Principles of downstream techniques in Bioprocess – a short course
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Lecture – 15
Electrophoresis

In this class we are going to talk about electrophoresis and SDS page. They are not really downstream process but they are techniques which are used for purifying proteins and identifying their sequence as well as trying to understand the structure. So they are very useful techniques used in analytical processes.

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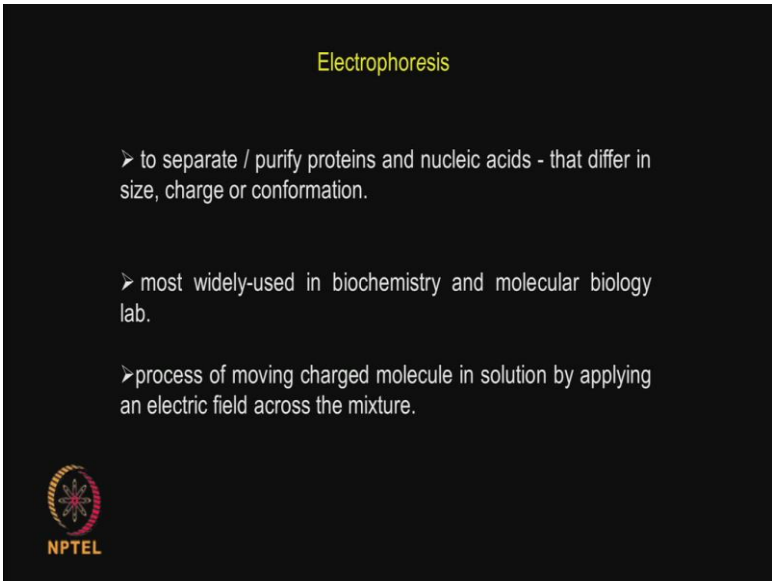

So what is electrophoresis? It is used for separating and purifying proteins and nucleic acid based on their size, charge or confirmation. It is used mostly as I said in biochemistry lab or molecular biology lab. So what you do is, you try to move the proteins, the charged molecule in a solution, by applying an electric field across the mixture. So based on the charge that's present on the protein, based on the electric field you are applying, based on the time protein separate out, either in one dimensional or a 2 dimensional matter. So we will look at it in more detail.

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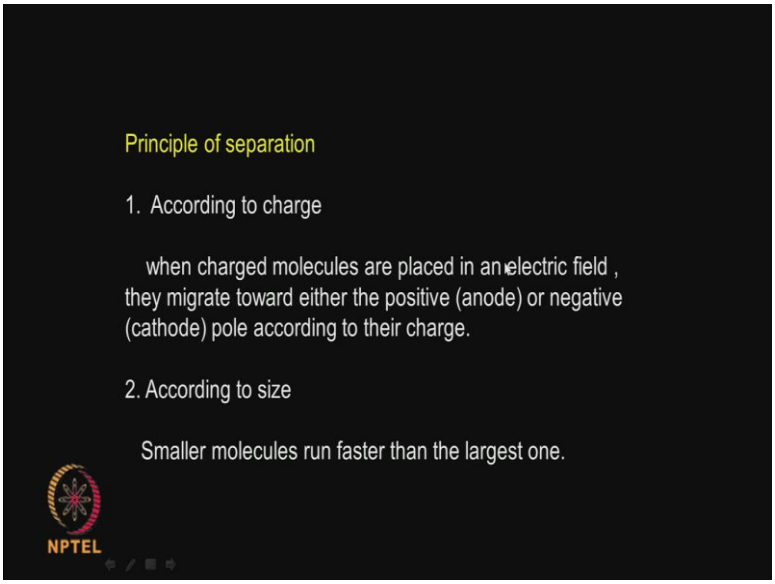

So what is the principle? It is based on the charge. According to charge when charged molecules are placed in electric field, they will migrate, either the anode or to the cathode, depending upon the charge that is present on the protein. According to the size, so smaller molecules will run faster than the larger one. So smaller molecule will try to go faster whereas the larger molecules based on the molecular weight will try to move slower.

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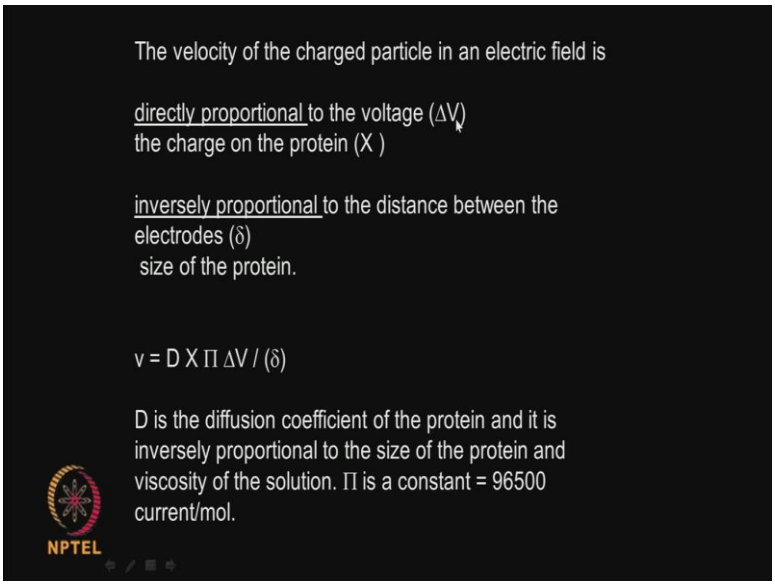

So the velocity of the charged particle in electric field is directly proportional to the voltage, the charge on the protein. So higher the voltage, higher the charge. The charge on the protein is same. Protein can have different charges then inversely proportional to the distance between the electrodes. How far the electrodes are placed and also the size of the protein. So larger the protein, velocity will be lower. Smaller the protein, velocity will be faster. So they are given by this relation velocity v on the left hand

side d is the diffusion coefficient okay.

X is the charge on the protein, pi is a constant, this is given by 96500 current per mol. Delta v is the voltage difference and this delta is the distance. So as you can see the velocity is directly proportional to diffusion coefficient directly proportional to the charge and directly proportional to the voltage, you are applying but it is inversely proportional to the distance between them and the diffusion coefficient is the function of the size of the protein.

Okay it is function of size of the protein, viscosity of medium and so on so if I have 2 proteins with the different charges, then they will move at different velocities. So after a certain time, they will be attaining different distances from the starting point and that is what it is this is the principle by which they are separated.

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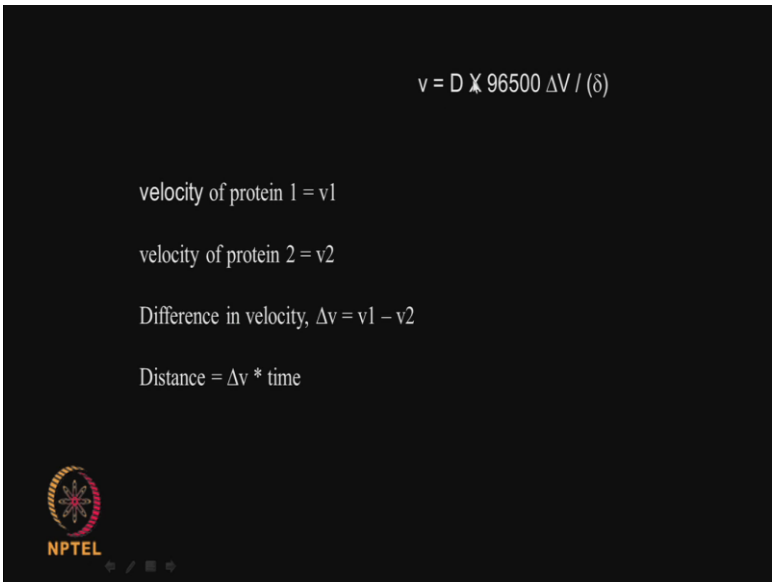

So the velocity of the protein 1 will be v1, so v is given by this, so that v1 is calculated based on the charge on the protein as well as diffusion coefficient of protein 1. So velocity of protein 2 will be v2, again v2 will depend upon the diffusion coefficient that is inversely function of a size and also the charge so the difference in velocity will be delta v = v1 - v2. So the distance will be distance traveled in certain time will be delta v multiplied by time. So if I give longer and longer time, I will achieve longer and longer separation okay and so the separation depends upon the diffusion coefficient which is inversely proportional to the size and directly proportional to the charge the protein has acquired.

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Okay this is the principle by which this type of separation takes place. So what are the instruments and reagents you need? You need a buffer, you need a power supply of course supporting media, detection and quantification okay these are the various reagents and instruments we require. So the buffer this carries the applied current, it establishes the pH, determines the electric charge in the solute, so high ionic strength of buffer produce sharper band, low but it produces very high heat.

So temperature could be very high. So commonly used buffer are Barbital buffer, tris EDTA for protein Tris acetate EDTA and Tris borate EDTA 50 mol/L for a pH of 7.5 to 7.8. So this type of buffer are commonly used.

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So support material used in electrophoresis is, it could be paper, it could be starch, it could be agarose,

polyacrylamide cellulose acetate and so on. Some of them are hydrophilic, some of them are hydrophobic. So we can use different material depending upon a requirement.

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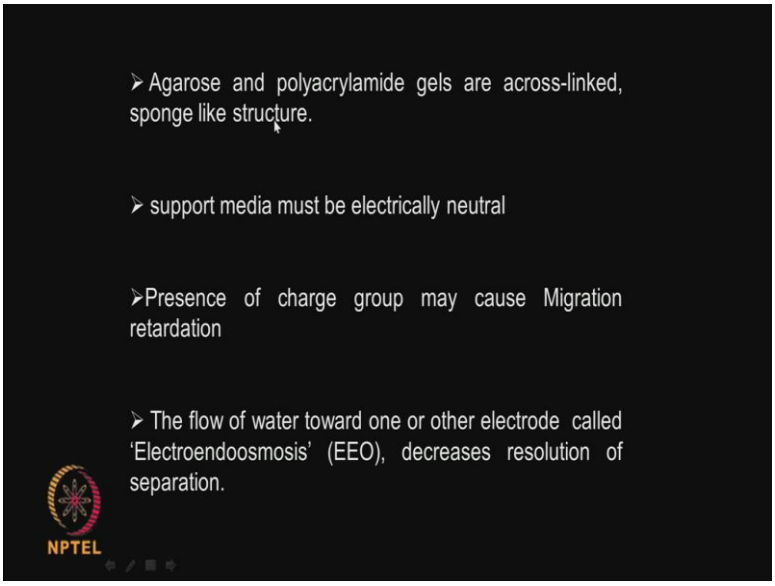

So agarose and polyacrylamide gels are across-linked, sponge like structure. So the support media should be electrically neutral because you don't want the voltage or which you are applying to get drained. Presence of charge group because migration, retardation, again the flow of water toward one or other electrode is called electroendoosmosis EEO. This decrease the resolution of the separation, so if there are any charge group present in your gel that is going to retard the movement of the protein.

Because the protein are moving because of the voltage that is applied at a distance on the 2 on 2 plates and also if the water flows from one or the other electrode, then it is called electroendoosmosis so it decreases the resolution of the separation also. So these 2 are problematic in addition, I also mention that heat generated is also a problem because if you apply very high voltage, this you get very sharp beads and then heat is also liberated.

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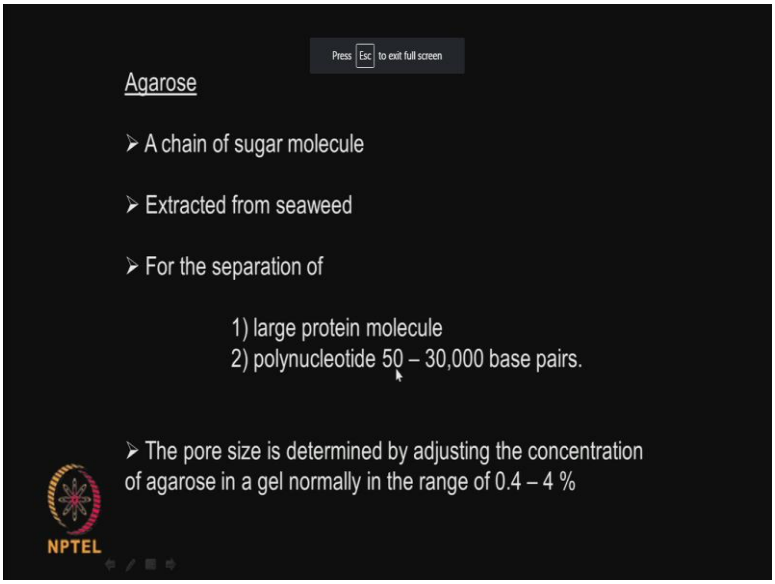

What is agarose? It is a chain of sugar molecule. It is extracted from seaweeds is very good for separating large protein polynucleotides 50 to 30000 base pair. So the pore size is determined by adjusting the concentration of agarose in a gel. Normally it is in the range of 0.4 to 4%.

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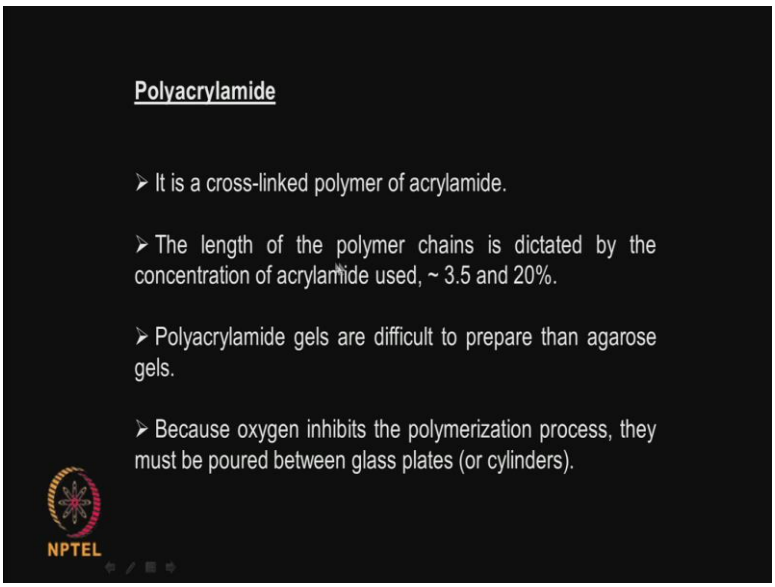

Polyacrylamide. It is a cross-linked polymer okay, it is synthetic polymer, the length of the polymer chain is dictated by the concentration of the acrylamide. Generally it is about 3.5 to 20 %. These gels are difficult to prepare than agarose gels because oxygen inhibits the polymerization process. They must be poured between the glass plates okay so we can go to agarose the gels or we can go to polyacrylamide.

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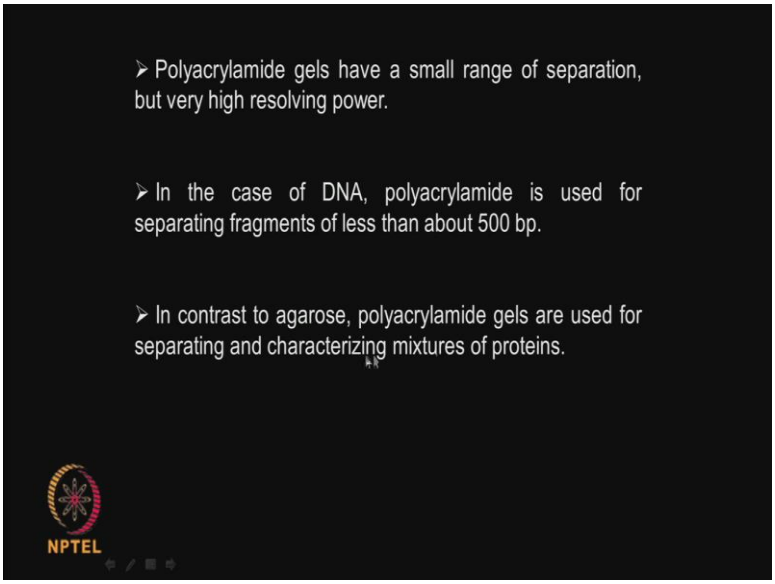

Polyacrylamide gels have a small range of separation but it is got very high resolution in the case of DNA, polyacrylamide is used for separating fragments of less than about 500 base pairs in contrast agarose, polyacrylamide gels are used for separating in characterizing the mixture of proteins. So if you want to separate characterize large number of proteins then its good.

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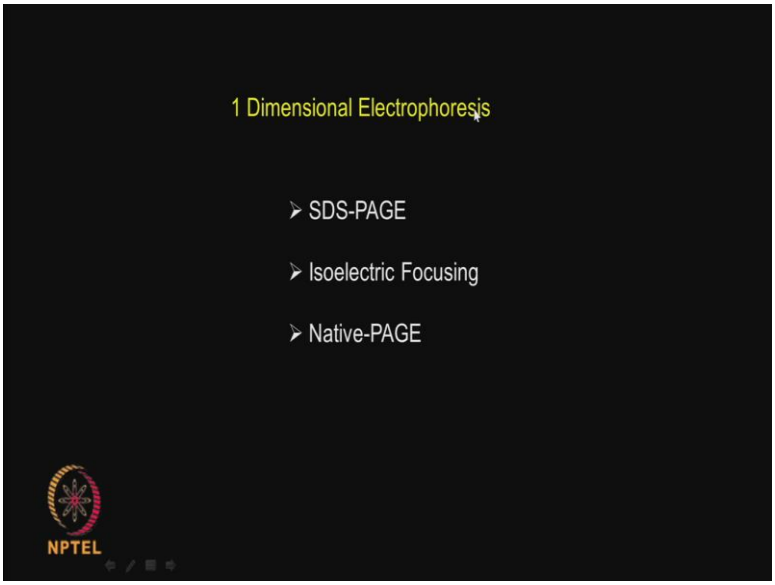

So we have a 1 dimensional electrophoresis and we also have 2 dimensional electrophoresis. I'll briefly touch upon that. So the 1 dimensional electrophoresis consists of SDS page, isoelectric focusing, and native page. These are the three techniques okay. SDS page, isoelectric focusing and native page.

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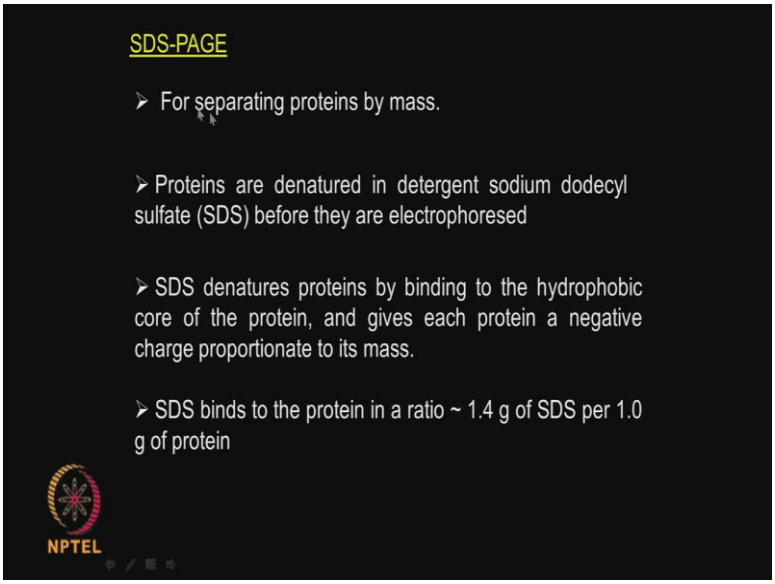

So SDS page for separating proteins by mass, proteins are denatured in detergent, so why do you call SDS? Sodium dodecyl sulfate. This is surfactants sodium dodecyl sulfate. So what it does? It denatures the protein, so the protein sort of binds or it forms agglomerate. So that's what happens actually. SDS denatures proteins by binding to the hydrophobic core of the protein and gives each protein a negative charge proportional to its mass okay SDS binds to proteins in a ration of 1.4 grams of SDS / 1 gram of protein that is how SDS works actually.

So basically you are denaturing the protein that means protein will lose its three dimensional structure and SDS attaches to the hydrophobic core of the protein. So by doing that, there is negative charge given to the protein based on its mass, so the separations will be based on the charge which is in turn based on its mass.

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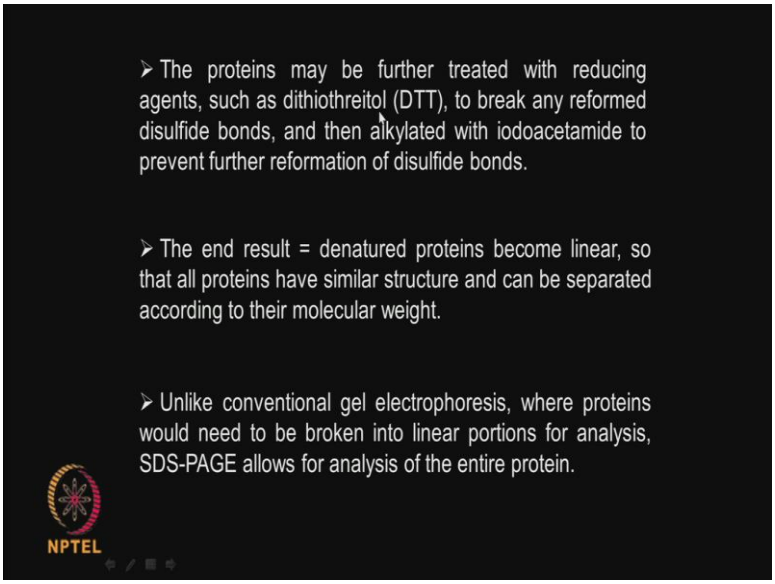

The protein may be further treated with reducing agents such as dithiothreitol, break any reformed disulfide bonds basically we want to break the protein. When you do SDS page, the protein loses its confirmation, so loses its activity alkylated with iodoacetamide to prevent further reformation. So the end result will be a denatured protein. It become linear, so all the proteins will have similar structures and so they are separated based on their molecular weight.

So in SDS page for separating proteins, only on the molecular weight, so you are not looking at the three dimensional structure confirmations and so on actually. So unlike conventional gel electrophoresis, where protein need to be broken into linear portions, in SDS page we can look at the entire protein in the linear form. That is the advantages okay.

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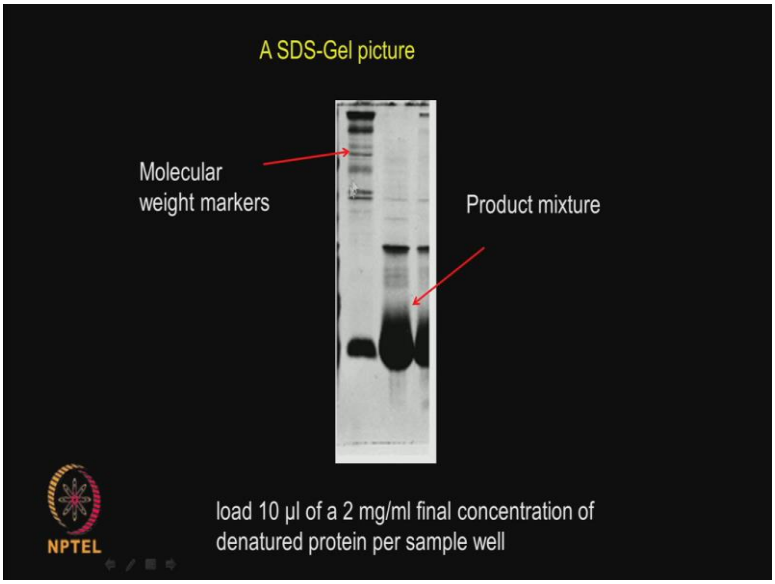

So typically this is how it will look like. Okay you have the molecular weight markers here. So you can buy standard molecular weight marker and each marker will represent certain molecular weight okay. Then we have the product mixture, we will load it here. So generally you load about 10 micro liters of about 2 mg / ml denatured per protein per sample and when you apply the voltage protein after certain time proteins will start traveling upwards and each band will represent a molecular weight from this molecular weight marker we can read out what type of protein molecular weight protein in this mixture.

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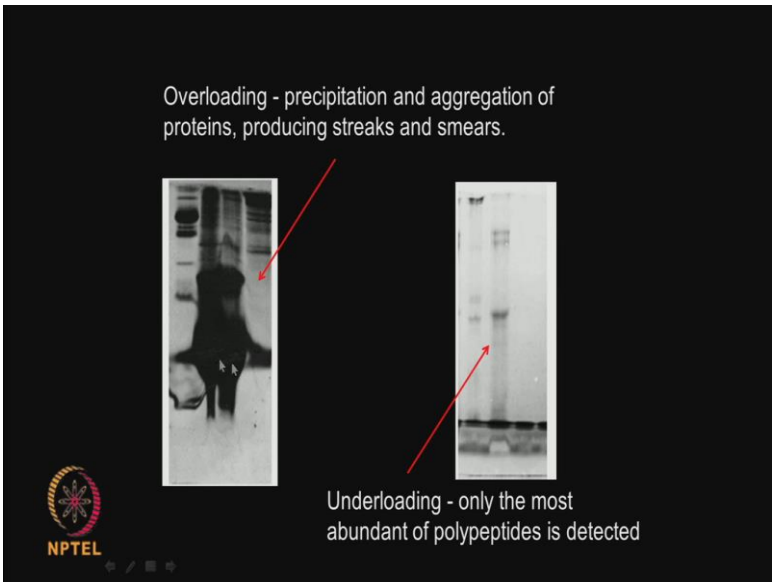

But there are many issues, sometimes it is called overloading, sometimes it is called under loading. So for example this is overload precipitation and aggregation of proteins, so it is producing streams in spheres. This is in under loading; you have not loaded enough protein here so we see very light bands.

And only the most abundant polypeptide is detected okay the less ones are not detected because you are under loaded your protein.

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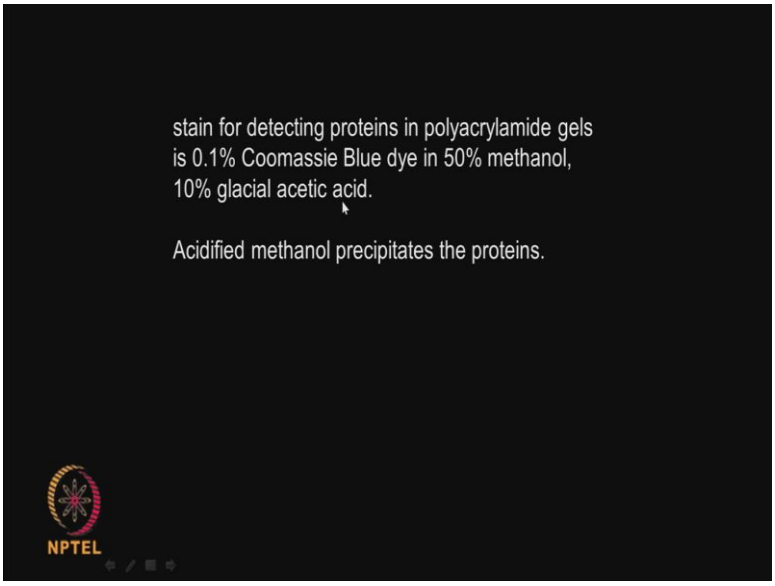

What are the stains for detecting your protein in polyacrylamide gels we can add 0.1 % coomassie blue dye in 50 % methanol 10 % glacial acetic acid. Acidified methanol will precipitate the proteins actually.

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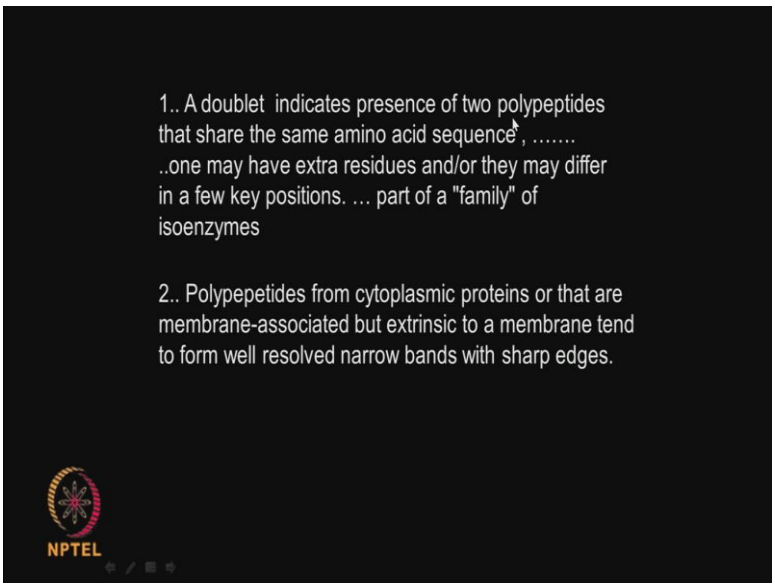

A doublet, if you have in SDS page is increased; it indicates presence of 2 polypeptide that share the same amino acid sequence okay. So a doublet one may have extra residue and or they may differ in a few key positions only. So they are part of a family if you see doublets polypeptide from cytoplasmic proteins are that are membrane associated but extrinsic to a membrane tend to form well resolved narrow band with sharp edges. So ideally you should see narrow band with sharp edges then we can

say they are completely cytoplasmic proteins.

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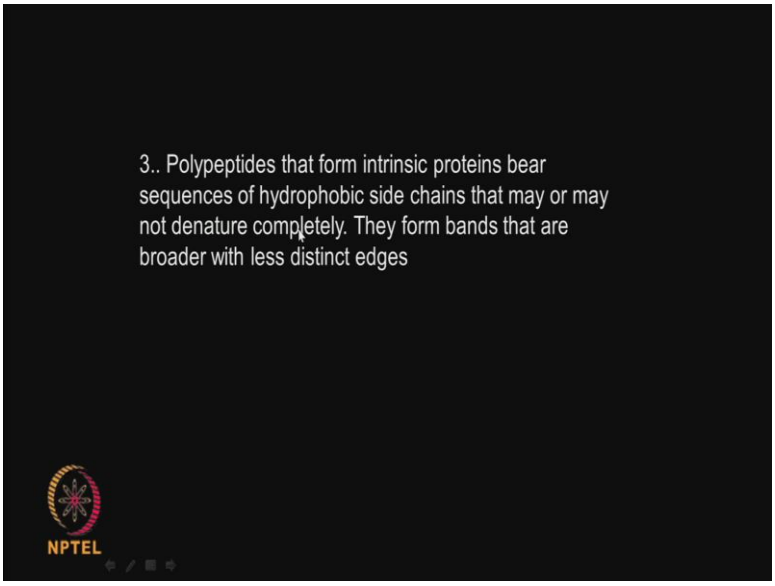

Polypeptide form intrinsic proteins bear sequence of hydrophobic side chains that may or may not denature completely. So they will form bands that are broader and the edges will be less distinct.

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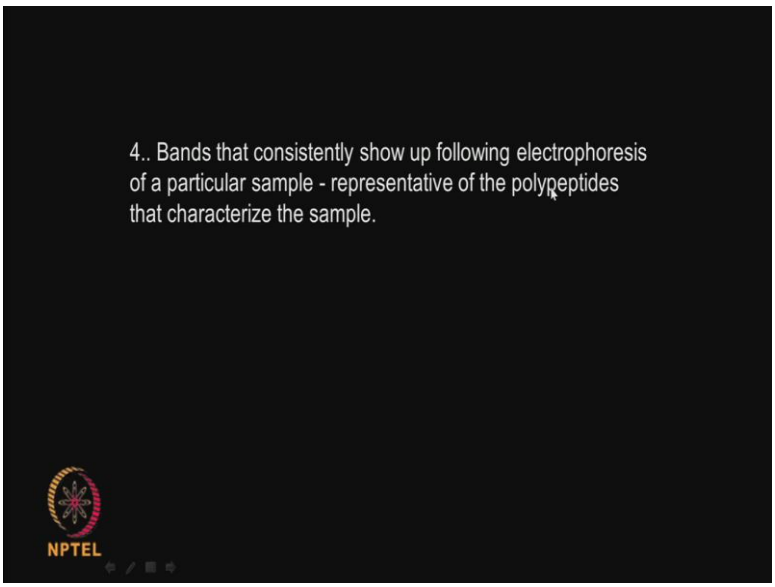

Bands that consistently show up following electrophoresis of particular sample that means the sample is characteristic of that particular polypeptide so that polypeptide that sample is characteristic. So it keeps coming and again and again when we run electrophoresis.

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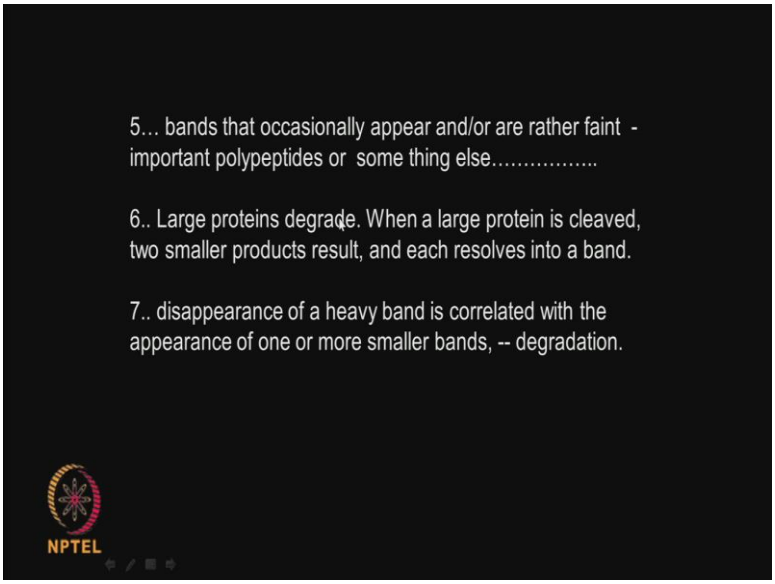

Bands that occasionally appear and or rather faint it means you have some important polypeptide or something else which we don't want and large proteins degrade okay. When it they degrade to smaller products each dissolve into a band okay. Disappearance of a heavy band is correlated with the appearance of one or more smaller bands that means degraded or degradation of that large band led into something right into smaller fragments.

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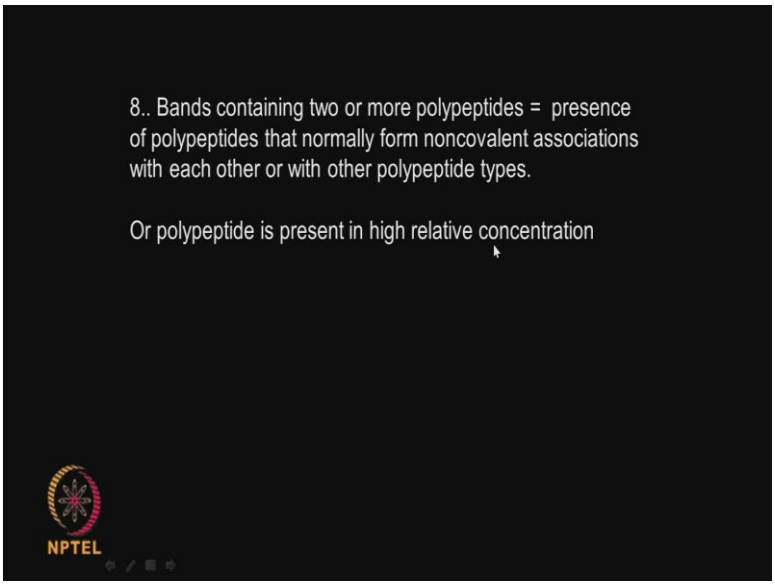

Bands containing 2 or more polypeptide that means presence of polypeptide that are normally form noncovalent association with each other okay or polypeptide is present in high relative concentration.

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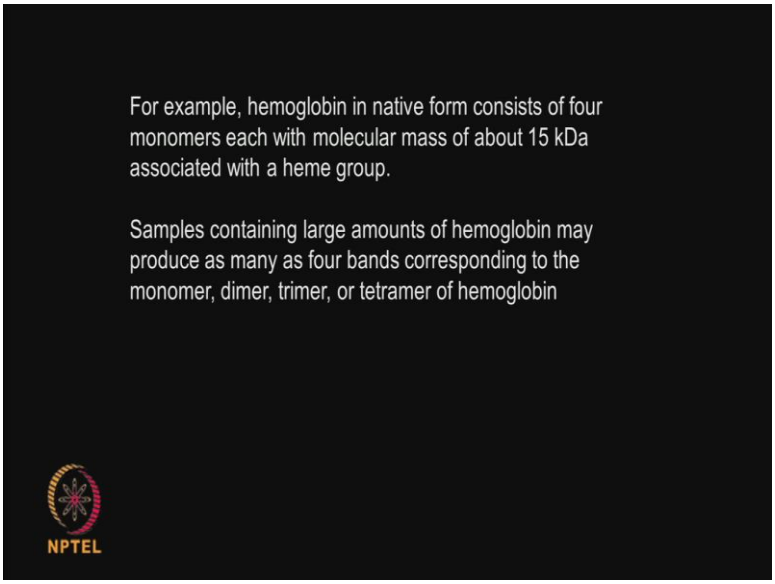

So these are various techniques by which you could conclude, by looking at those bands that appear in your SDS. For example, if you have a hemoglobin native form consists of 4 monomers, each with 15 kilo dalton, they are all associated with the heme group. So when you run that you will see 4 bands corresponding to monomer dimer trimer or tetramer of the hemoglobin. So will have 4 different bands each having different molecular weight and each one will be characteristic of the monomers present in the tetramer.

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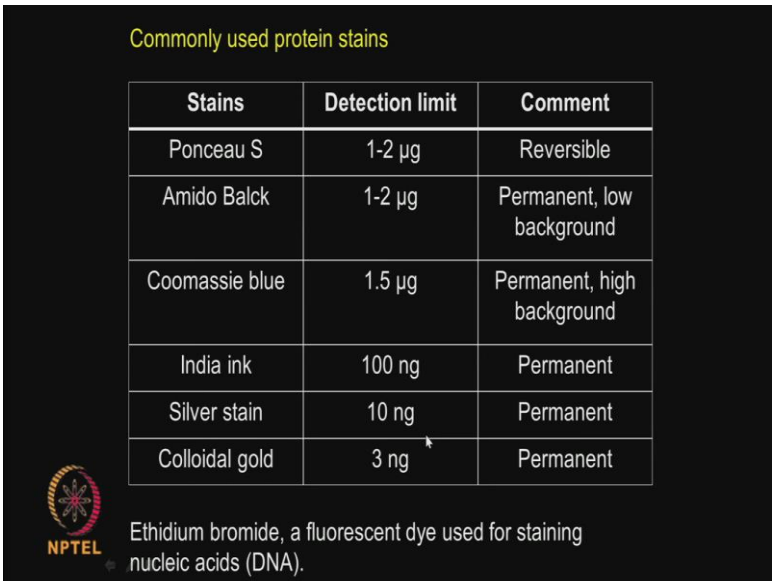

So commonly used protein stains, okay if you want to detect protein at the 1 to 2 micro gram or in the nano gram range okay, so we can use different types of stains. Okay we will not go too much into that but as you can see for detection in the nanogram level or in the microgram level. We can have different types of stains actually. So if you want to stain nucleic acids, then we need to use a fluorescent dye like

ethidium bromide, so large number of protein stains are available.

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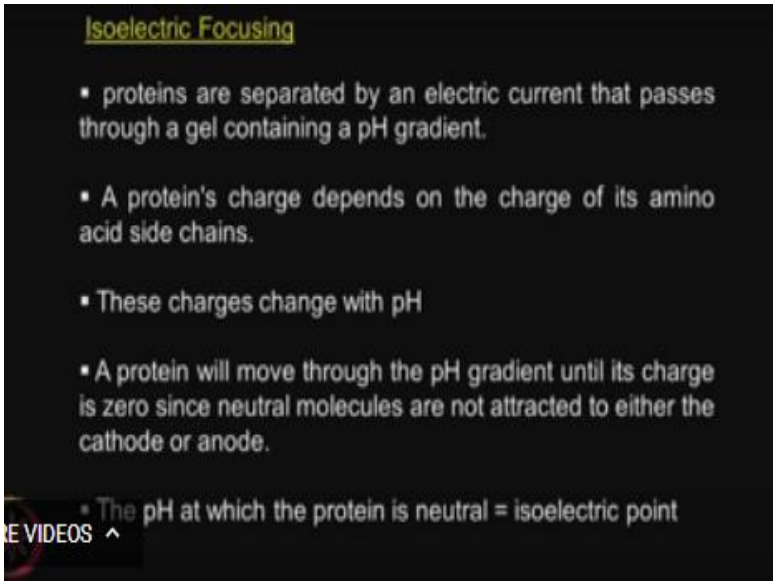

Then comes isoelectric focusing, we all know what is isoelectric point right. The pH at which the protein completely looses all it is charge okay. So it passes through a gel. It containing a pH gradient, so the charge on the protein depends on the amino acid chains, the chain these charges changes with pH, if the protein will keep moving through the pH gradient because of the electric current, because of the electric charge but as soon as the charge is neutralized.

When it reaches the pH, isoelectric pH, it will not move okay it will stop there and that is how you achieve separation. So if you have proteins with different isoelectric pH, they will move in a pH gradient and then they will stop as soon as they reach neutral pH or neutral charge condition.

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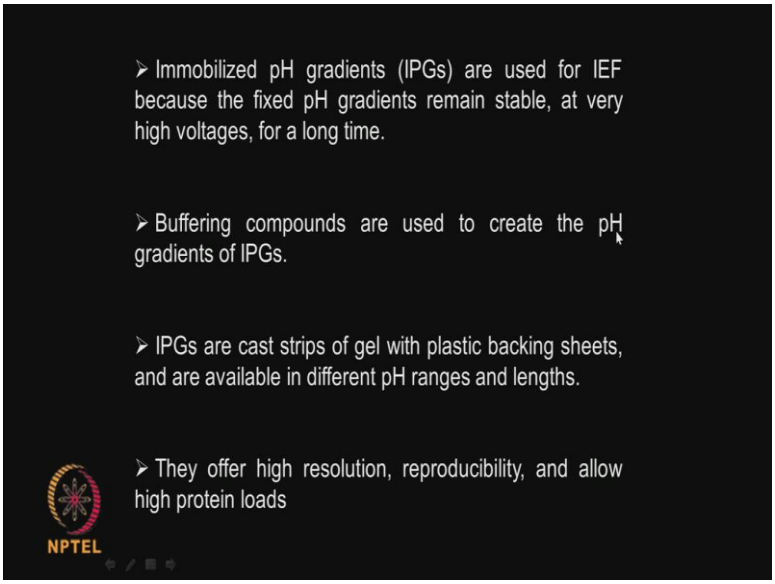

Immobilized pH gradient are used for isoelectric focusing okay because the fixed pH gradient remains stable at very high voltages buffering compounds are used to create the pH gradients immobilized pH gradients are cast strips of plastic backing sheets. They offer high resolution reproducibility and allow high protein loads okay.

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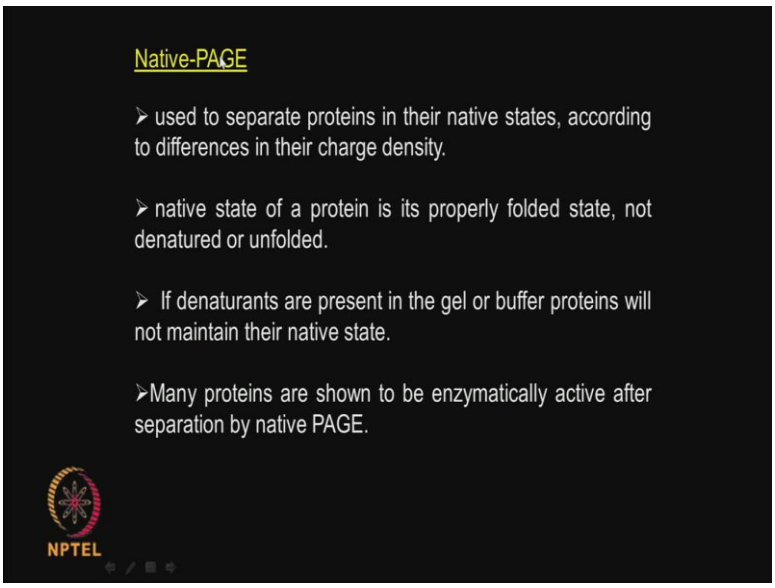

Then comes native page okay. The advantage of native page is we can get the protein in, it is active state unlike the SDS page. We completely destroy the activity of the protein and make it linear. The native page, the protein still retains it is activity okay because your are separating base at native stage you are according to their differences in their charge density. Native state of a protein is properly folded state. So it is not denatured okay but if you are adding denaturants then the protein or buffer proteins or gels.

Then it will lose it is native state. So the native protein we can take out the bit protein and then look at activity using some biochemical assays whereas in SDS page the protein uses it is activity completely.

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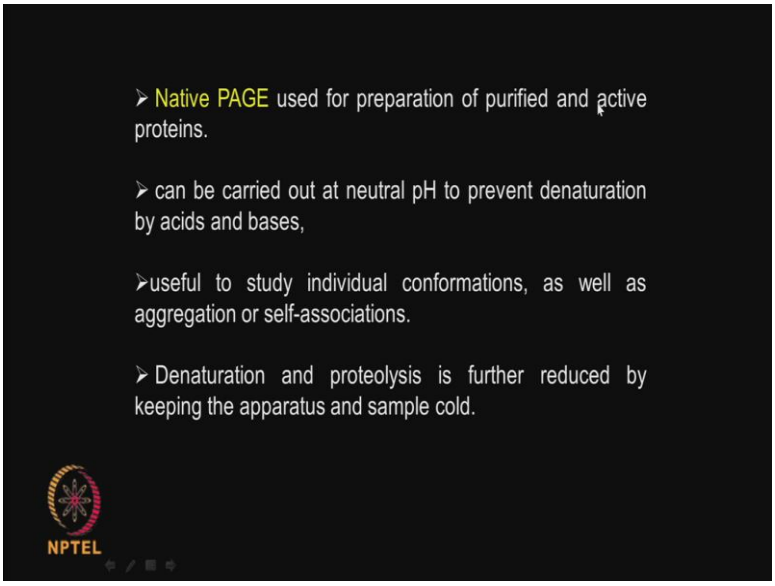

Native page is used for preparation of purified and active proteins. Okay so in the lab, if I have many proteins, I want to recover a particular protein, with the activity so sometimes I will run a SDS page look at molecular weight, then I will run at the native condition. So it can be carried out at the neutral pH to prevent denaturation because acids and bases can denature proteins. Okay so we can study individual confirmations, aggregation, self associations and so on and if you need to carry out better in cold condition, the apparatus should be in the cold condition; so that the protein doesn't lose it is activity.

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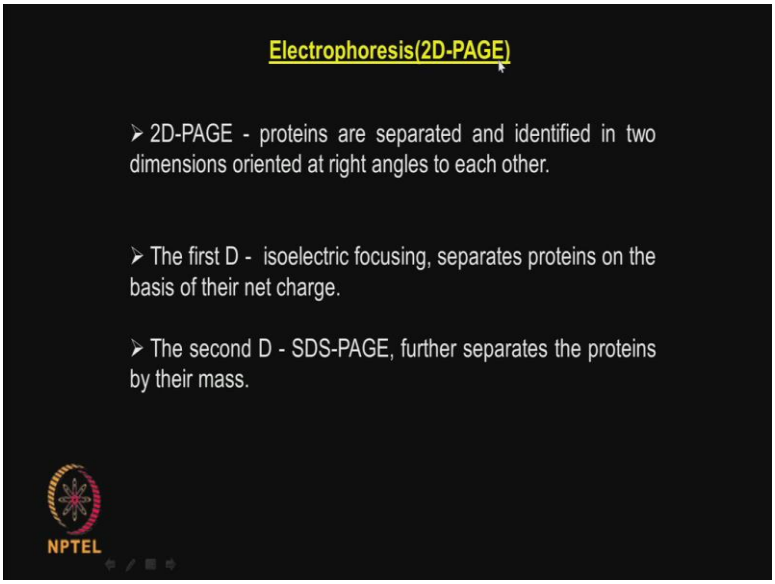

Okay now we talked about 1 dimensional, down we have 2 dimensional electrophoresis. Okay the proteins are separated right angle to each other in two dimension, the first dimension could be isoelectric focusing. That means it may separate the proteins based on the net charge, the second dimension could be based on the mass. So initially you separate based on the charge, then you separate on a SDS page so that the proteins get separated based on the mass.

So instead of getting a linear protein movement will get a protein movement in a 2 dimensional okay. So and then we can pick out protein of our interest and go use maldi or a mass spec and then find out what is the molecular weight of that particular protein. That is the advantage of 2 dimensional electrophoresis okay. So you run the isoelectric focusing, that's the first 2 dimension where the protein is separated based on the charge. The second dimension, the protein is based on molecular weight, the SDS page and then you can look at the molecular weight of the protein.

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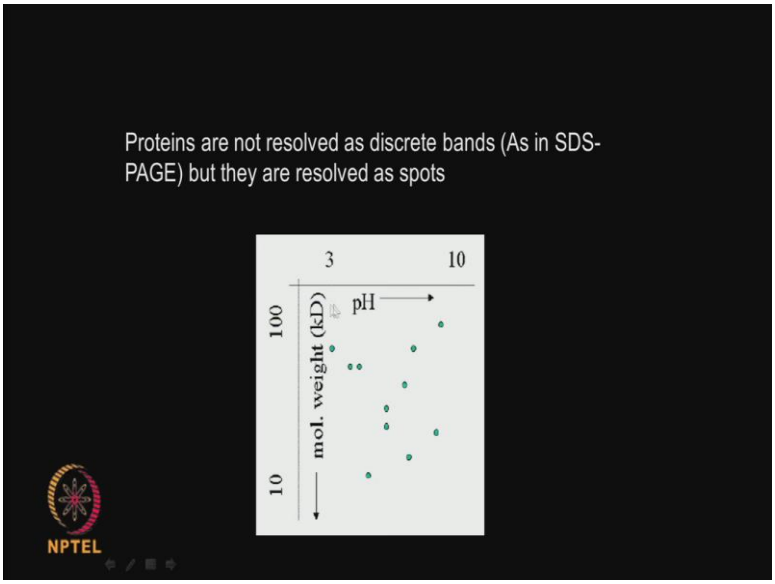

So proteins are not resolved as bands like in one dimensional SDS page and so on but it is dissolved as spots. So you have pH in one direction and you have molecule in another direction. Okay as you can see they get separated based on their isoelectric pH and then here they get separated based on their molecular weight okay this is how it will look okay.

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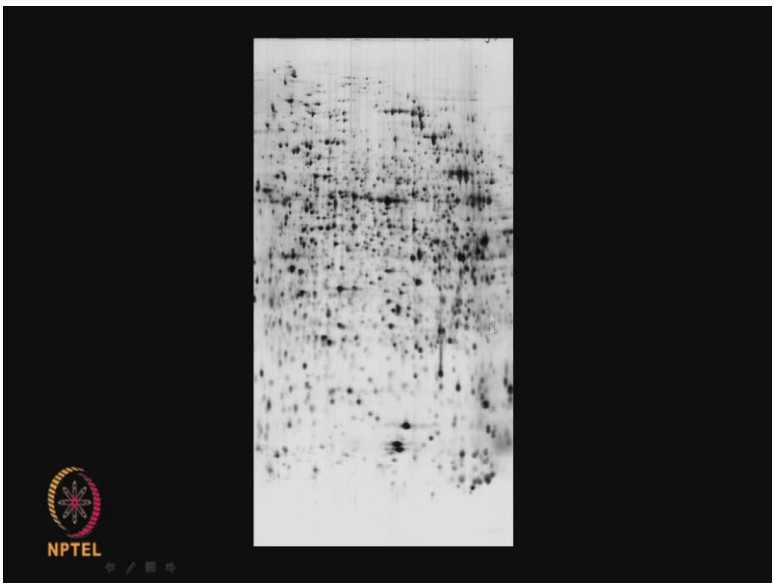

Now look at it. So complicated, a typical picture, we have proteins sometimes the problem is, this could be several proteins all combined together although we may think it is only single protein. So it may be very difficult to say this is single protein and most abundant protein may come out as dark spots, others may come out weeks very light spot. So it is quite complicated if they want to now take out one of these and then run maldi to find out the molecular weight and look at some secret.

So we have to be sure that this is only single protein and not to make sure so theoretically these spots could be mixtures of proteins. But this is how proteomics is carried out in 2 dimensional page where we separate out in one dimension based on it is isoelectric pH and in the second direct dimension you separate out based on it is molecular weight. So the proteins would have lost their activity here especially when you run through this SDS page but uh this is very useful technique if I want to look at the individual protein and characterize it in more detail actually.

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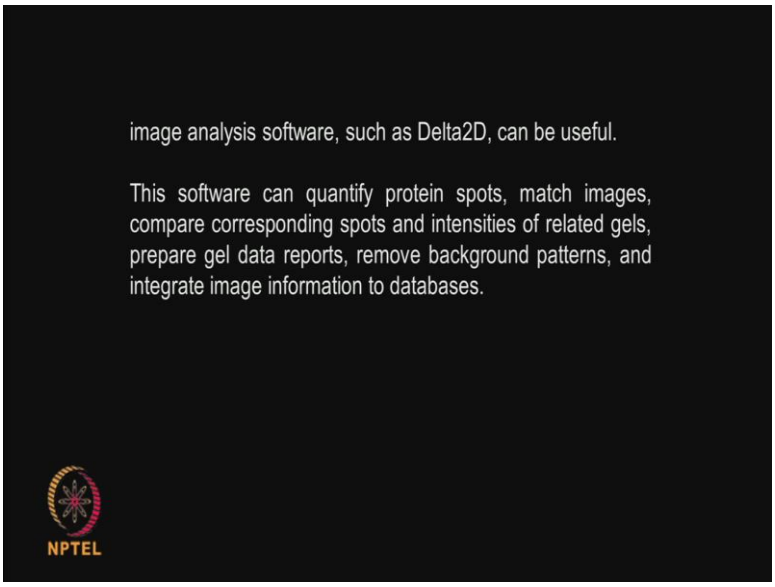

Okay there are many software that are available by which can help you, it can compare with existing database and then say possibly this is the protein. So there are many software like one of them is called delta2d. So it can use to quantify protein spots by looking at this spot and by looking at the enhanced image we can see a protein concentration could be of this order. Match images. We can match it with the other images in the database and say this protein could be this much.

And compare corresponding spots and intensities of related gels. For example I'm having the protein from a tissue sample before treatment and after treatment and I can compare those two 2d gels and then I see some proteins have disappeared so I can say may be enhancement or suppression because of treatment. Prepare gel data reports, remove background patterns, so for example if there are proteins from one set and another set constant we could remove them and just see those proteins which have appeared suddenly okay.

Or those proteins which have disappeared suddenly after treatment for a particular disease and then go into databases and try to identify, what that could be or take it take out this parts. Okay nowadays there

are something called a spot pickers, which can using an electronic system pick out, a specific spot and without human touch directly injected into a maldi using a solvent and then identify the molecular weight and the sequence of those proteins so such advance studies could be done with that 2d gel which I showed you actually.

So today we looked at very important set of techniques which are normally used by a molecular biology, biochemistry lab, trying to identify proteins based on their charge, based on their molecular weight and the movement of their diffusion coefficient and so on actually.

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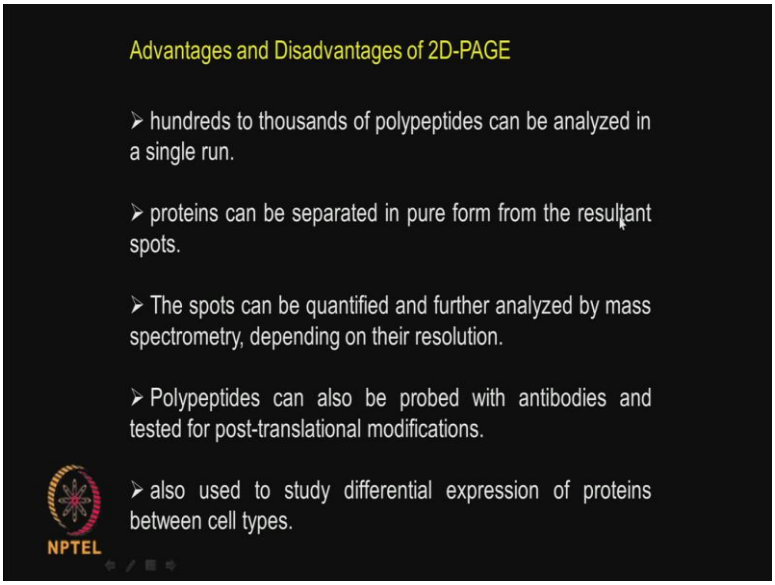

So what are the advantages and disadvantages to this 2d page. Hundreds to thousands of polypeptide can be analyzed in a single run, that's an advantage right. Proteins can be separated in pure form from the result spots. Because as I said you can just pick up and then do whatever studies you want. The spots can be quantified; we can say the quantity in concentration. It can be further analyzed using mass spec.

Polypeptide can also be probed with the antibodies. Yes you can add antibodies and look at post-translational modifications. We can look at differential expressions of protein between cell types. So I can compare to cells from and then say some spots have appeared some spots have disappeared and so on.

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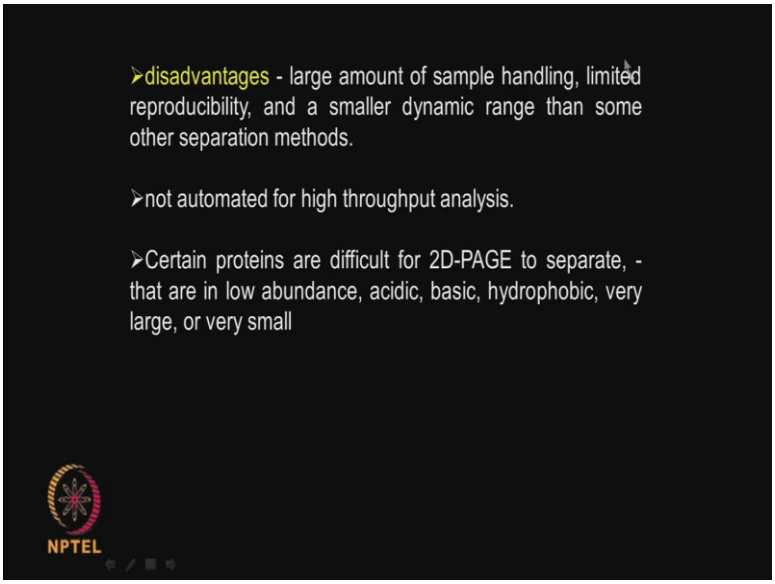

And disadvantages, large amount of sample handling as I showed you, there can be 1000s of spots okay limited reproducibility if I take another sample from the same spot and run 2d am I get a totally different pattern okay. When I get a smaller dynamic range than some other separation methods so not automated for high throughput analysis very difficult for high throughput and certain proteins are difficult for 2d page to separate which are low in abundance if the quantity is very low.

It is difficult, acidic, basic, hydrophobic, very very large or very very small. We may not be able to identify in a 2d electrophoresis. So these are the advantages and disadvantages of a 2d electrophoresis actually okay so…Thank you!! Thank you very much of for this lecture.