Experimental Biochemistry
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Lecture - 16
Spectroscopic Techniques Summary

Hello welcome to the end of week 3. So in this week we learnt about Spectroscopic techniques. So, in this lecture I am just going to again summarize what we have learnt so far in week 3.

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So, primarily we learnt about two techniques 1 is the UV Visible that is absorption method of determining amino acid and protein properties and then also we learnt about the Fluorescence spectra of amino acid and proteins.

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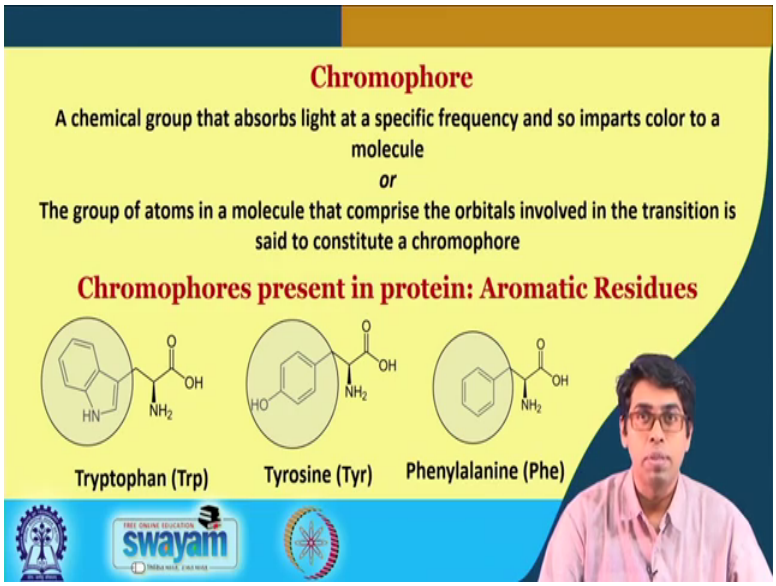

So, one of the important concepts that was introduced in this week was that of a chromophore. So, chromophore is a chemical group that absorbs light at a specific frequency and so imparts color to a molecule ok. So, whenever we think of color we think of a color in the visible range, but a chromophore can also be something that will not give a color in the visible range. So, it can emit energy at a range which is beyond the visible range, in this case we saw absorption spectra that we are collecting in the UV range ok.

So, again a chromophore can be a group of molecules that comprise the orbital's involved in the transition and is said to constitute a chromophore. For example, in case of proteins we saw that 3 amino acids have chromophores that absorb in the UV range 1 was tryptophan the other 1 was the tyrosine and the third one was phenylalanine you will see that they all have aromatic side chains and it is the side chain that is responsible for the absorption in the UV range and the same side chains also give us the Fluorescence spectra.

So, essentially in this week's lecture we focused on these three amino acids and again what we saw is that Tryptophan is the one that gives us the maximum intensity followed by Tyrosine and finally Phenylalanine has the least contribution whether it is UV absorption or fluorescence.

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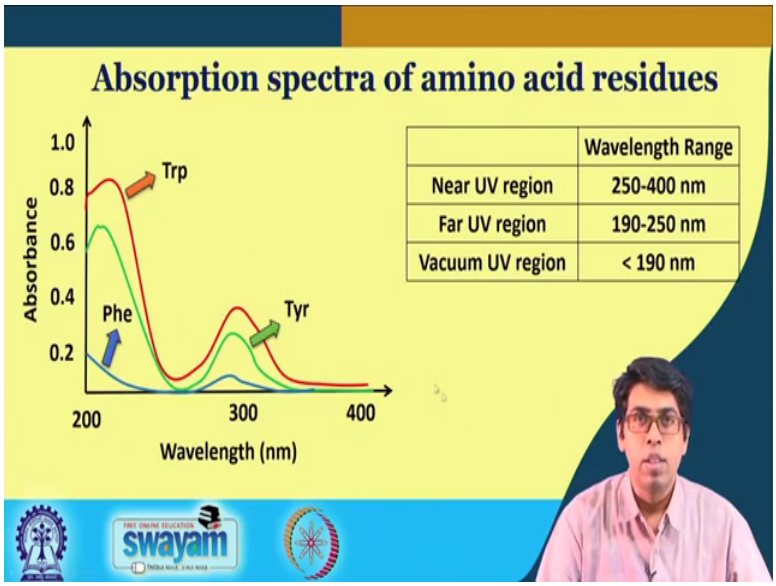

So, if we look at an individual amino acid not in the context of a protein. For example like we did in the lab were we made solutions of amino acids and we collected the absorption spectra. So, in that case curves like this are obtained. So, again tryptophan gives you the largest intensity at around 280 nanometers versus two 200 nanometers followed by Tyrosine and Phenylalanine gives you the smallest intensity.

So, we can divide these range 200 to 400 nanometers in this 3 different regions. So, under so less than 190 nanometers will be your vacuum or vacuum UV region 190 to 250 nanometer this region is the far UV region and the typical measurements that we have been doing and you will see in the subsequent weeks also you will stick to a range which is in which we will call the near UV range, primarily we will make all our measurements at 280 nanometers.

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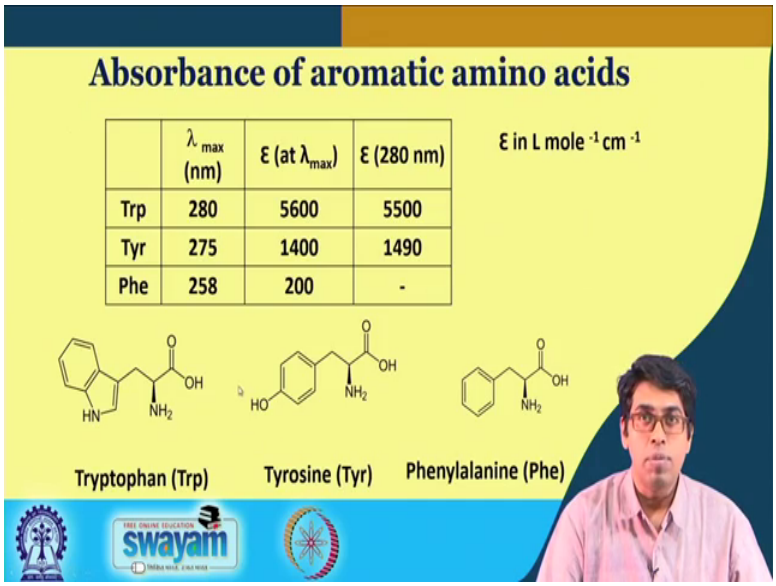

So, tryptophan has the highest absorptivity at 280 nanometers followed by Tyrosine and Phenylalanine. So, you can see that phenylalanine has the least contribution when it comes to the UV absorption and this is a concept that we will revise it again in the subsequent weeks. So, using this molar absorptivity we can calculate the concentration of a protein or a amino acid solution. So, we will learn how to use that in the subsequent lectures and we will see that we can determine the concentration of proteins solutions very precisely using this technique.

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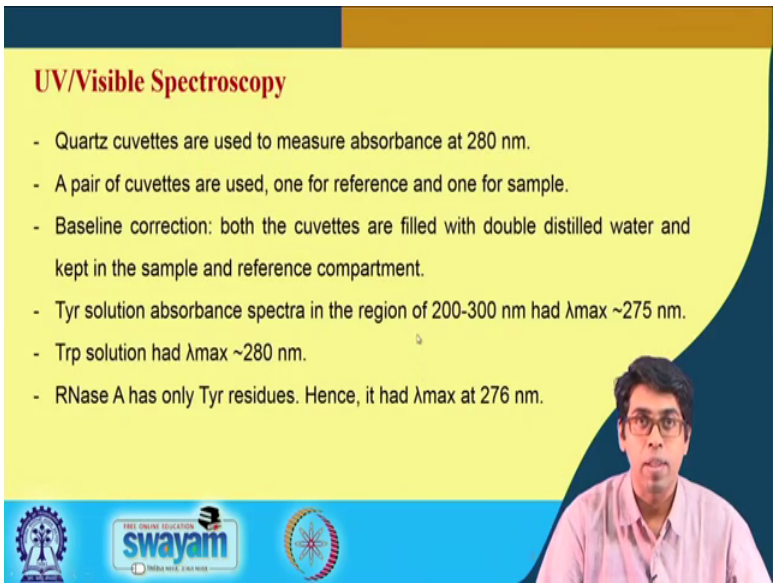

So, in the lab portion we saw how you will measure the absorption of absorption spectra of an amino acid solution. So, since we are in the near UV range a normal glass cuvette will not work. So, we use the special type of cuvette called the Quartz cuvette a pair of quartz cuvette were used one was used as reference and the other one was used for sample, the first step that was done was baseline correction.

So, every instrument from time to time the baseline keeps on changing, so this is basically just like in the weighing balance you saw that we would set the weighing balance at 0, before we make any measurement this is something very similar to that where you set the instrument at a 0 value and once both the reference and the sample cells are set to the same baseline 0 then you can start making your measurements.

So, once the baseline correction was done then the cuvette which used to which should have the sample the that was the sample was added to that and for the reference one you would add water or buffer. So, it should be exactly the same buffer in which the protein or your amino acid is dissolved. So, that takes care of any contribution that the buffer or the buffer salts etcetera can make in the absorption spectra.

So, ones you collect your spectra tyrosine give us spectra the range of 200 to 300 nanometers with a lambda max of 285 nanometer. Whereas, Tryptophan gives give us a spectra with a lambda max of around 280 nanometers, so these were done by using just the amino acids solutions and this and we saw that this show up in the expected range. But when we do actual measurements with proteins then the proteins do not have just Tyrosine or Tryptophan they will have mixture of Tyrosines and Tryptophans ok.

And depending on the mixture you will see that the lambda max will vary we used one example of a protein that is RNase which has only tyrosine residues and it showed that since there are no tryptophans the lambda max for this amino acid was very close to that of a tyrosine solution. So, for tyrosine solution we got a lambda max of around 275 nanometers and for RNase A which has only tyrosine residues and not Tryptophans the lambda max was at 276 nanometers.

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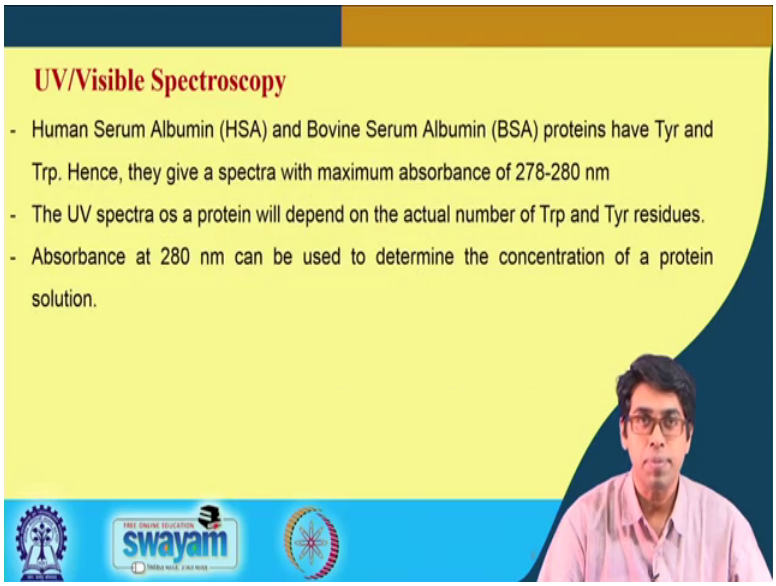

We chose two more protein samples the HSA and the BSA and these proteins have both Tyrosines and Tryptophans. So, now in this case we saw that the maximum absorbance of the spectra was now shifted more towards the 280 nanometer. So, it was in this range of 278 to 280 nanometers. So, as I mentioned before that the actual spectra will be depend on the number of Tryptophan and Tyrosine residues in the protein and once you measure the absorbance of a protein you can use that to determine the concentration of that protein. We will come across that in the lectures in subsequent weeks.

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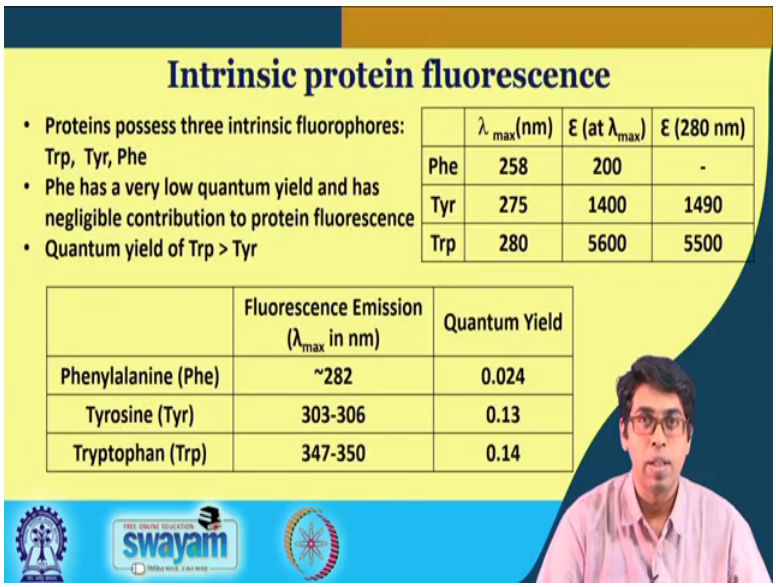

So, next we learnt about the intrinsic protein fluorescence. So, you will see that the fluorescence emission was recorded at higher wave lengths. So, even though the excitation was done in this near UV range, the emission spectra was collected at different wavelengths for this 3 different amino acids. For Phenylalanine it was around 280 for Tyrosine it was around 305 nanometers and for Tryptophan it was around 350 nanometers.

So, when we learnt about fluorescence we also learnt about an important parameter that is quantum yield. So, quantum yield is something that gives you the ratio of the photons that were emitted versus the photons that were absorbed ok. If you look at quantum yield for phenylalanine it has the lowest quantum yield and it is actually very small.

So, it means that if I have a protein which has Phenylalanine Tyrosine and Tryptophan the contribution from Phenylalanine will be almost negligible and the fluorescence spectra will be mostly dominated by Tyrosine and Tryptophan and again since the epsilon value for Tryptophan is almost 3 types or more than 3 types that of Tyrosine. It will be dominated by tryptophan if you have equal number of tyrosine and tryptophan residues.

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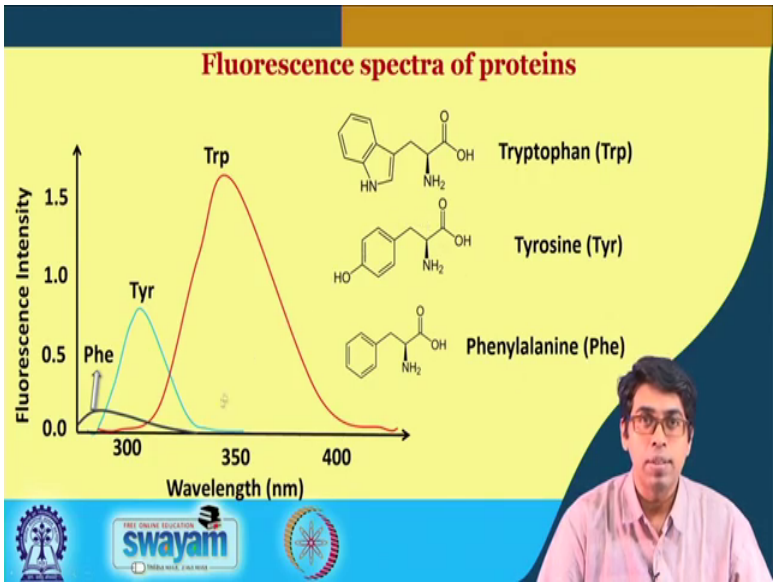

So, this is a typical fluorescence spectra of these three isolated amino acids and again you can see that it is scaled by their quantum yield and the epsilon value. So,

Phenylalanine has the least fluorescence followed by Tyrosine and then Tryptophan and you can see that the maximum lambda values also shifts depending on this amino acids.

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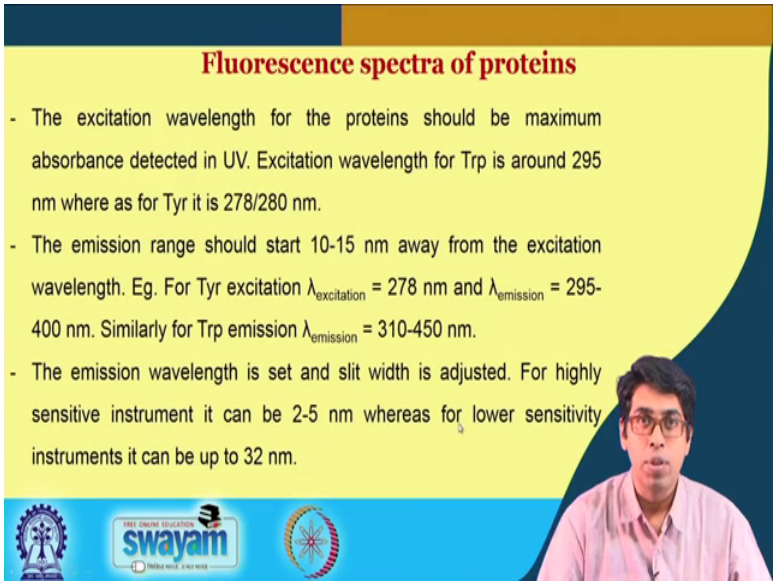

So, when we collected the spectra for again for the proteins what we saw is so first we did it for Tryptophan, so the excitation wavelength for protein should be the maximum that is detected in the UV. So, the idea is that you just collect the UV absorbance if the excitation if the protein has tryptophan then its excitation will be close to 280, so you can use 295 nanometer and for Tyrosines you can use around 280 nanometers.

So, whatever excitation wavelength we use the emission range should start reflecting into 15 nanometers away from the excitation wavelength, so this is something that depends on the instrument. For example, if an instrument is highly sensitive then we can use this range as only 2 to 5 nanometers, but for a lower sensitivity instrument these difference can be go up to 32 nanometer.

So, this sort of sets the lower limit from where you can starts carrying your spectra. So, for tyrosine the emission range that is used is around 295 to 400 nanometers and for tryptophan the range is 310 to 450 nanometers.

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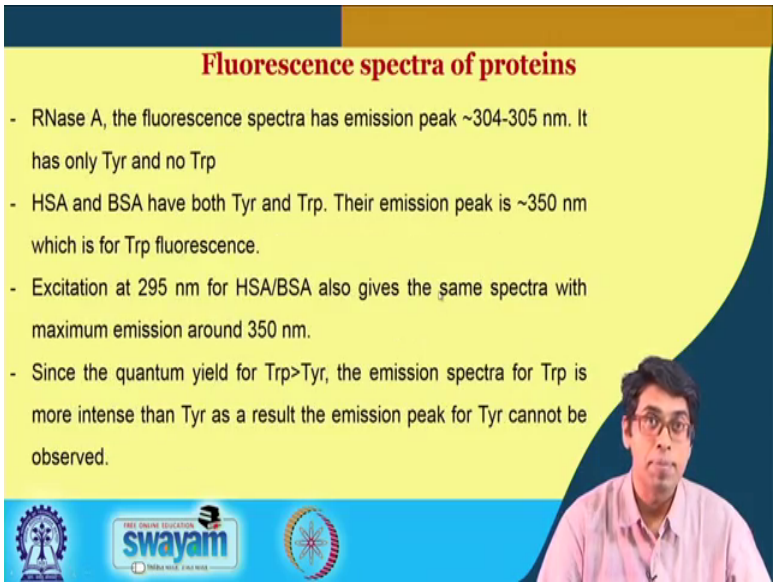

For RNase A we saw that the emission peak was close to 300 nanometers and this is the expected again because it has only tyrosine residues and no tryptophan residues. So, it sort of mirrors the emissions spectra of the tyrosine residues on the other hand for HSA and BSA the emission peak was at 350 nanometers. See now you know that tryptophan has gives much more intense fluorescence spectra compare to tyrosine.

So, even though there are both tyrosine and tryptophan the spectrum for HSA and BSA was dominated by tryptophan resulting in a emission peak at 350 nanometers. So, depending on the whether we so irrespective of whether we excite at 295 nanometers or 300 or 280 nanometers the both this proteins gave the same spectra with a maxima at around 350 nanometers, this is because the quantum yield for Tryptophan is greater and also it gives you more intense spectra compared to Tyrosine.

So, we will the applications of these techniques in the coming weeks when we start dealing with actual examples and you will see that while we are when we work with unknown proteins we want to know their concentration we want to know their absorption spectra, we want to know their fluorescence spectra and then also you will see that when we are purifying proteins these techniques become very useful. So, we will keep on revisiting these methods again and again in the coming lectures.

Thank you.