Analytical Technologies in Biotechnology
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Module - 4
Electrophoresis
Lecture - 4
Isoelectric Focusing (IEF), 2-D Gel Electrophoresis and Protein Detection Methods

In pervious lecture, we discussed about continuous and discontinuous buffer systems. Also, we have discussed about the, SDS page electrophoresis which is a form of discontinuous system. It was developed by Laemmle, but before that Einstein and Debye's have developed the particular non-denaturing discontinuous system. This lecture, we are going to discuss some more techniques which are utilized for analysis of proteins.

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These are isoelectric focusing and 2 D gel electrophoresis. Also, we are going to discuss about the detection methods. That is, how the protein is detected in a gel and also preparative electrophoresis. That though, it is an analytical technique, but you can always you can always purify very high quality protein here, by preparative gel electrophoresis.

Alright, so we will begin our discussion with isoelectric focusing. Now, isoelectric focusing is an electrophoretic technique, employed for separation of amphoteric

molecules. For example, proteins are amphoteric molecules and this done in ph gradient its separates proteins, based on their different isoelectric points. Proteins or any other amphoteric molecules, all amphoteric molecules will have a particular isoelectric point.

Now, the net charge on an amphoteric molecule depends on the ph of its local environment. So, the amphoteric molecules like as, we will be discussing more about proteins, we will focus on proteins here. They carry a positive charge. So, any amphoteric molecule will carry a positive charge or a negative or a zero net charge, depending on the ph of the environment or surrounding. Now, what is isoelectric point?

Now, isoelectric point is defined as, the ph at which a protein has no net charge, that is the number of negative and positive charges balance each other, they are equal and this nature of the proteins net charge. So, the isoelectric point is a characteristic physico chemical property of the protein.

Now, I think all of you know that, proteins are positively charged at ph values below their pi and they are negatively charged at ph values above their pi and like the charge, amount of charge will depend on how far they are from the pi value or the ph surrounding ph, how far it is from the pi. So, they will, as they will contain a particular charge, net charge at particular ph, depending on their pi values they will migrate to respective electrodes in an electric field, until the protein or the molecule focuses at the position in the ph gradient where, its net charge is zero.

That is isoelectric point. Now, proteins migrate to their respective steady state position. Where ph, in an, ph gradient equals pi from anywhere in the system. That means that, even if you load the protein or amphoteric molecule anywhere in the in the ph gradient, in the gel they will migrates to its pi or isoelectric point, where ph equals their isoelectric point. So, separating proteins according to their net charge is accomplished by generating a ph gradient in an electric field.

So, the first thing in the isoelectric focusing is, to create a ph gradient in the gel. We will discuss that. Now, the effect of proteins size on mobility is minimized here. To do that, the electrophoresis carried out in gels with large pore sizes and if you could recall the low concentration acryl amide gels, like say four percent or around three point five or so or say one percent agros.

So, large pore sized gels, if u could recall, such as, low concentration poly acryl amide gels, around three point five percent or four percent or you can use agro gels, which are one percent agro gels. So, the large pore size, minimizes the molecular sewing. So, and so those restrictive or the migration patterns are not impeded by the pore size here. So, this typically, the protein will run on its charge.

Now, how a ph gradient is created in the gel? A ph gradient is generated with synthetic carrier ampholytes, what are these synthetic carrier ampholytes? These ampholytes are mixture of polyamino poly carboxilite compounds and they carry different charges, net charge. And they are about very small, 300 to 1000 Daltons in size. These are multi charge organic buffer molecules with closely spaced pi values. So, and they have high conductivity.

So, in a mixture of ampholytes there will be a lot of compounds, different closely spaced pi values or isoelectric point values. So, initially the ph of an ampholyte solution, if you make, will be the average of the pk values of the mixture, but on application of electric current, what will happen is, they will cause the ampholyte to migrate towards the electrodes, according to their charges. So, ampholytes, that have pk value above the ph values will be positively charged and those with pk values below the ph will be negatively charged.

Now, as the ampholyte migrate on application of electric current this will result in charges in the local ph, this will result in, changes in local ph, due to the buffering action of the ampholyte. This change in the local ph will affect the change, affect the charge on the ampholyte. So, as they migrate there will be changes in the local ph and they will certainly affect charge on the ampholytes, depending upon their pk values. So, the ampholytes will continue to migrate, until they reach a position in which the local ph equals their pk, that is no net charge or we can say pi value.

So, the result is ph gradient in which the most basic ampholytes are founded the cathode which is, contains a dilute alcoholic solution like NaOH and the most acidic ampholyte are the anode which contains the dilute solution of say S three two four. So, what you have done is, you created a ph gradient in the gel carrier. Ampholytes with defined, ph range can be purchased from different commercial providers. They come in different in ph ranges like, you can go from ph three to ten or you can go from say four two eight and

different ph ranges are of level and their commercially can be purchased from commercial providers.

Now proteins if you say, proteins also contains both there multi charge molecules, they are also ampholytes or amphorates, but they will migrate within the ph gradient until they reach ph, reach a ph equal to their isoelectric point.

So, problem with this, that concentration of ampholyte is higher, but proteins here, cannot be used for creating ph gradient as such. Ph gradient created by ampholyte, mostly the concentration of ampholyte is about to two percent, which is appropriate. And, as concentration, if is lower, that is say below one percent, it will result in unsuitable ph gradient, and above three percent ampholytes solution will be very difficult to remove from the gel and may interfere with proteins staining. So, usually two percent ampholyte concentrations are taken.

Carrier ampholytes are needed, since the protein concentration is generally not high enough to establish a stable ph gradient. So, proteins are not, cannot be used as ampholytes, although we are utilizing the gel to analyze the proteins here. So, and there cannot give a stable ph gradient due to low concentration and the isoelectric points of the proteins, may not be uniformly distributed along the ph gradient. So, in ampholytes you have mixture of ampholyte with lot of different types with closely spaced pi values, so they are more suitable for ph gradient.

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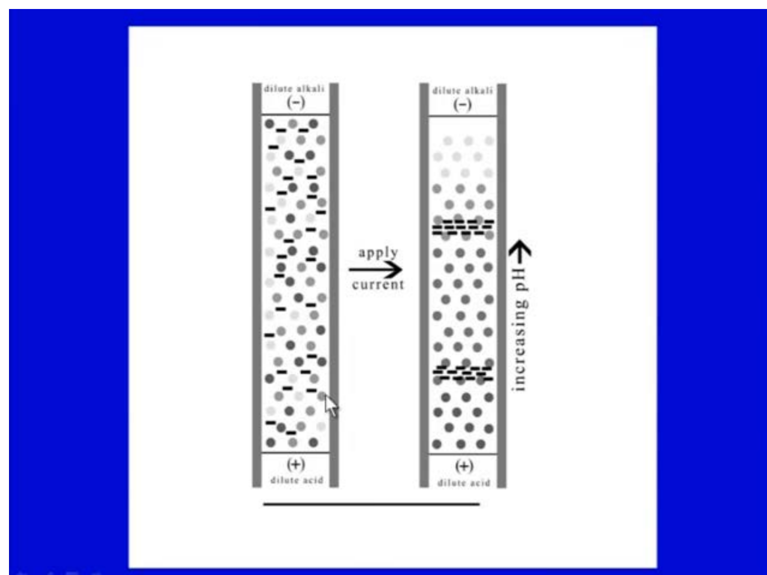

This figure shows here, if you could see that, you have casted the gel here. Now, this gel contains say lot of different ampholyte. There is dilute alkali, that is negative electrode here it is a positive electrode. As you apply the electric current, they will certainly migrate to their respective places and there they will concentrate there. So, that will be the way, how ph gradient gel can be created. Let me show you, on your screen, these isoelectric gels could be both, it could be simple tube gels as well as it could be slab gels. Now, what is done if in a, it is in a tube gel or in a slab gel.

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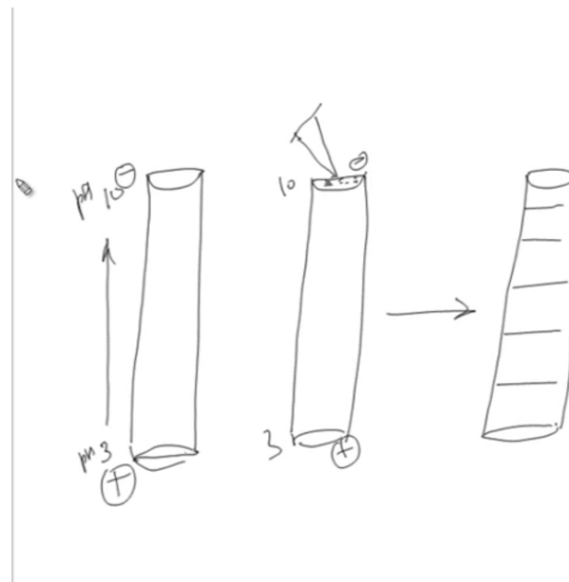

So, first thing is that, in a tube, in a tube gel for example, you have you have the gel in here. Now, this gel contains ampholytes, when you cast the gel, it already contains ampholytes all over the place. So, when you when you apply the electric current, then these ampholytes migrate to their respective places. As they move, the ph gradient is created, as we have explained. And so can have say ph gradient from say, these are two electrodes, then you can have ph gradients from say three to ten or so. Now, here you have a ph three, here you have a ph ten. It will be, you have an increasing ph in this order all right. So, they will be like as they migrate and they settle down at their pi values.

Which you can then, utilize it for this gel, can be utilized for isoelectric focusing. So, what is done next? So, here what you have to do is, then you have to load your sample protein. Now, remember protein could be loaded anywhere like I said, but could be loaded on one side. You have particular ph here and negative and positive electrodes. So,

when you load the protein, the protein and as you apply the electric current, the protein will, this protein sample will migrate according to their pi values. They will migrate towards or they will settle down, you can say focus at the particular side where there pi values, where ph equals pi values.

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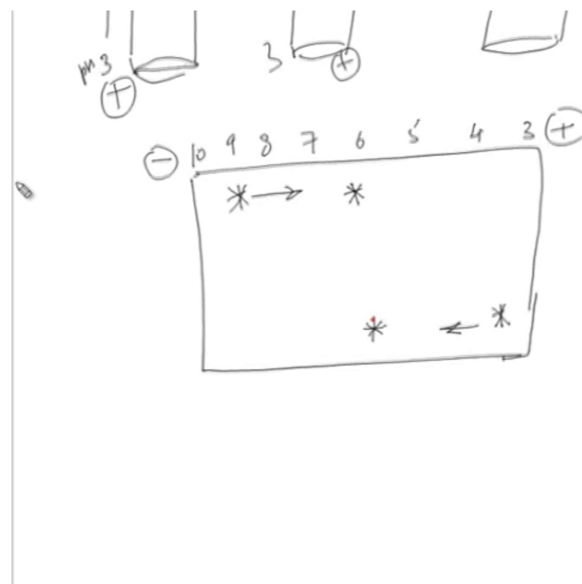

So, when you apply the electric current, then what resultant, you will get is that you get is different bands which could be visualized by staining action. Same will happen in a slab gel. Like a slab gel like for example, I can just show you here. In a slab gel, say there is negative, positive electrode and say the ph is, there is an increasing ph here.

So, if you have say, ph starts from, now you have a protein which you can have either, load here same protein, if you can load here and if the protein pi is say around six then this will move, migrate towards this side. And this will migrate towards this side and finally, they well settle down at ph six or they will focused a particular pi value. So, this is how you will, you can you can focus, you can see that because of the ph gradient due to ampholytes, the protein loaded anywhere will come and focus at the particular place in isoelectric focusing. So, that is why, it is the name is so.

Alright, let us return to our discussion. So, isoelectric focusing is the very high resolution method and it can resolve proteins which are differing in pi values say around or less then point zero five ph volts. So, even if there is spaced by point zero five ph units, they could be separated on isoelectric focusing gel. The technique is performed in non-

denaturing condition, most of the time and it is quite rapid. Like for example, proteins say enzymes and antibodies, anti gels or other proteins, they will retain their activities in these non-denaturing conditions, but many times denaturing agents could be added, but those denaturing agents, like they could be non-ionic detergents or urea can be utilized.

This happens in certain cases, particularly like say proteins may precipitate near there pi value or you want to solubilize membrane protein, then you might require addition of say urea or nonionic detergents even in two dimensional electrophoresis these non-denaturing agents will be utilized. So, this method can be analytical, like it could be just for analysis and not for purification. As well as, this method could be preparative also. Now, typical analytical gels take around say ninety minutes to at two hours or so to run, depending on the voltage condition. Whereas, preparative gels may take more time, say around four hours to complete.

So, here as we have seen that the main points or in I E F is that, you have to first make ph gradient gel and in that ph gradient gel, you can run your protein samples, which carries the particular charge at particular ph according to its pi value. Then, it might migrate to pi value in the gel and it will focus there.

For example, say if diffuses little bit ahead or it is remaining behind, then also after some time it will focus on them, like if it diffused little farther, then it will come back and focus. So it is very good technique. It is a high resolution technique. Now, like I was saying it could be analytical as well as preparative technique. Like you can, use it for just for analytical proposes or you can purify the samples.

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Analytical IEF

In analytical I E F, it is mostly carrier out in large core acryl amide gels and these are like five percent t and three percent c. Hope you remember what is t and what is c? t is total gel concentration like acryl amide concentration and c is the cross linker percentage in total concentration of acryl amide, that is acryl amide. It could also be run on agro gels, like one percent agro gels. So, traditionally earlier one to two millimeters thick gels were used, but then high cost of ampholytes makes the procedure very expensive. So, what was done? Introduction of thin or ultrathin I E F gels was proposed, were introduced and these were like say around or less point four millimeters or around point one five millimeter gels were introduced.

They have considerably reduced the cost of running I E F gels. To prepare these ultrathin layers gels, what is done is that, carrier ampholytes and it will be of suitable ph range as for your requirement. So, you have to take care that, your protein pi lies in that particular ph range and it could suitably in such a mixture, suitably isolated and differentiated. Also, carrier ampholytes will be mixed with acryl amide solution and initiator system. So what is initiator system here? Poly acryl amide gels, here in I E F for polymerized by photo polymerization and also chemical polymerization, but so here initiator system is includes riboflavin, which could be around five microgram per ml, if you could recall riboflavin is used for photo polymerization, rather than like a free radical.

Here, you have riboflavin, then you have ammonium persophate also and timid also. Ammonium persophate around point zero one five percent. And timid point zero five percent. Now, why we are use, why all these three chemicals are used here? It will results in more complete polymerization and it has been seen, when all three initiators are present, then the polymerization is much better.

So, the polymerization takes place for almost more than two hours and where in second hour particularly, direct light from a florescent lamp or other source the gel will be exposed to that light and so photo polymerization. So, you can say photochemical polymerization can occur in here. So, this is done in I E F gels. Now, for most analytical isoelectric focusing, the horizontal poly acryl amide slab gels are used. Even in agro gels, they are horizontal gels are utilized. But, poly acryl amide is most commonly used.

Now gels are cast on glass plate or they can also be cast on specially treated plastic sheets. Now, it is a laborious and you have to work very carefully when you are casting the gels. So, many times, we speak casted or gel strips, with different ph rates ranges are available commercially. You can directly buy. Of course, it will be expensive here, it is a very hard to, you can make gels, but it is very hard many times to reproduce the results, which could be done in commercially available gel strips or gels.

So, once you casted this on a glass plate like we have, said to mix all the sticks like acryl amide, ampholyte solution, initiators and then it has to be polymerized. Once its polymerized, as we have discussed earlier, also it could be polymerized taking two plates and then one plate is moved, all different way then, electrolyte strips are for, are saturated. Now, these electrolyte strips are saturated, for both anode and cathode. So one like one point one molar phosperic acid is utilized for one anode and NaOH, which could be from one to one molar NaOH is at cathode.

So, you have positive and negative electrode buffers you can say and they are placed on the gel. And they are in contact with the power supply. So, they are like these electrolyte strips, make contact and they are maintained an this contact is maintained by electrodes of platinum wire. Now, I E F can also be done in tubes, like I was saying, you can do it in slab gels, but you can also do it in tubes and this constitutes if you are doing it in tube gels, tube gel also constitutes the first dimension of two dimensional gel electrophoresis.

After the gel is cast, the potential difference is applied which allows the formation ph gradient between the anode and cathode. So, what is done? Once you have made, you have prepared this, all this apparatus and this particular agreement is made, then first thing is, the casted gel contains ampholyte, they are scattered just like that.

So, a potential difference for some time is applied, say about thirty minutes or may be around that time. The ampholytes will migrate to their respective pi values and this will create formation of ph gradients will be done between the anode and cathode, so that is the first part of running the gel. Once the ph gradient has been made, then sample could be loaded. Now, suitable sample can be prepared by dialysis or gel filtration and you have to see, what is the chemical buffer concentration? Other things have to be looked into.

So, they could be prepared by dialyzing for suitable in suitable buffers. Now, samples once ph gradient is in place, samples can be applied to these thin I E F gels. In tube gels, you will be applying it to one hand actually, but in like say thin I E F gels, they are directly placed by, you can what you can do is you can soak the filter strips in the sample. They will, when you are soaking, the enough contain sample. You make sure that, they contain enough sample. These buffer strips, containing the sample could be directly placed on to the gel.

Alternately you can, one can also load say a few micro-litre of sample, can be placed directly on to the surface of the gel. Maybe there might be a few cassettes sort of, where you can put the gel, where those things could be put onto either the middle of the gel or on one side of the gel. Then, once you loaded the sample, at the through buffer strips or directly few micro litre on to the gel in certain cassettes. Voltage is again applied and for about a say thirty minutes or so. What it does, is it allows samples to electrophoresis out of the paper or from those cassettes into the gel. So, applying voltage makes sure, that your sample is loaded on to the gel.

Now, to achieve the rapid separation, then once you have loaded the sample, you can run the gel for like say at a particular voltage and like said, to achieve the rapid separation relatively high voltage are used. And, when you use high voltage certainly the heat generation will take place. So, gels are run on cooling plates, to minimize this heating. So, once gel is run then, the gel could be visualized on by staining, like say if you are

staining it with coomassie blue stain, then around point five micro gram of protein is required for dye staining and fifteen nano gram of protein per ((Refer Time: 27:29)) is required for silver staining, that is the sensitivity range of the method.

Now, staining solution for protein in I E F, they consists of point zero four percent of coomassie brilliant blue are two fifty or with containing point zero five percent crocein scarlet in twenty seven percent ethanol plus ten percent acidic acid, gels are soaked in staining solutions for one hour. Then staining is done, but with several changes of forty percent ethanol and ten percent acidic acid. Silver staining can also be done for higher sensitivity.

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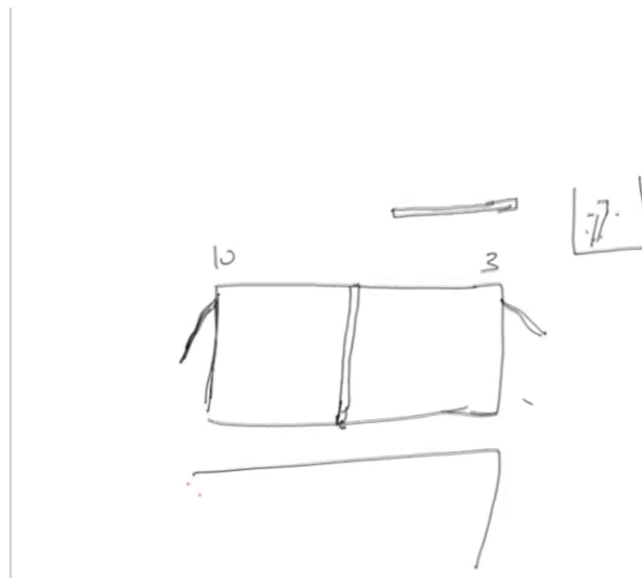

So, let me just show you little bit, that how this whole thing was done actually. So, what is done is, you have a very thin gel of certain size, which is there and this gel will be, they will be like Vicks or paper filters, like I said, where u will have phosphoric acid and NaOH at different electrodes. So, they will be connected here, like all over here, thorough platinum wire to the power supply. Now, loading the sample, could be done like, you can have buffer strips, with thin buffer strips and these buffer strips are so thin, thus, sample solution. So, you can soak these buffer strips in sample solution and then they could be put, say around one side or many times you put them in the middle also.

Simply, you can put them in middle of the gel, then on either side. And this is a ph gradient gel. Say, we say three to ten or different ranges could be neutralized. Now, what

is done is, this strip contains the solution and this remains in the here. And as you apply the voltage, then from the strip, from the, this buffer filter strip, the solution will get into the gel, this thin gel.

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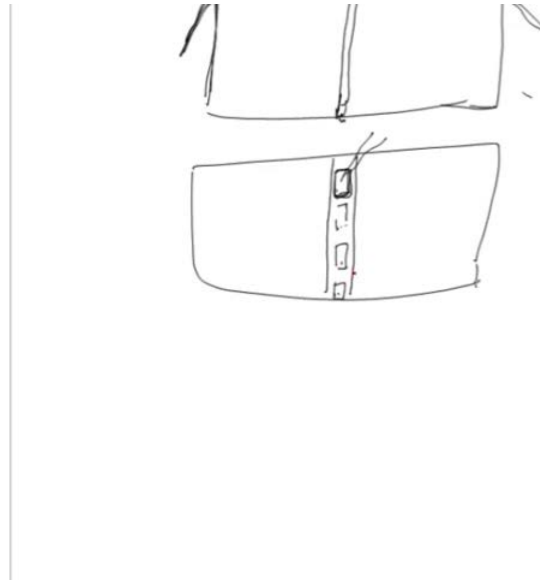

There could be another way is like, what you can do is, rather than having the particular buffer strip, they could be simple, like obstacles you can put in here, on both side. Samples in very small amounts, like you can have separate, like you want to load, more than one sample here. They will like slots sort of here. Now, here remember these are not wells as such actually, rather you have slots, which you are making, the physical material.

You have put in like say, cassettes sort of or empty cassettes or hollow kind of plastic cassettes kind of, then sample could be loaded in here. So, you can load your samples, separately in each of the slots and then it could be run. And you can perform, the run and as you run it for thirty minutes, this will electrophores into the gel. So, this was about the gel, analytical gel electrophoresis, likewise the preparative isoelectric focusing can also be done.

So, what we have seen till now, analytical isoelectric focusing, which is done on ultra-thin or very thin gels or in tube gels, where you are not really aiming to purify the sample, but in preparative isoelectric focusing, you will be working on like spatial cells or auto pores which are utilized. They could be like in solutions or other wise and large

amount of proteins can be like, could be separated in that and could be subsequently for different application. So, we are not going into details of that, but it is possible in isoelectric focusing, to purify the protein and you get very good quality, that is in terms of purification like homogenous sample. You can utilize it for different application.

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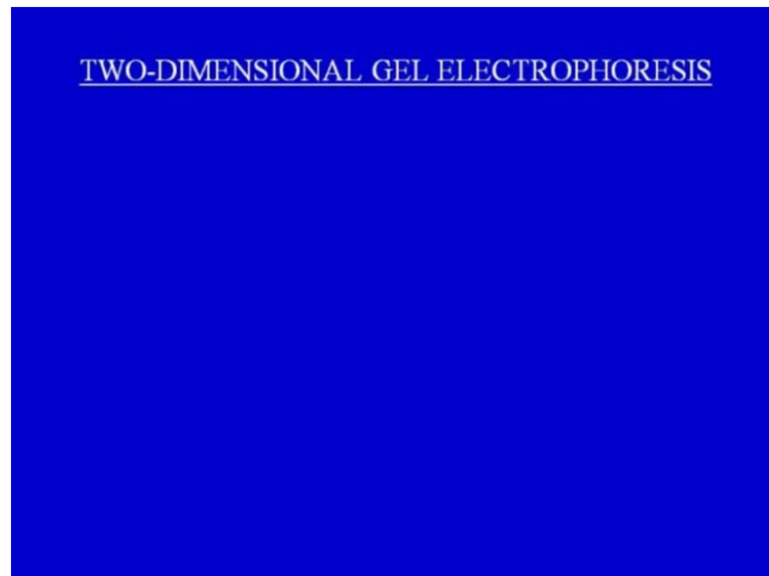

So, we move on to the next technique, that is two dimensional gel electrophoresis. This technique combines the technique of isoelectric focusing, which separates the protein in a mixture according to the chart. Then next, will be the size separation technique at ninety degree. Now, remember the two dimensional gel electrophoresis means, any technique which combines two runs at ninety degrees or at perpendicular to each other, like you with one technique, you running one direction and then you, same gel could be run at ninety degrees like you turn up in perpendicular direction. But, most widely used combination is the isoelectric focusing and SDS page.

So, two dimensional gel electrophoresis, which we are going to discuss about is about, this particular one. That is first the separation of protein occurs on isoelectric focusing, on the bases, on their pi values and then they run on at ninety degrees on SDS page gel. Now, the first dimension is isoelectric focusing, is carried out in poly acryl amide gels in narrow tubes and this could be say one to two millimeters internal diameter, in the presence of ampholyte, like I showed you, that first you have to create the ph gradient.

Here, you will use, denaturing conditions. Where, eight molar urea and non-ionic detergents could be used. Now, the denatured proteins, separate here, according to their isoelectric points and once that is done, then the gel is extracted from the tube. You can just apply it, slight pressure to one end and it will come out. Then it incubated for fifteen minutes in buffer, containing SDS. Now this, what happens here is that, binding of SDS to the denatured proteins occur here and then they are placed along the staking gel.

So, you have casted the SDS page gel which is, discontinuous gel. So, you place this gel, which was in tube and which has separated the proteins, from the basis of their pi values. You will just lay it on the stacking gel and what is done is, you will pour some molten agros here, over the gel. In electrophoresis purpose. Now, once agros has set, then the electrophoresis is started and the SDS bound proteins will run into the stacking gel and then separate according to size in resolving gel. So, that is a very simple way of running a two dimensional gel.

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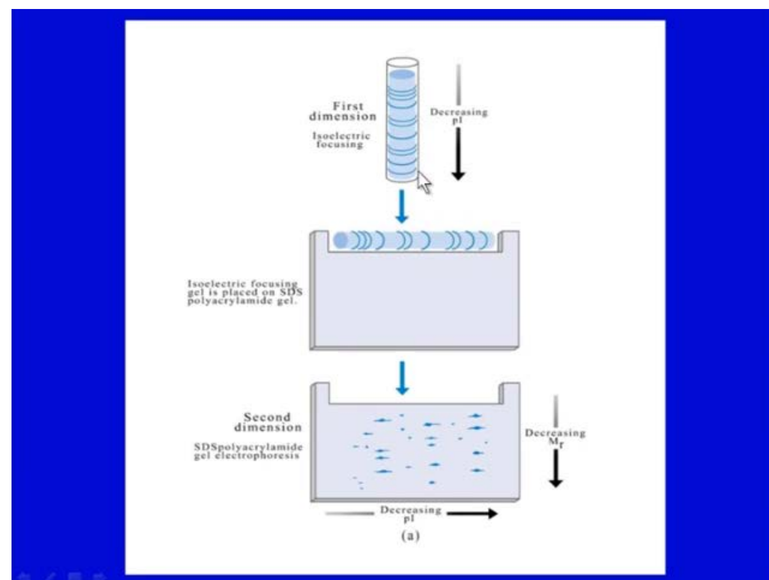

What you have here is, if you can see here in this figure, first thing you have done is isoelectric focusing has been performed. Then in isoelectric focusing, you can see various bands here, of proteins, which you can see after staining. If you do not want to stain, it is also alright, but one time, you can see that it is doing well, but what happens is you will lay this gel and it will run, it will run in this direction and now it will run in this direction, that is at ninety degrees to the pervious run.

So, you have put it on the staking gel and then it will run, it could be a gradient gel or step gradient or continuous gradient gel, where your pore size will decrease as you along. What will happen then? The proteins will run as per they are, one time one basis of charge. Now, they will run on the basis of their size.

So, smaller molecular weight protein will run faster and larger molecular protein will remain on the top. So, that is how, you will be able to run the same sample in two direction and gives. Now, what is advantage of this is that, you can handle lot of proteins at a time. Like say you would like to do protein analysis of particular tissue sample or cell sample, I want to see the expression, the particular protein and you can compare the native one and where we have, you have put in cloned new protein, in a, in a particular system and want to see the expression, then comparison can be made and the extra band width, which is shown of a particular protein could be made out.

Likewise in certain diseased condition, the normal cell and diseased cell could be compared for their proteomes, that is total proteins and then again gives you lot of information. Likewise unknown protein sample, from a particular system could be analyzed.

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Now, here you can see, this is how two d gel will look like, after the two dimensions. You can see there are whole lots of bands of proteins, like spots here. These are the spots, remember to be, they could be many or few thousands spots here, which can be

handled at a time. This protein spots, you can pluck the spot and this could be analyzed on mass spectrometric which can give you the sequencing results and you can identify the proteins in here. So, this is a, you can say high throw put procedure to analyses lot of proteomes from different system actually. And very useful technique, in combination like I E F and SDS page. So, two dimensional gel electrophoresis is one which is very good technique for and it is widely used with mass spectrometric.

Now, when you have run these proteins on different kinds of gels, now let us little bit discuss about the detection methods, the protein separated in gels are detected mostly by staining with dyes or metals. Coomassie brilliant blue r two hundred and fifty is the most commonly used for routine work but, coomassie brilliant blue g two fifty is also used for low molecular mass polypeptides. Then, sliver staining is the most sensitive method for proteins and as well as nucleic acids and is used for assessing high purity of sample.

Then, there is another copper staining, which is rapid and sensitive method for SDS page only. Where, proteins are not fixed, you can illute also protein. There could be other methods, florescence and radio labeling. Now, all methods are performed with precautions, taking clear, they are done at room temperature, gentle agitation of shaking on orbital shaker and gel and staining are contained a glass container or plastic container.

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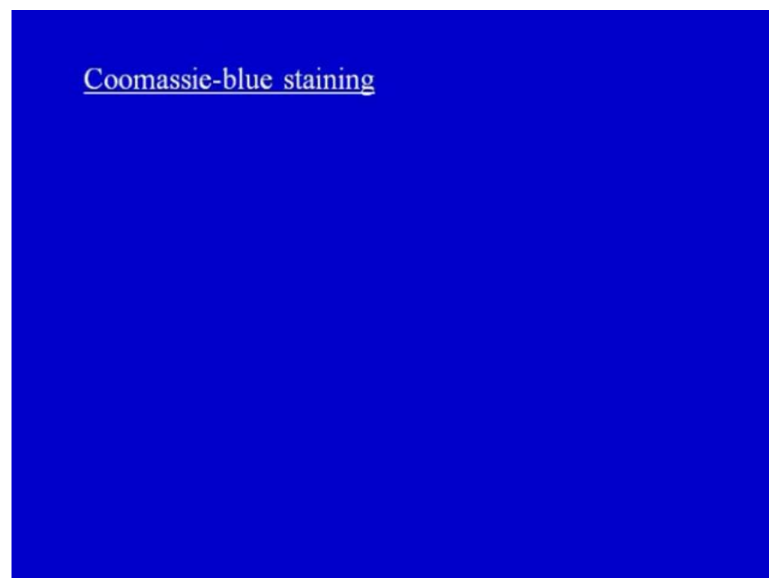

Now, coomassie blue staining is a very popular method and very routinely used method. So, what is done here? Staining solution consists of point one percent coomassie blue r

two fifty plus forty percent methanol. Now, this methanol and acidic acid also, fixes the protein in the gel that is, precipitates the protein in the gel. So, that it is fixed here. That why is called fixed fixing the protein. After electrophoresis gels are incubated, with agitation, in staining solution for thirty to sixty minutes then, after that the excess dye is removed by de-staining the gel in acidic acid and methanol. Solution same percentages, with several changes.

So, that the background dye is gone and then you conclude clearly, see the protein bands. Gels contain low molecular mass polypeptides are fixed in fifty percent, methelon and ten percent acidic acid for one hour, then stain with point zero two one percent coomassie brilliant blue, g two hundred and fifty and ten percent acidic acid. Then distain with ten percent. So, for smaller molecular with polypeptides, g two hundred and fifty is more common. In terms of sensitivity protein bands containing point one to one program could be seen in here, in coomassie brilliant blue staining.

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Then, silver staining is almost like hundred times more sensitive than, coomassie blue staining. It can detect like ten to hundred nano gram of protein or nucleic acid per band in a particular band. Method is based upon binding of sliver ions to proteins and reduction of the silver ions to metallic silver. So, fixation of proteins in gel is performed with methanol and acidic acid and then methanol is removed by washing with ethanol ten percent five percent acidic acid. Here, then proteins are oxidized in a solution of

potassium di chromate dilute nitric acid, then excess oxidizer is washed of the gel with water and gels are treated with sliver nitrate solution.

So, the sliver ions bind to the oxidized proteins and then they subsequently reduced to metallic silver, but treatment with alkaline formaldehyde. So, color development happens that could be stopped with five percent acidic acid after sometime otherwise, lot of background might or lot of diffused bands might be seen. Another method of or silver staining is available in a kit form and it requires single staining and development step and it could be used for both proteins and nucleic acid.

After fixation of fifty percent methanol and ten percent acidic acid five percent glycerol and after washing with water, gels are treated with solution containing silver amine complex, bind to ((Refer Time: 42:47)) asset. Then, sliver ions transfer from this to proteins or nucleic acid. Then, they could be reduced to metallic sliver with formaldehyde. So, silver ions do not accumulate in the gel and that gives the less background. Again, you have to stop the color development in proper time. Photo development, can also be done like light could be used to reduce the silver ions to metallic silver.

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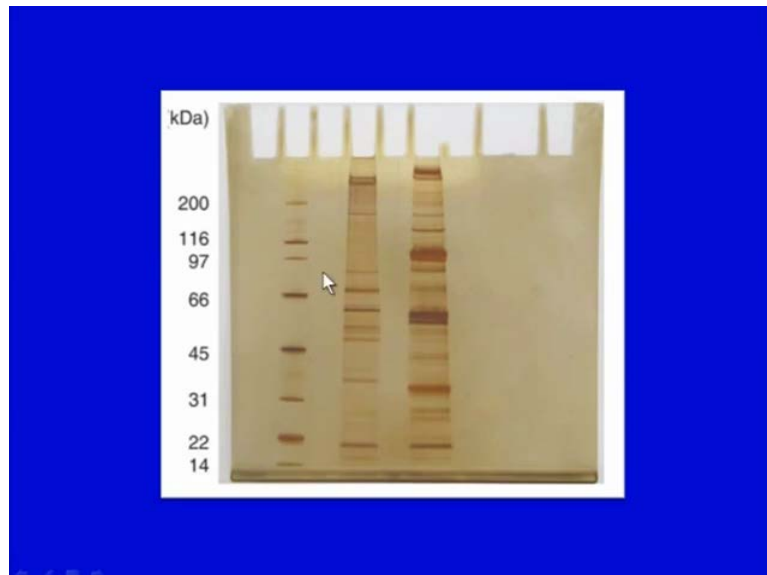

This is how the gel looks like, in when you have silver staining. Like I said, it has a very high sensitivity or the highest sensitivity more than the coomassie brilliant blue staining. Then, copper staining is a very rapid single step staining of the only SDS features.

Staining is performed by incubating gels in point three molar copper chloride for five minutes, followed by washing with water. So, what will happen is that, blue green precipitates of copper hydroxide in the gel will be seen, except where there is concentration of SDS. So, where proteins bands are there and their proteins contains lot of SDS, so, there copper will not are these the blue green precipitous will not be seen.

So, again the black background, you can see clearly, the protein bands and the proteins are not permanently fixed. So, they can be illuted here, but the problem is that, electrophoretic patterns will not last for long. So, you have to, when you dry the gels, they will be gone. So either it should be store them in water or photograph it right away or you can restrain with coomassie blue. Sensitivity is there somewhere in between the coomassie blue and silver staining.

So, copper staining is also very good method, where you will not do preparative electrophoresis. There are methods also like fluorescent dyes, that bind non-covalently to proteins they could be utilized like say UV elimination or others you can used fluorescent dyes. Also, you can attach, but that will affect charges.

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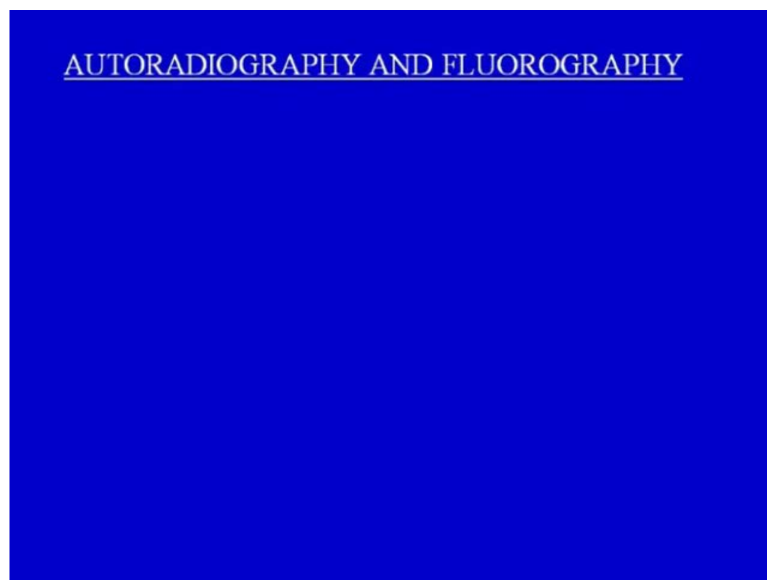

Then autoradiography could be used, or fluorography could be used. If you rememeber we have discussed this in, section where we have spoken about autoradiography and radiography, like in gels you can put radioactive material. And then through autoradiography, it could be seen. They could be enhanced by fluorography, where

exposure of film to the secondary light emitted by either, by intensifying screens or floors etcetera could be used.

So, these methods also could be used utilized but, they are not so commonly used methods. Enzymes can be detected on the basis of their activity, after running the gel but, it should be non-denaturing gels Substrate could be added to the on the reaction, which can form insoluble products, that could be seen here. Like in for example, proteins and others, this could be utilized,

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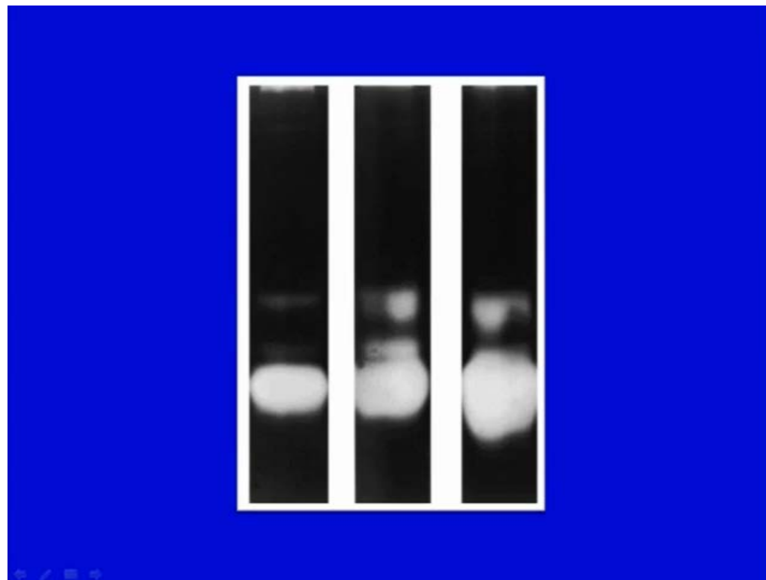

This is how it looks like, when you are doing those activity patterns could be seen. Through different methods of staining, we are not going to discuss those in here. These are gelatin, the gels contains gelatin and like for example, protein they contain certain part of gelatin and could clear bands could be seen in here.

Quantification as such is not possible but, quantification in the gels done by comparing with standards like known amount of the sample and then unknown protein could be compared by visualization or digitally. So, that could be a method to, like by generating standard curve, you can really quantitate it. Also radioactive proteins can be exercised and then on liquid synthelation counting, that could be done to quantitate the protein. Scanning densitometers is usually convenient method to quantify the particular amount of protein. So these are few methods of quantification.

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PREPARATIVE ELECTROPHORESIS

Now, little bit preparative, like this electrophoresis technique is most, more of an analytical technique, but you can use it for electrophoresis as well. So, here in preparative electrophoresis, this could be two methods, one is that you illute it from the gel and or another would be can do continuous electrophoresis.

So, in illusion, like you can do it by two methods, one is the simple diffusion or passive diffusion method, where what you do is you can cut the gel from the band, from the gel protein band gel or nucleic acid band and you can macerate it. You can leave it in the buffer and the protein will diffuse into the solution, but recovery is very low. The process is very slow, then protein is in very diluted form.

Other way is, electro illusion, that is you force the protein thorough electric current to migrate to and out of the gel. Like say, you can put them in a dialysis bag and then can run. Can apply electric correct and might come out of the gel, because they have to run particular electrode, particularly for nucleic acid. Then there could be contains flow electrophoresis. Where, it could work like a column, that you continue the electrophoresis, until all of the proteins migrate off from the bottom of the gel.

If you could remember, we have to switch of the gel, when you are having say bromophenol blue or dye reaches the bottom, but here you allow it go through. The chamber is sealed with dialysis membrane to prevent the proteins migration to the tank buffer. Then, that could be even, you can put uv monitor or fraction collector those

things could also be done in here. Remember this, gels for preparative electrophoresis are not thin gels, as we are utilizing, they are much thicker than gels.

So, that more amount of could be loaded and good amount of and you can do it, in like one could be done in tube gels, continues flow and thick gels could be passed and lot of protein cast and lot of protein samples could be of high quality, could be purified in here. Purity is quite high here.

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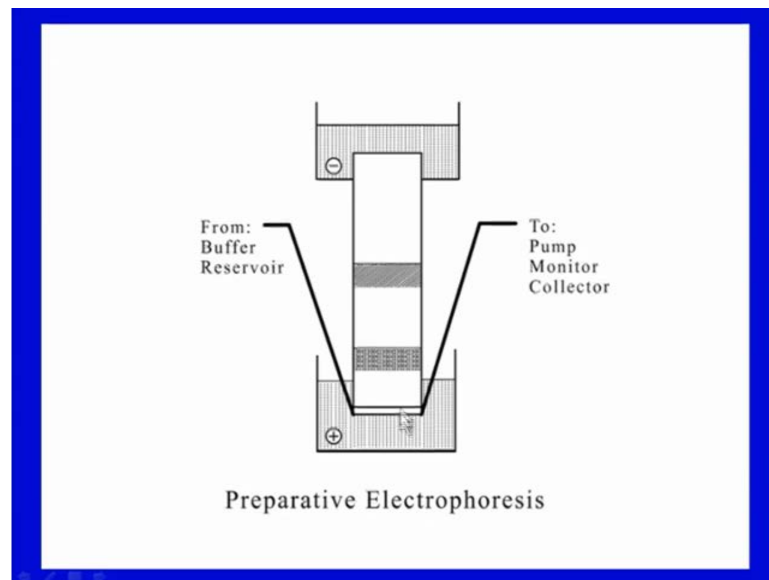

This is simple thing, like you have gel and there is a buffer reservoir here, but, you collect it and this would be directed to a pump or monitor. This is how over run the gel but, of course, you do not allow it pass to the into the tank buffer. So, this is how preparative electrophoresis can be performed, where you can recover lot of samples actually.

So, we can summarize todays lecture here. What we have gone through, todays one is isoelectric focusing, which is the very important technique and you can here purify or you can separate protein which are differing from point zero five to ph units. That is a quite good. On the bases isoelectric point, you can separate these proteins, analyze them, even you can purify these proteins in preparative isoelectric focusing ph gradient is made through ampholytes and then once the ph gradient is made, proteins migrate to the particular ph, which equals to their pi and they focus on their.

This could used for both analytical method and preparative propose two d gel electrophoresis, the most commonly used which combines I E F and SDS page and it is widely used for analysis of proteons, like proteins from a system. Even comparing saying two systems, minor differences like say over expression of proteins or normal in disease cells or otherwise it could utilized for lot of the application and it is combined with mass spectrometry.

Also, we have seen the detection methods where coomassie brilliant blue staining, silver staining or copper staining, could be done. With silver staining is most sensitive of the thing. And preparative electrophoresis, is one other method though, chromatography is more common, because you can use, you can purify large amounts but, in here also you can purify the sample with the very high purity, through preparative electrophoresis. So the next lecture, we will move on to the electrophoresis of nucleic acids.

Thank you.