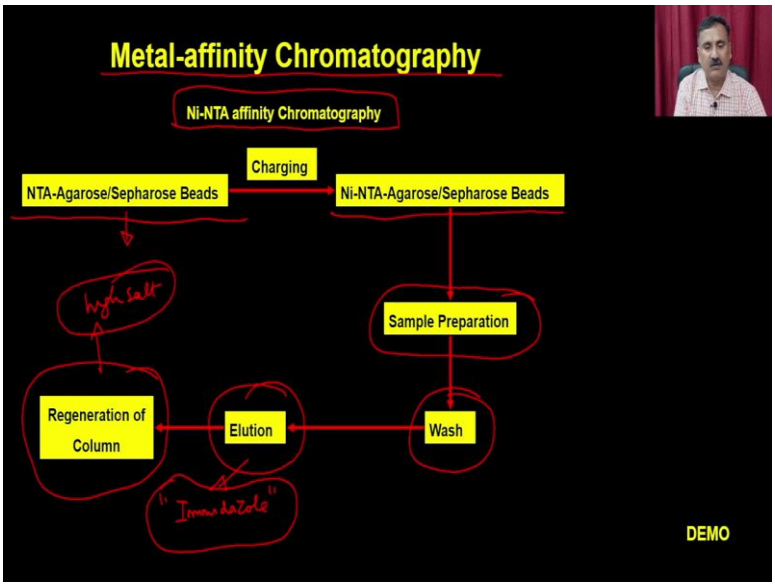

Then, you could also use the another affinity chromatography which is called as metal affinity chromatography. So, in a metal affinity chromatography, we are going to discuss about the nickel NTA affinity chromatography. So, first what you have to do is you have to prepare the column with the help of a step which is called as charging steps.

So, what you the nickel NTA what you are going to get from the vendors is the plain columns ok. So, you are going to get NTA agarose or the sepharose beads, then you will within a charging step you are going to first load the metals onto these beads. Then, you are going to load the sample and then you are going to have the washing steps and once the washing step is over then you can do the elution.

You can have the multiple options, the mostly people are always using the imidazole as the as the ligand for competition. So, you can when you do the imidazole different concentration of the imidazole. Imidazole is actually going to have a competition with the histidine which is bound to the nickel and that is how it is actually going to help you to elude the column.

Once done it is this, you can actually regenerate the column with the help of washing the column with a high salt concentrations and that is how the same column can be reused again and again. So, we have prepared a small demo clip to explain you how you can be able to perform the nickel NTA, affinity chromatography.

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Sir, protein purification first we have to inoculate the culture into this larger volume of ELB flask, then we will induce it. So, first I will show you how to inoculate. This is the single (Refer Time: 36:52) grown overnight culture. So, we can use for the inoculating into large cultures. So, this process should be done in accepting conditions so; that means, we have to use laminar airflow for this purpose.

So, and 12also, we have to remember we should include suitable resistant marker like ampicillin or (Refer Time: 37:20) these kind of antibiotics. And, this is depends upon what vector what resistant you are carrying you are having. So, in this case we are using the ampicillin as a antibiotic. So, let us start.

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We have lysidine cells using sonication. Now, we have to centrifuge the lysate to get superannuated. So, that superannuated we load onto nickel NTA column and purify the protein. So, I will transfer into 50 ml centrifuge tube, then 8 centrifuge tube.

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While the centrifugation is going on, we have to wash the column using first. This is in 20 percent ethanol. So, we have to wash first with water, then equilibration buffer. So, let it drain completely that 20 percent ethanol, then we will add water, double distilled water.

So, at least 5 column values of water should be added to remove complete completely and next we will equilibrate with the lysis buffer the buffer which we used for the lysis of the bacterial cells. Before equilibration of the column, we have to charge the column. Nickel NTA, there are two types of beads are there.

One is already readily charged beads which comes from company and another one is we have to charge. They will give only anti-agarose beads. So, here what we will do is we will charge the beads with the nickel and then we will equilibrate. We already washed the column with water and fine to normal NaOH again with water.

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So, now we will equilibrate. So, this is nickel hexachloride solution. So, we will keep in this condition at least 20 minutes to charge the beads. After that we will remove nickel NTA, we will elude the nickel solution and then I equilibrate with the lysis buffer. So, after 20 minutes we will rotate the nickel solution. Next, we will equilibrate with the lysis buffer.

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We have to wash at least two column volumes to remove any free nickel which exists in the base. So, after equilibration next step, we will load the lysate and then incubate for binding.

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This is paraffin, I am going to close this (Refer Time: 41:34) I am going to keep it here.

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So, once column packing is over, we will kept it in ice and we will keep in this condition for at least 2 hours for binding. So, that (Refer Time: 42:11) protein, we will bind to the nickel NTA and in further steps we will encode it.

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After incubation with beads, we have to follow another 3 steps to get completely purification done. First step is we have to wash with the equilibration buffer. First after the beads taken out from ice, you have to you have to remove the outlet. So, that all the flow through other than beads will be taken out.

And, the next step is we have to wash with the incubation buffer and the third step is we have to elute the sample, elute the protein, (Refer Time: 43:08) protein using indazole containing buffer. All for all these buffers, the pH should be adjusted prior hand. Not like you have to first you take the buffer; lysis buffer and you have to add indazole, it is not like that.

It may increase the pH of the buffer. So, after combining all the lysis buffer with the indazole, then we have to adjust the pH; so, that throughout the procedure the pH will not change. So, this is the flow through whatever we are getting is flow through. In next step, we will wash with the in lysis buffer. In this step, we are going to wash with the lysis buffer or equilibration buffer. So, I will just this is the lysis buffer. Before doing this, we have to observe the beads we should not directly load onto this.

You just have to pour through corner through the wall of the column. Otherwise, it may disturb the beads so, protein may also declined. So, this we have to keep in mind, while doing this washing. While, doing purification, we have to remember that every time you are introducing new buffer, you are introducing new buffer that time you have to collect

the fraction and this can be used for the running SD phase and testing the purity of the samples.

And, also the flow through part and washing part what we have collected, we have to keep it safely. After verification of the gel only we have to throw. Say you are getting only 10 percent of the protein in your purified fractions and 90 percent of the protein eluting in the flow through that time you can reuse the flow through for purification, purification and purifying the protein.

And so, you have to collect the fractions in a small microcentrifuge tubes and we have to save those fractions. They will end same.

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So, we washed with the equilibration buffer and we also collected the flow through. Now, it is time we will wash with the 20 millimole of imidazole. So, this will remove any non-specific proteins binding to the beads.

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So, we will wash with the 20 millimole imidazole containing buffer, then we will eluting subsequently eluting 250 millimolar imidazole contained in the buffer.

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In final step, we are going to (Refer Time: 48:58) with the 250 millimole imidazole containing buffer. So, what we are going to do is we have to incubate beads with this buffer for some time and collect the fractions. Now, we have to collect the fractions.

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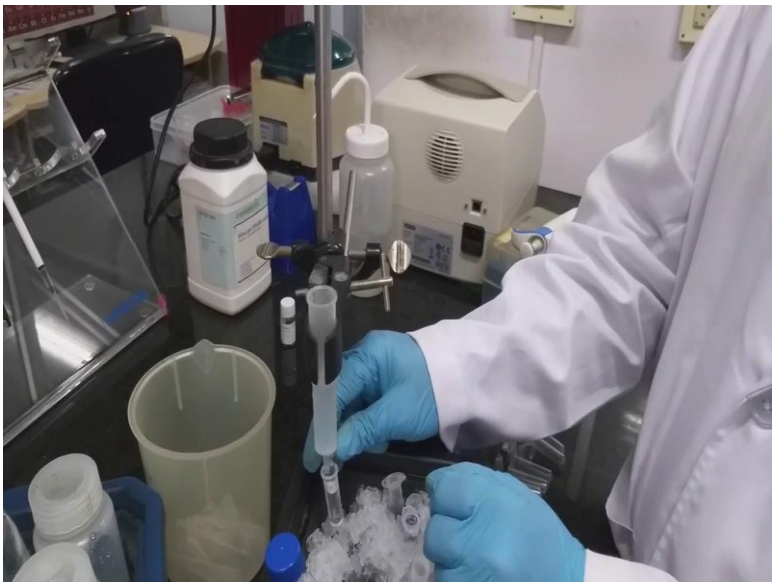

After eluting the complete fractions, we have to wash the column with water, then 0.2 normal sodium hydroxide solution. Then again water, after final wash with the water, then we have to store the beads in 20 percent ethanol.

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So, I will wash it and store it in the ethanol.

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When the washing is going on, we have to take 50 microlitre of each fractions and run ton SDS base that will give the purity of the fractions. We have to heat the samples before loading on to SDS base. And, also, we have to keep these all these fractions what we have collected at 4 degree Celsius for further confirmation of the purity.

Once the purity is confirmed, we have to dialyse those productions against the our buffer of interest. Then, we use for the further studies.

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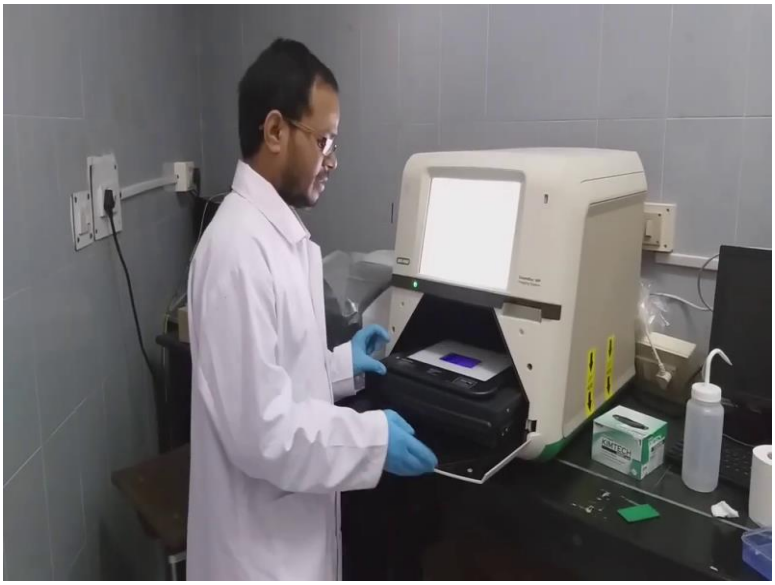

So, we purified the protein using nickel NTA column. We run the gel and stained or de-stained. So, now it is time to document the gel. So, we have to identify whether we got any single band fraction or not. So, this is the gel I kept on a white tray. Now, just close it.

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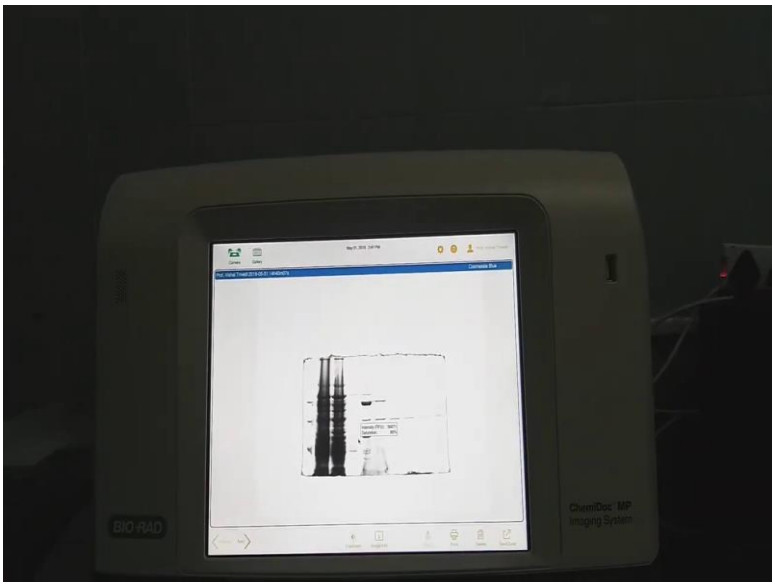

So, we have loaded marker and this from this side. Second one is the load. This is flow through wash 1, wash 2 and these fractions are eluted fractions; 1, 2, 3, 4, 5 serially. So, as we can see the eluted fractions showing a band corresponding to this protein. But, the molecular weight can be calculated using the software image lab software.

So, as we can see in the protein corresponding to this purification, histived 1, it is going most of the fraction in the flow through. So, we can use as I said in the video earlier, we can use this flow through fraction again for purification of the protein. You can incubate this flow through with the same beads and you can repurify again. So, that will increase the productivity getting the protein.

So, these are all other bands whatever we are seeing in the protein, these eluted bands. Those are because of the contaminates or degraded protein. Contaminates sometimes may come because of histidine to 3 or 4, histidine having in folder state, that will give possibility to bind to nickel NTA column. And also washing, vigorous washing should be done.

If you do not wash properly with a high amount of imidazole, that will give you this kind of non-specific binding. So, with this, we will complete the video. So, I hope it will help you to help you in your work, for during protein purification, or help you to understand how protein purification binds. Thanks for watching.

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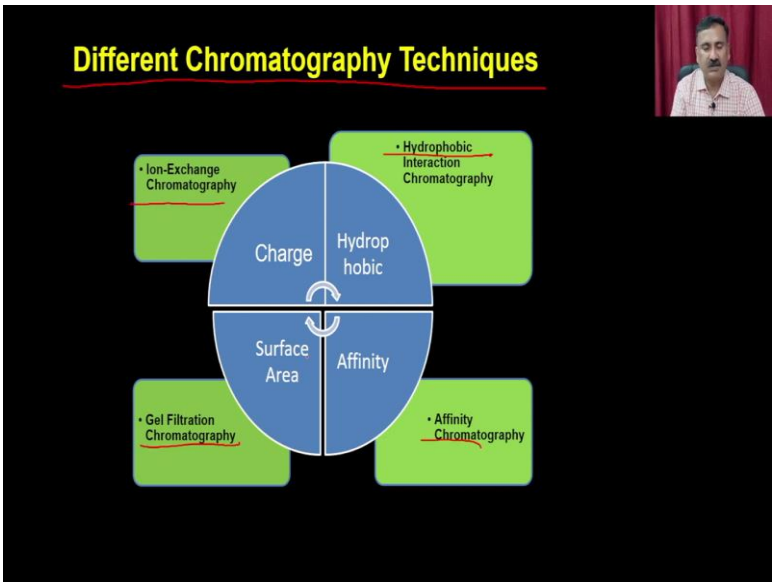

So, this is all about the different chromatography technique, what we have discussed. We have discussed about the ion exchange chromatography, HIC, affinity, and we have also discussed about the gel filtration chromatography. Now, the question comes how you can be able to use these different chromatography techniques for purifying the proteins or the enzymes?

So, we have taken couple of examples to explain you how you can be able to utilize these different chromatography techniques for the purification of the enzymes.

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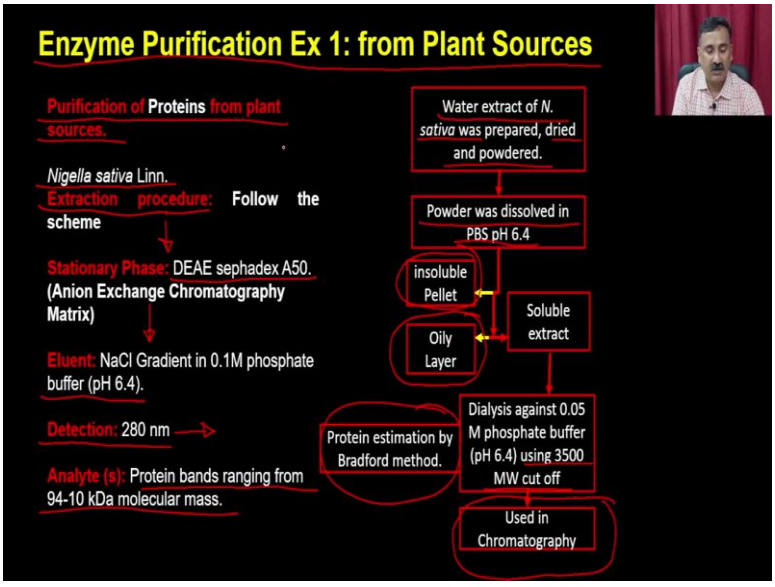

So, first example is that how you can be able to do the enzyme purification from the plant sources. So, purification of the enzyme from the plant sources. So, we have taken an the example for the plant, which is the nigella sativa. And you first, you are going to do the extraction of the substance or the protein. So, you can follow the scheme. So, you can prepare the water extract of the an sativa and you can try it in make the powder. So, powder is going to be dissolved in PBS 6.4.

So, that is actually going to give you two fractions. It is going to give you the insoluble pellet or it is going to give you the soluble extract. So, within the soluble extract, you are going to have the oily layer or the soluble extract. This soluble extract is actually what you are going to use and you are going to dialyse that against the you know the 0.05 molar phosphate buffer using the 3500 molecular weight cut off.

And as a result, it is actually going to remove all the contaminating substances and you can actually be able to estimate the protein consideration with the help of the Bradford. And, this is what you are going to use in the chromatography. After this, you are going to perform the ion exchange chromatography.

So, you are going to use a pack the column with a DEAE-Sepharose and you are going to then elute with the NaCl gradient at pH 6.4. And you can do the detection of the protein at 280 nanometres and the analyte band, protein ranging from the 94 to 10 kDa. You can actually be able to use for the monitoring the protein, what you have isolated from the plant sources.

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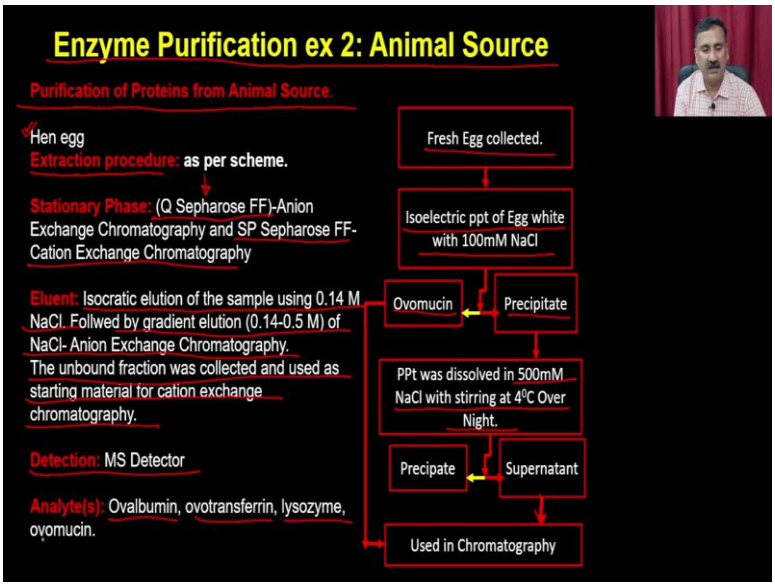

Then you can also, we have taken another example, which where you can actually be able to use that for the animal sources. So, purification of the protein from the animal sources. So, we have taken the hen egg. So, first you are going to do the extraction. So, what you are going to do is you take the fresh egg, which you are going to collect it.

Then you do the isoelectric precipitation of the egg white with the help of the 100 millimolar NaCl. So, that is going to give you the two proteins, that is ovomucin and the precipitate. This precipitate, you can just dissolve into the 500 millimolar NaCl with a stirring at 4 degree overnight. And, that is actually going to give you the some amount of precipitate and the supernatant.

And, that supernatant you can use for the chromatography into the further use in the chromatography. That supernatant you can load onto a Q Sepharose, an ion exchange chromatography and followed by the SP Sapharose, which is the cation exchange chromatography. So, remember that in the previous example, we have only used the single column of the ion exchange chromatography he.

We have you we you have we have used the two column in tandem actually. So, isocratic illusion of the sample using the 0.41 molar solution followed by the gradient illusions of the NaCl from the anion exchange chromatography. The unbound fraction for collected and used as a starting material for the cation exchange chromatography. You can use the mass spectrometry to detect the illusion of this particular protein from the column.

And in the analyte, what you will see is you are going to get purification of the ovalbumin, ovotransferrin, lysozyme and at the ovomucin.

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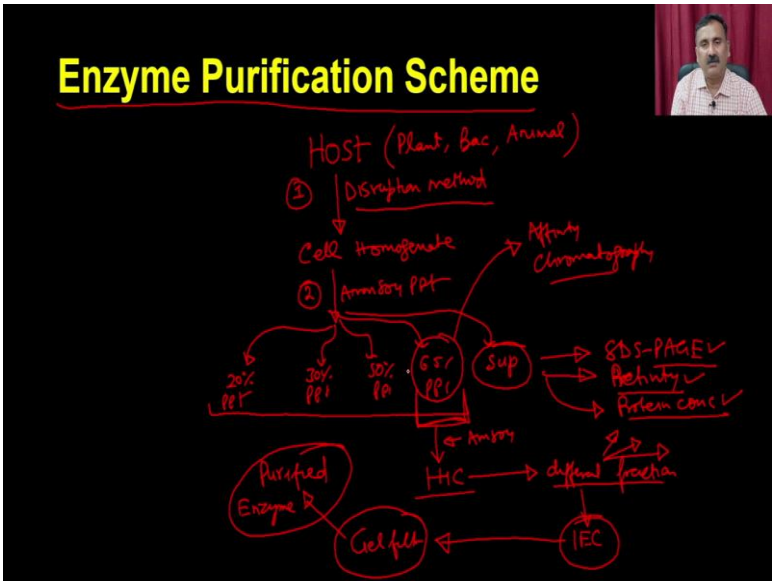

Now, whether you are dealing with the plant sources or whether you are dealing with the bacterial sources or whether you are dealing with the animal sources. The purification strategies are going to be very very fixed. For example, first you are going to take the host cells depending upon whether it is a plant, bacteria or animal; you are actually going to first utilize the disruption method ok.

So, first you are going to select the disruption method. You can do the sonications, you can do the thermolysis, you can do the osmotic lysis or you can do the homogenization. Then, what you are going to do is you are going to get the cell homogenate or cell lysate right. This cell homogenate, first thing what you can do is you can do a ammonium sulphate precipitation ok.

And, that ammonium sulphate precipitation is actually going to give you multiple fractions. It is going to give you like the 20 percent precipitate. It can actually give you 30 percent precipitate, give you the 50 percent precipitate, 65 percent precipitate and also a final supernatant.

All of these can be analysed onto the SDS page. So, you can actually be able to check the you know purification of the enzyme on the SDS page and you can also do the activity of

the enzyme. So, you can also do the activity of the enzyme and you can also do the protein concentration ok. And, that is actually going to be a guiding force whether the purification is going in the right direction or not ok.

Because, if you are doing some procedure and if it is giving you no activity, for example, there will be an activity loss, then the purification is not good actually. So, then you have to modify the scheme actually. Now, anyway, once you are done with ammonium sulphate precipitation, what you can do is you can take all these fractions. Suppose you have the protein in this particular fraction ok. So, if you take this fraction and directly you can take this and perform the HIC.

So, you can add some more amount of ammonium sulphate, right? Depending on how much ammonium sulphate need for you further you have to add. So, that you can actually be get the you know the exposure of the hydrophobic patches. And, then you can actually be able to perform the HIC. HIC is also going to give you the different fractions and all these different fractions can be another again analysed for the SDS page for the activity and for the protein concentrations.

Now, from HIC different fractions, you can just pull the fractions where you have the protein, right or wherever you have the activity. And, then these fractions can be first further loaded onto the anion exchange chromatography followed by the gel-filtration chromatography. And, ultimately what you are going to see is you are going to see a purified enzyme, if you are going to optimize all these steps in a meticulous way.

I do not have examples of discussing all these, but you can actually be able to get an idea and you can you know go with this particular scheme. So, first you are going to first step you are going to use the going to select the disruption method. So, that the host cell is going to be get disrupted. Second step, you are going to do the ammonium sulphate precipitation.

So, that you are going to have this suitable fraction where the major amount of activity and the protein concentration is present. And, then from this fraction you can be able to do the hydrophobic interaction chromatography. Because, the major advantage of the HIC just after doing after the ammonium sulphate precipitation is that you do not have to waste the time for the dialysis.

Because, if I want to do the ion exchange chromatography after the ammonium sulphate precipitation; I have to dialyse and remove the salt, right? So, that is not required for HIC. Once you have got the different fractions, you can select the fractions and that selected fraction you can put it for the ion exchange chromatography.

And, then from the ion exchange chromatography selected fractions, you can take where the activity is very high. You have the minimum number of bands and your or your protein concentration is also very high. And then you can put it onto the gel-filtration chromatography and while doing so, all this you can be able to get the purified protein.

If you have the any kind of antibody ready right, then what you can do is you can take the 65 percent cut. For example, you have the major activity in this fraction and you have the antibody which actually can recognize your protein. Then, what you can do is at 65 percent cut, you can actually do the affinity chromatography directly.

Because, most of the time affinity chromatography we should not do from the crude extract because crude extracts is actually going to give you the non-specific binding. So, better you do the one round of ammonium sulphate precipitation. So, that some of the protein which are you know not good for the affinity chromatography can be removed.

So, this is all about the different chromatography technique and the schemes what you can actually be able to follow to purify the enzymes. What we have discussed? We have discussed about the antibody generations in the mice or the rabbits. And, then we also discuss about the nickel NTA affinity chromatography also.

So, with this I would like to conclude my lecture here. In our subsequent lecture, we are going to discuss about how you can be able to utilize these enzymes in a different types of a applications or operations.

Thank you.