Experimental Biotechnology
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Module No # 04
Lecture No # 18
Ion – exchange chromatography (Part – 2)

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(Video Ends: 01:00)

Hello everyone, this is Doctor Vishal Trivedi from the department of biosciences and bioengineering IIT Guwahati. And so for in the previous lecture we have discussed about the basic principle of ion exchange chromatography. And in addition, we have also discussed about the how to perform the generalized ion exchange chromatography what are the different steps you have to take to successfully complete the chromatography.

And how to choose the different types of matrix and what is the importance of isoelectric point and as well as the other parameter which you have to consider by using a suitable matrix for protein; purifications. In addition we have also briefly discuss about the several applications of the Ion exchange chromatography for the industrial usage as well as for the daily uses. So in that context we have also discuss about the protein water purifications how the Ion exchange chromatography can be used to purify the different component of the water, so that the water will be free of the salt and heavy metals and the fluorides and all the kind of contaminants.

And now in today's lecture we are going to discuss about the how a ion exchange chromatography can be utilized to solve the different types of research problem.

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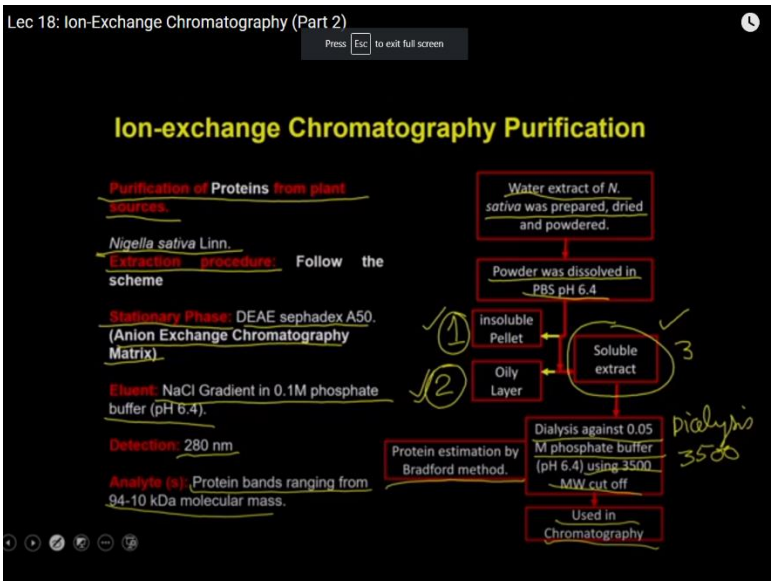

So one of the major application or the utilization of ion exchange chromatography is to purify the or to process the complex protein mixture and from there you can be able to select the protein of your interest simply using the ion exchange chromatography. So we will go through with the several examples how you can process the samples and what are the different precautions you have to take.

So that it will give you an idea about optimizing the protein purifications of your own choice as well as it will give you the idea how to perform the chromatography if you have the similar kind of samples. So in this case I have taken the first examples where we are going to purify or we are going to learn how to purify the protein from the plant sources. So the first example is that where we are planning to purify the proteins from the plant sources.

And for this example we have taken a plant which is called as Nigella sativa. So in the first step itself you have to perform the extraction. So in the extraction procedures you have the different systematic scheme where you have to first make a watery extract of the plant and then you dry and keep the powder and then you can take the suitable amount of powder and dissolve it into a PBST at pH 6.4.

Now once dissolves this into PBS you are going to get and when you regressively mix this solution along with the PBS you are going to get 3 fractions. In the fraction number 1 you are going to get the insoluble palate the palate which is not going to be which is going to settle down

after you making the powder dissolving the powder in the PBS. The second is there will be oily layer which is present on the top and then you have a soluble extract which is the fraction number 3.

So once you have dissolve this powder into PBS you regressively mix the powder then carefully first you remove the oily layer from the top with the help of pipette. And then you spin this mixture and that actually is going to give you the insoluble pellet as well as the soluble extract. This soluble extract can be dialyzed against the 50 millimolar phosphate buffer pH 6.4 and using a 3500 molecular cut off dialysis bag.

So the dialysis membrane what you have to use in this case is going to be off 3500 Dalton molecular weight cut off. Once you are done with this you can estimate the protein because you know that the every column has a suitable binding capacity. So you cannot load more than what is the binding capacity of a column? So you have to estimate the protein using the Bradford method and once you know that you have the sufficient quantity of the protein then you can take this and use it in the chromatography.

So the chromatography will be done with the help of the pipe packing the matrix into the column. In this case we are using the DEAE sephadex A50. DEAE sephadex you remember it is a anion exchange chromatography matrix. So once the protein is bound then you can do the washing step and after the washing you can elute this with NaCl gradient in 50 ml phosphate buffer pH 6.4.

And whatever is coming out from the column can be detected by the spectroscopy method by measuring the OD's at 280, nanometer and that actually will give you the chromatogram known and what fraction you have the protein and then subsequently what you can do is? You can analyze this fraction in a SDS page and that actually will give you a protein bands of the different molecular weight.

Now depending on what is your choice? Or what is your protein of interest? You can be able to refine and fine tune these protocol and as well as you can be able to get the desirable protein in a very well high quantities.

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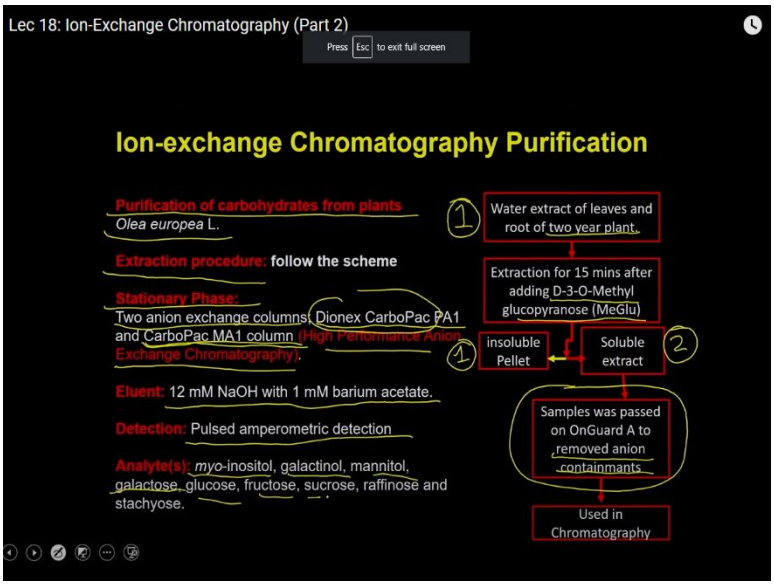

Now in the next we are next examples we are going to purify the carbohydrates from the plant sources. So for this what we want to do is? We want to do the purification of the carbohydrate from the plants for this we have taken an example of Olea Europea and the steps are remain the same. So you have to first do a extraction procedure. For the extraction you have to follow a particular scheme.

So what you have to do is you have to first take the leaves as well as the root of this plant. And then you have to first prepare the water extract the only advice or the procedure is that the leaf or the root whatever you take should not be taken from the very old plants. So it should be very up to the 2 year old plant. And then what you do is you extract the watery extract with the D30 methyl glucopyranose by and you extracted for 15 minutes after that you are going to get 2 fractions.

You are going to get insoluble pallet as well as the soluble extract. Now the soluble extract you can just pass through a guard column. So, that it should remove the impurities because as you remember we have while we were discussing about the preparation of the sample it was very much advisable that you should not have the particulate matters. Especially in this case where; we have to use the guard column so that you will remove all the contaminant. So that it should not interfere in the subsequence steps.

After that you can do the chromatography. So in this case we are using the not only 1 column but that 2 columns. So you can use the 2 anion exchange column one is called Dionex CarboPac P1 and the other one is called as the CarboPac M1 column both are the HPLC Anion exchange columns. And the elution so what you have to do is? First you load the dissoluble extract which you have pass through the guard column first to this column.

And then you do the elusion with the help of the 12 millimole NaOH with 1 millimolar barium acetate. Once you got the fractions, both fractions can be loaded onto the next column and that is how you can be able to fine tune the purifications. The detection can be done with the help of the pulsed amperometric detections and that actually is going to detect the carbohydrates, and ultimately you are going to get all the carbohydrates what is present in this particular plant.

And depending on the choice of the carbohydrate which you are interested to purify you can be able to fine tune the purifications with the changing the changing in the variant curve. As well as the changing in the pH of the resolution as well all other parameters and that is how you will able to achieve the purification of the carbohydrate from the plants.

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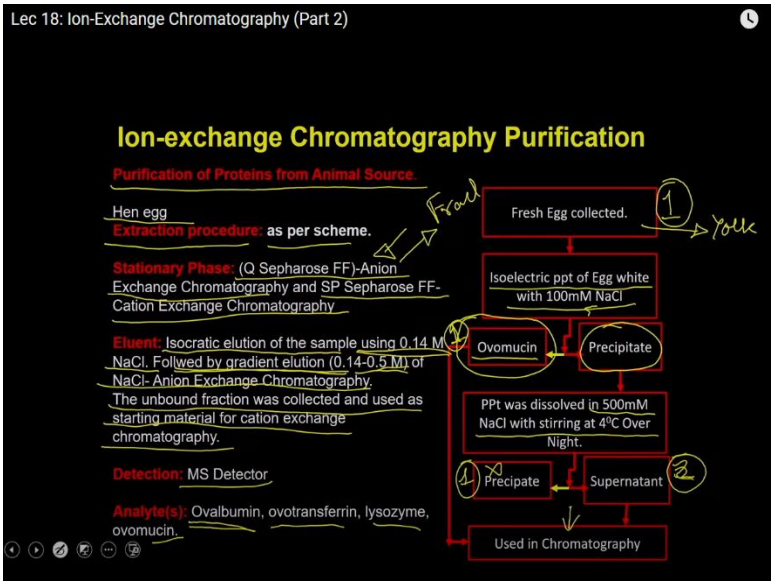

Now the third example is because so for what we have discussed we have discus about the plants. Now we will discuss about the animal cells we will discuss about the how you can able to isolate the protein from the animal source for this example we have taken the examples of the

hen egg. So the our objective is to purify the protein from the animal source. We have taken an example of hen egg.

And the first step as it is that we have to follow the extraction procedure. So in the extraction procedure what we have to do is we have to first collect the fresh egg, and then we have to break open the egg we have to remove the yolk part. So the first part is that you have to remove the yolk part. You have to just take the white egg white. And then you have to do the isoelectric precipitation of the egg white with the help of the 100 millimolar NaCl.

Once you do that you are going to get 2 fractions. In the fraction one supernatant ovomucin and you are going to get the precipitate. This precipitate you can further dissolve into 500 millimolar NaCl with the stirring at 4 degree for overnight. So once you dissolve this precipitate into 500 millimolar NaCl with stirring at 4 degree it will again give you the 2 fractions. The fraction number 1 is the precipitate which you are going to discard and then you are going to get the supernatant.

The supernatant so the supernatant 2 as well as the supernatant 1 is going to pool together because this will contain the protein especially the ovomucin. And this one is going to contain all other proteins which are the part of a egg white. And then you pull these to so you pull the supernatant 1 you pull the supernatant 2 and then you use them into the chromatography.

For the chromatography in this case because we have to fractionate the all the proteins present in the egg white we are using the 2 column 1 is called Q Sepharose fast flow, which is the anion exchange chromatography column and then you are using the SP Sepharose fast flow which is a cation exchange chromatography. So in this what we are going to do is first you are going to load the sample on to the Q Sepharose fast flow.

And then you are going to elute the sample with the isocratic elution of the sample using the 0.14 molar NaCl. Following this you are actually going to do the radiate elution with the help of 0.14 to 0.5 molar of NaCl in the case of anion exchange chromatography. So that actually is going to give you the different fractions containing a protein. And the unbound fractions so what will happen is once you load the sample on to Q Sepharose fast flow and you will going to do the

elution profile you are some of the protein you going to bind and some of the protein is not going to bind.

The unbound fraction whatever is going to you are going to get from the Q Sepharose is going to be loaded on to the cation exchange chromatography which is the SP Sepharose chromatography. And then you have to follow the similar elusion scheme which means you are going to first do the isocratic elusion of the sample using 0.14 molar NaCl and then you have to do a gradient elution from the 0.14 to 0.5 molar.

And ultimately you are going to use the mass spectrometry as the detector to detect the massive of the protein whatever is coming from the either the anion exchange column or the cation exchange columns. And ultimately you are going to detect the different proteins like ovalbumin, ovotransferrin, lysozymes and ovomucin. And because all these protein are of different molecular weight you will be able to detect them while they are coming from the elution.

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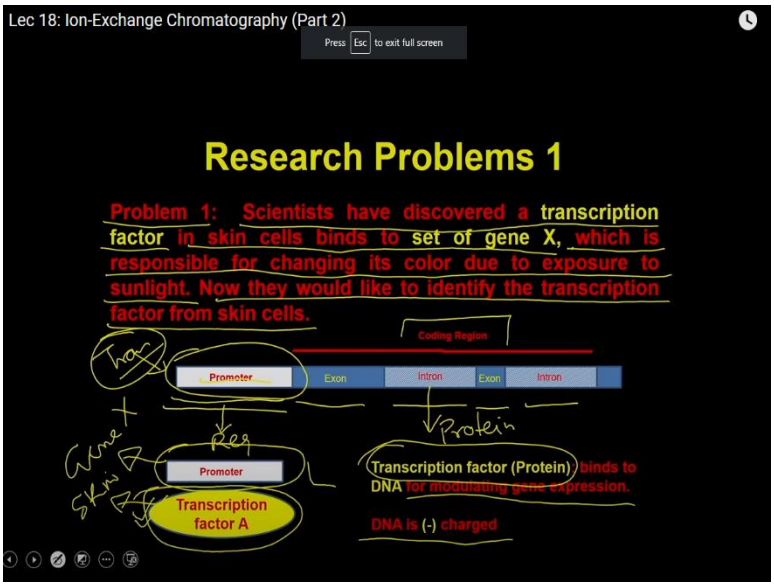

So these are the research problems where we are using or we are using the ion exchange chromatography to only purify the particular proteins you can be able to have the multiple such examples. But overall what you have to do is you have to clarify the samples you have to do at extraction procedure so that your recovery of the protein for these samples be as high as possible.

And then ultimately you also have to perform the ion exchange chromatography either the anion exchange chromatography or the cation exchange chromatography or in some case we have taken an example where you have been using the combination of anion as well as cation exchange chromatography to purity the many factors from the single source. Now what we are going to discuss we are going to discuss about the research problem where you can be able to utilize the ion exchange chromatography.

So this is the problem 1. The problem 1 is that the scientist have discovered a transcription factor in a skin cells binds to the set of gene X, which is responsible for changing its color due to the exposure to sunlight. Now what they want? They want to identify the transcription factor from the skin cells. So what is the problem? Problem is that the scientist have speculated that there are transcription factors which are actually binding to set of gene or set of gene x.

By doing so actually changing the downstream expression profiling; in such a way that actually, changing the color of the skin. So now before going into the solution of this problem what you have to understand is what is the design; of the particular gene? And what is the function of a transcription factor? So a particular coding gene in a ((()) (17:59) cells is containing different component.

For example it contain promoter, then it contain the exon or the introns together this region is called as the coding region. So these are the only region which we are interested to discuss. Now, this is the region which actually gives the protein part and whereas this is the region which is actually playing the role of regulatory role. Which means the expression of this particular protein is going to be regulated by the promotor because the promotor is going to bind the transcription factors.

And by doing the by binding of the transcription factor to the specific promotor which are associated which are bound or which are present to the next to the gene it actually regulates the expression. But what are the transcription factors? The transcription factors are the proteins which are actually binding to the DNA. And that is how they are actually modulating the gene expression.

What you have to understand here is that they are not binding to the DNA but they are only binding to the promotor regions. So to solve this problem or to under to decipher the whole equations or to identify the transcription factor what you have to do is because DNA is negatively charged and the transcription factor has the positive charge region because of that the transcription factor as well as the promotor is binding through the electro static interaction.

And so you can be able to purify or exploit this phenomenon which means you want to do this you will need a promotor means promotor of this particular gene and what you need is? You need a source from where you are going to get the transcription factor. So in this case the promotor is anyway belonging to the gene X and the transcription factor you are going to get from the skin cells ok.

So what you have to do is? You have to first break open the skin cells you have to recover the mixture of transcription factors and then you have to also clone the promotor of this particular gene. So, that you are going to have the multiple copies of this promotor. Then what you are supposed to do?

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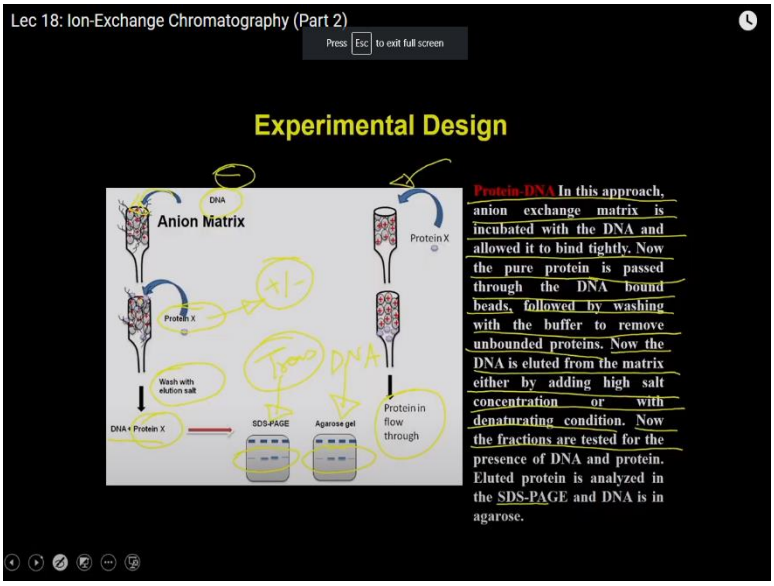

You have to design an experiment so that you will be able to control all the parameter so that you will not be able to get the false positive. So in this case what you are going to do is you are so in a experimental design what I will what you will do you will take a DNA you will bind it to the anion exchange column because the DNA is negatively charge. So it will bind to the anion

exchange columns and then what we will do is we will take up this transcription factor and allow them to flow ok.

And we will choose the pH in such a way so that the protein X or the transcription factor what you are taking is going to be containing no charge, which means it will not bind to the column on its own. Then what you do is you wash this with the (()) (21:52) wash with the salt and then elute it with the salt. So what will happen? When you elute with the salt the DNA as well as D transcription factor is going to be eluted.

Then what you are going to do is you take this transcription take the eluance and analyze on to the SDS page as well as the agarose page. The agarose is going to give you the pattern for the promotors where as this one it is going to give you the pattern for the transcription factors. Now the transcription; factor the multiple transcription factors which are going to be present in your lysate.

So if the transcription factor will bind to the DNA or if a particular set of fractions are going to give you the binding then the pattern of these proteins on to the SDS page is going to match with the pattern of the promotors ok. So in this approach the anion exchange column is incubated; with the DNA and allow it to bind tightly. Now the transcription factor is pass through the DNA bound to the beads followed by washing with the buffer to remove the unbounded protein that transcription factors.

Now the DNA is eluted from the matrix either by adding the high salt or with denaturating condition. Either of these conditions now what you are going to get? You are going to get a different fraction which you can test on the SDS page as well as the agarose and that eventually will give you the pattern. That pattern is going to match if they are actually binding to the promotor region of that particular gene.

As a control you can also run the lysate on to the empty column without the DNA. So whatever the transcription factor will bind to the beads and will come through into the elution profile those are the transcription factor you can easily ignore because it is not that the transcription factor is only bind. The other protein, will also going to bind to the DNA for example the single standard DNA binding protein or (()) (24:15) or DBA polymeric.

These are the proteins also will have a very high affinity for your promotors or probably a smaller stretch of DNA. So those proteins are also going to bind. But they will not going to show you a pattern because their binding is going to be non-specific they will bind to every region to the DNA. Whereas the transcription factor is going to have a specific affinity and they are also they are only going to bind to the particular DNA.

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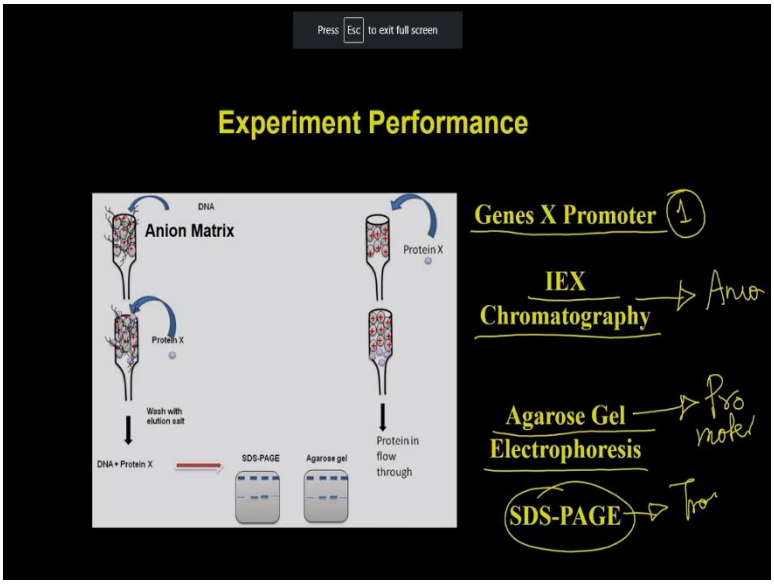

To execute this DNA these experiments you need to perform 4 things. What you have to do? First you have to clone the promoter of the gene X, then, you have to do the ion exchange chromatography which means in this case you have to do an anion exchange chromatography. Then you have to do the agarose gel electrophoresis to see the pattern of the promoter DNA. And you also have to do the SDS page to see the pattern of the transcription factors.

And ultimately by doing this experiments you will be able to identify the particular transcription factors if you fine tune the purifications you will able to or if you add some more purification tools for example if I if use the gel filtration or affinity chromatography you will be able to remove the out layers and that is how you will be able to identify the particular transcription factor utilizing this approach.

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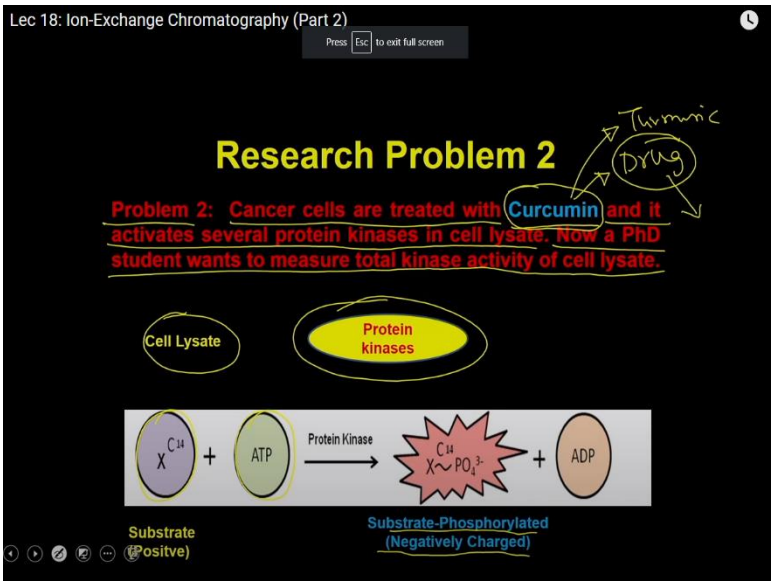

Now let go move on to the next problem and in the next problems the problem 2 is that the cancer cells are treated with curcumin. So, curcumin is a drug or curcumin is actually a phytochemical chemical which is present in the turmeric. So but curcumin is a very well-known anti-cancer compound. And it activates the several protein kinases in the cell lysate. Now a PhD student wants to measure the total kinase activity of the cell lysate.

Which means the objective is that the particular student wants to measure the kinases activity which is present in the cell lysate. Now what you have? You have the cell lysate which contains the different types of protein kinases and what these protein kinases are doing? They are actually converting the substrate with the help of the ATP to substrate phosphorylated phosphates which means they are actually generating the negatively charged molecules or negative charges substrates.

So in this case what you can do is? You can easily perform the ion exchange chromatography to purify this negatively charged or phosphorylated substrate and then you can be able to measure the kinases activity or the cell lysate.

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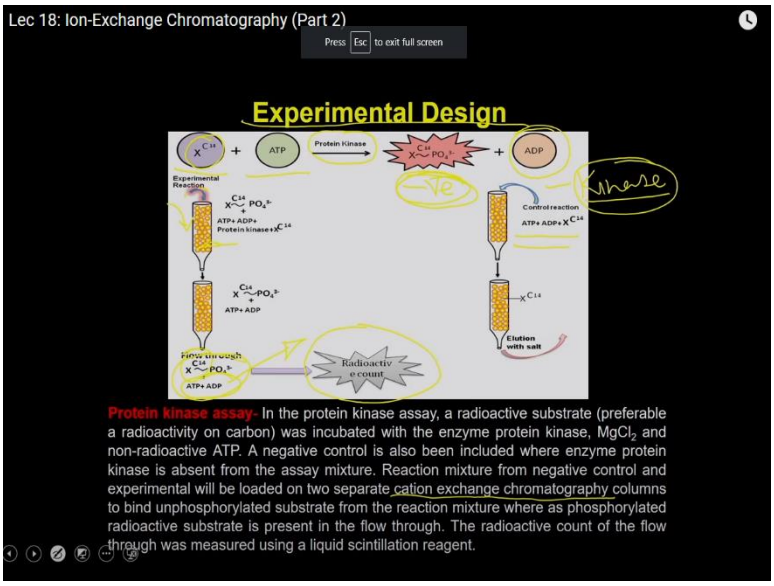

Now what you have to do is so what is the experimental design is that you take the substrate ok radiolabel the substrate with the help of the carbon 14 ok. So that actually going to make this substrate; detectable with the help of the radiography. Then you incubate it with the ATP so what will happen? Protein kinases which are present in the lysate is going to phosphorylate and they are going to give you a negative charge.

And in addition it is going to generate the ADP. Now what you do is you take this complete assay mixture and load it on to a the ion exchange column. So in this case we are takin the ion exchange column like the cation exchange column ok. So what will happen the cation exchange column is going to bind everything except the negatively charge substrate. Which; means you are going when you load the protein when you load this experimental assay system it will bind everything except the phosphorylated substrates.

Which means and in addition it is also going to release the cold ATP and cold ADP. But cold ADP is hardly matters because what you are going to measure is the radioactivity associated with the carbon 14. So carbon 14 which is non-phosphorylated is going to be trapped on to the column itself. Whereas the phosphorylated carbon 14 or phosphorylated substrate is going to be release from the column in the form of flow through and then you can take up the flow through and measure the radioactivity.

Ultimately you can also do elution with the salt and you can be able to measure the radioactivity to confirm that the column has trapped the radioactivity. As a control you can also do a control reaction where you can do everything but you can remove the kinase from the reaction. So if you do the removal of kinase whatever you are going to get the counts these are going to be the vessel level counts. And these counts are going to be subtracted from your experimental counts.

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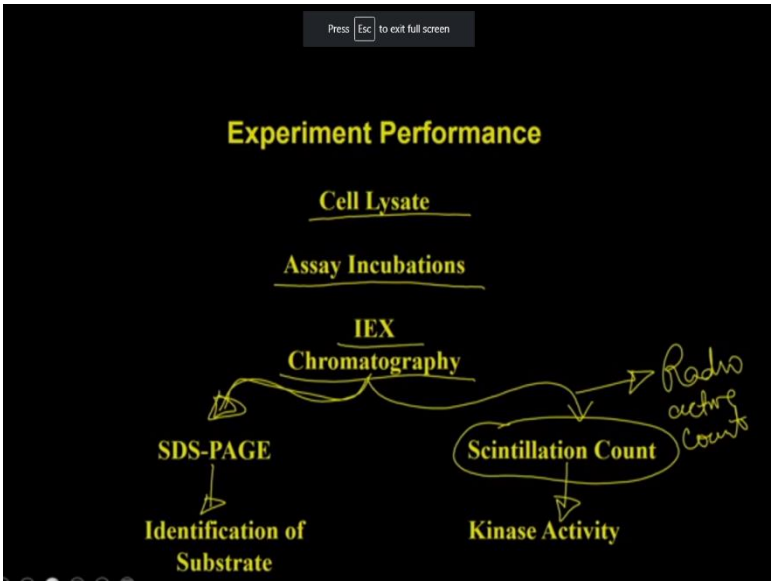

So for this particular experiment to perform this experiment you have to prepare the cell lysate, then you have to incubate the cell lysate with the radio labelled carbon 14 substrate and then you also have to add it to the ATP and incubated it for some time. And then you have to perform the ion exchange chromatography. After this ion exchange chromatography whatever the flow through you are going to get that flow through can be analyzed in 2 different ways to get more and more different information's.

So what you can do is this you can analyze in 2 different ways. One you can easily collect the you can take up the fractions and you can do the flow through and you can collect the radioactive counts. And that you can collect the radioactive count in the scintillation counter and that actually is going to give you the kinase activity associated with the cell lysate. On the other hand you can also analyze this flow through into a SDS page and SDS page followed by the identification of the substrate.

So if you do the SDS page and then you further you do the auto radiogram or if you do the western plotting you can be able to even identify the substrates. So we started with the single experiment but the single experiment can be diverged in a multiple experiment and that is how you can be able to get 2 different information's like what is the kinase activity associated with the cell lysate.

And what are the different substrates which are present or which are going to be get phosphorylated by these kinases. So this by diverging the thumb one single reaction you can be able to get more and precise information about the experimental condition. So with this we would like to conclude our discussion about the ion exchange chromatography. What we have discussed? We have discussed about the basic principle of ion exchange chromatography.

We have also discussed about the different steps what you have to do and what are the crucial parameter you have to consider when you would like to utilize them for running the ion exchange chromatography. And also how the different parameters are crucial to choose the specific matrix as well as the specific pH at which you have to perform ion exchange chromatography and what are the different buffers which are available for cation exchange chromatography as well as the anion exchange chromatography.

And in today's lecture we have also discussed about the potential of ion exchange chromatography in the solving different types of problem, and how you can able to use the ion exchange chromatography to purify the protein from the complex mixture. So with this I would like to conclude our lecture here. Thank you.