Lecture 4 Determination of protein concentration by ultraviolet spectroscopy

Aim:

To determine the concentration of a given protein using ultraviolet (UV) spectroscopy

Introduction:

Estimation of protein concentration in a given protein preparation is one of the most commonly performed tasks in a biochemistry lab. There are several ways of estimating the protein concentration such as amino acid analysis following acid hydrolysis of the protein; analyzing the changes in the spectral properties of certain dyes in the presence of proteins; and spectrophotometric estimation of the proteins in near or far UV region. Although dye-binding assays and amino acid analysis following acid hydrolysis of the protein can be used for estimating the protein concentration for both pure as well as an unknown mixture of proteins; UV spectroscopic quantitation holds good for the pure proteins. If a protein is pure, UV spectroscopic quantitation is the method of choice because it is easy and less time-consuming to perform; furthermore, the protein sample can be recovered back.

Absorption of ultraviolet radiation is a general method used for estimating a large number of bioanalytes. The region of the electromagnetic radiation ranging from ~10 – 400 nm is identified as the ultraviolet region. For the sake of convenience in referring to the different energies of UV region, it can be divided into three regions:

- Near UV region (UV region nearest to the visible region; $\lambda \sim 250 – 400$ nm)
- Far UV region (UV region farther to the visible region; $\lambda \sim 190 – 250$ nm)
- Vacuum UV region ($\lambda < 190$ nm)

This division is not strict and you may find slightly different wavelength ranges for these regions. We shall, in this course, stick to the above-mentioned definitions. Absorption of UV light is associated with the electronic transitions in the molecules from lower to higher energy states (Figure 4.1).

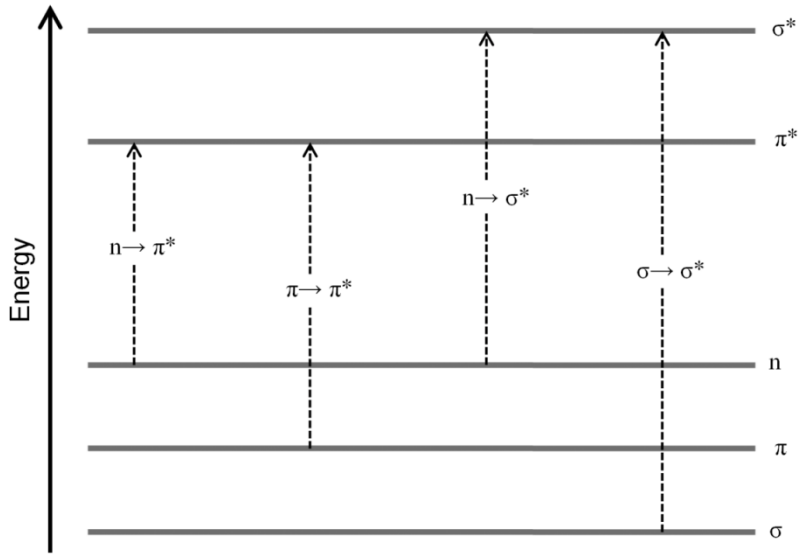

Figure 4.1 A diagrammatic representation of the energy levels of molecular orbitals; the vertical arrows represent electronic transitions.

As is clear from figure 4.1, $\sigma \rightarrow \sigma^*$ transition involves very high energy and usually lies in the vacuum UV region. Saturated hydrocarbons, that can undergo only $\sigma \rightarrow \sigma^*$transition, therefore show absorption bands at ~150 nm wavelength. Compounds that have unsaturation and/or lone pair of electrons *i.e.* the ones that can undergo $\pi \rightarrow \pi^*$ or $n \rightarrow \pi^*$ transitions, absorb at higher wavelengths that may lie in far or near UV regions, the regions of UV radiation the biochemical spectroscopists are usually interested in. The group of atoms in a molecule that comprise the orbitals involved in the transition is said to constitute a chromophore.Figure 4.2 shows an absorption spectrum of a peptide. The spectrum immediately suggests that the proteins can absorb both in near UV and far UV regions.

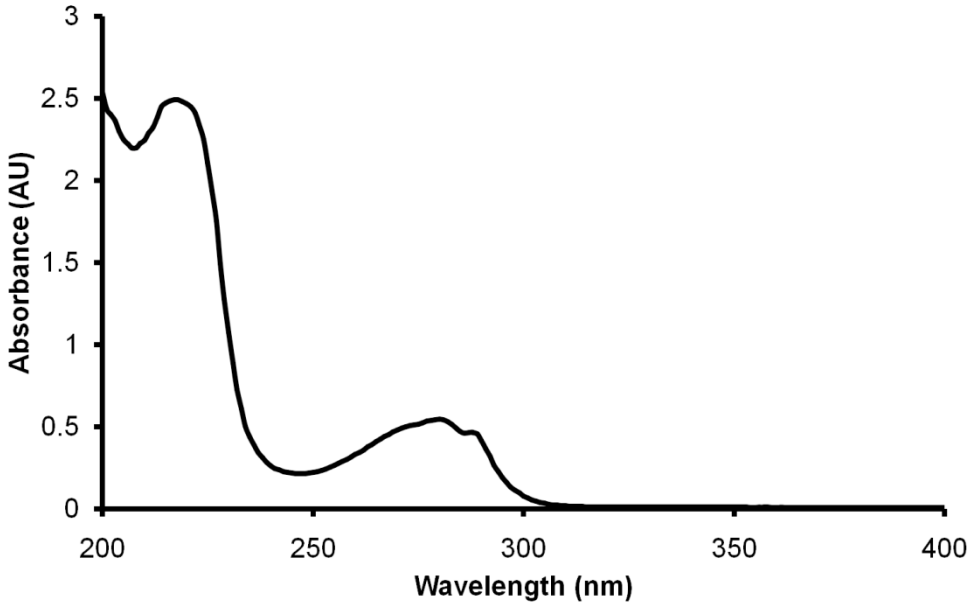

Figure 4.2Absorption spectrum of a peptide in the near and far UV regions.

Absorption of UV radiation is usually represented in terms of absorbance and %transmittance:

$$Absorbance\ (A) = -log\left(\frac{I}{I_0}\right) \qquad \text{---------- (4.1)}$$

$$\%Transmittance\ (\%T) = \frac{I}{I_0} \times 100 \qquad \text{---------- (4.2)}$$

where, I_0 and I represent the intensities of light entering and exiting the sample, respectively.

Absorbance of an analyte depends on the concentration of the analyte and the path length of the solution (Beer-Lambert Law):

$$A = \varepsilon cl \qquad \text{---------- (4.3)}$$

where, ε is the molar absorption coefficient, c is the molar concentration of the analyte and l is the path length of the cell containing the analyte solution. If molar absorption coefficient of the analyte and the path length of sample cell are known, concentration can directly be determined using Beer-Lambert law.

Let us see how protein concentration is estimated using near and far UV radiation.

Near-UV radiation

Aromatic amino acids, tryptophan, tyrosine, and phenylalanine and the disulfide linkage constitute the chromophores that absorb in the near UV region. Absorption of near UV radiation by proteins is usually monitored at 280 nm due to very high absorption by Trp and Tyr at this wavelength. Table 4.1 shows the molar absorption coefficient of the protein chromophores that absorb the light of 280 nm.

Table 4.1 Molar absorption coefficientsof protein chromophores at 280 nm

	$\varepsilon_{280}(M^{-1}cm^{-1})$		
	Trp	Tyr	S-S
Average value in folded proteins	5500	1490	125
Value in unfolded proteins	5690	1280	120

where, ε_{280} is the molar absorption coefficient at 280 nm.

It is therefore straightforward to calculate the molar absorption coefficientof a folded protein if its amino acid sequence or composition is known:

$$\varepsilon_{280} = (5500 \times n_{Trp}) + (1490 \times n_{Tyr}) + (125 \times n_{S-S}) \quad \text{---------- (4.4)}$$

For short peptides that are usually unfolded in water, the molar absorption coefficients can be calculated using the following equation:

$$\varepsilon_{280} = (5690 \times n_{Trp}) + (1280 \times n_{Tyr}) + (120 \times n_{S-S}) \quad \text{---------- (4.5)}$$

Far-UV radiation

The proteins and peptides that lack aromatic residues and disulfide linkage do not absorb the near UV radiation. The concentration of such proteins and peptides can be estimated using far UV radiation. Peptide bond is the major chromophore in the far UV region with a strong absorption band around 190 nm ($\pi \rightarrow \pi^*$ transition) and a weak band around 220 nm ($n \rightarrow \pi^*$ transition). As oxygen strongly absorbs 190 nm radiation, it is convenient to measure absorptionat 205 nm where molar absorption coefficient of peptide bond is roughly half of that at 190 nm. A 1 *mg/ml* solution of most proteins would have an extinction coefficient of ~30 – 35 at 205 nm. This means

that the result obtained can have more than 15% error. An empirical formula, proposed by Scopes [1] provides the $A_{205}^{1\,mg/ml}$ within ± 2%:

$$A_{205}^{1\,mg/ml} = 27 + 120\left(\frac{A_{280}}{A_{205}}\right) \quad \text{---------- (4.6)}$$

Alternatively, the concentration can be estimated using Wadell's method[2] that relies on the absorbance at 215 and 225 nm:

$$Protein\ concentration\ \left(\frac{\mu g}{ml}\right) = 144(A_{215} - A_{225}) \text{---------- (4.7)}$$

Materials:

1. A UV/Visible spectrophotometer
2. Pipettes
3. Pipette tips
4. Disposable microfuge tubes
5. Quartz cuvettes (suitable for wavelengths smaller than 205 nm)
6. Pure protein solution in a buffer (or in water)
7. The buffer the protein is dissolved in (will act as the blank).

Procedure:

1. Switch 'ON' the UV/visible spectrophotometer and allow it 30 minutes warm up.
2. Determine the number of tryptophans, tyrosines, and disulfide linkages present in the protein.
3. Determine the molar absorption coefficient of the protein at 280 nm using equation 4.4.
4. Take the bufferused for protein dissolution in the quartz cuvettes.
    a. The volume of buffer has to be sufficient enough to cover the entire aperture the light beampasses through and depends on the capacity of the quartz cuvette; typically cuvettes with 1 *ml* capacity are used.
5. Place the cuvettes in the reference cell and sample cell slots in the spectrophotometer.
6. 'ZERO' the baseline for the 250 – 350 nm range.

7. Remove the quartz cuvette placed in the sample cell slot and discard all the contents.
8. Add the same volume of the given protein solution into the cuvette and place it back in the sample cell slot.
9. Record the absorbance at 280 nm $\left(A_{280}^{Sample}\right)$and 330 nm $\left(A_{330}^{Sample}\right)$.
    a. Proteins do not absorb at wavelengths higher than 320 nm; any absorbance obtained at 330 nm therefore arises due to scattering.
    b. If the absorbance at 280 nm does not lie between 0.05 – 1.0, dilute the protein solution in the same buffer so as to obtain an absorbance in this range.
10. Switch off the spectrophotometer.
11. Take out the quartz cell and clean them using detergent solution and deionized water.

Calculation:

The absorbance at 280 nm is corrected for light scattering:

$$A_{280(corrected)}^{Sample} = A_{280}^{Sample} - 1.929 \times \left(A_{330}^{Sample}\right)$$

The amount of the given protein is determined using Beer-Lambert law (equation 4.3):

$$A_{280(corrected)}^{Sample} = \varepsilon cl$$

$$c(M) = \frac{A_{280(corrected)}^{Sample}}{\varepsilon(M^{-1}cm^{-1})\ l(cm)}$$

Notes:

1. If the given protein lacks Trp, Tyr, and disulfide linkages, the concentration can be estimated using A_{205} or A_{215} and A_{225} using equations 4.6 and 4.7.
2. If the protein solution is turbid, it will scatter light leading to inflated absorbance values. The solution should therefore be cleared either by filtering it through a 0.2 μm filter or through centrifugation.

References:

[1] Scopes, R. K. (1974) Measurement of protein by spectrometry at 205 nm. Analytical Biochemistry, 59, 277–282.

[2] Waddell, W. J. (1956) A simple UV spectrophotometric method for the determination of protein. The Journal of Laboratory and Clinical Medicine,48, 311–314.

Lecture 5 Determination of protein concentration by Bradford method

Aim:

To determine the total protein concentration in a given sample

Introduction:

As discussed in the previous lecture, the concentration of proteins can be estimated using various methods. We have discussed the concentration estimation of a pure protein using ultraviolet absorption in the previous lecture. For estimating the total protein in a complex protein mixture, one can use dyes that exhibit changes in their spectral properties on binding to the proteins. Bradford is one such dye-based assay for protein concentration estimation.

The principle underlying Bradford assay is the binding of the Coomassie Blue G250 dye (Figure 5.1) to proteins.

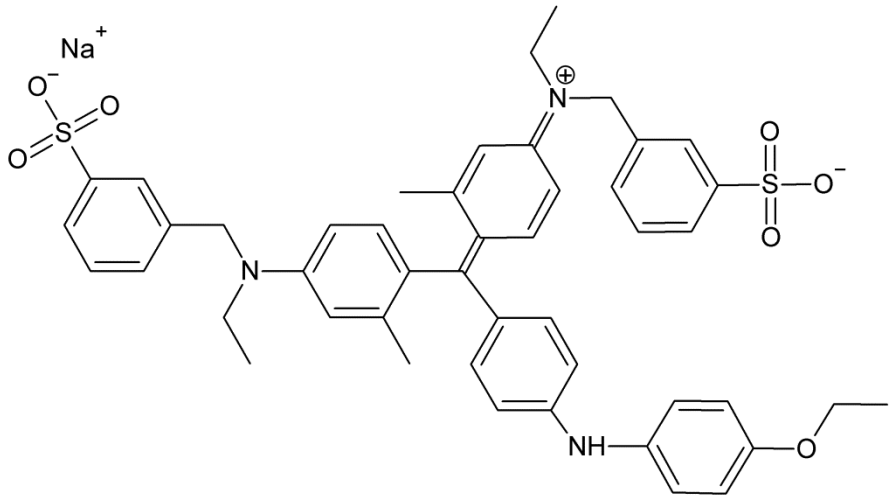

Figure 5.1 Structure of Coomassie Blue G250

Free Coomassie Blue G250 can exist in four different ionization states with pK_{a_1}, pK_{a_2}, and pK_{a_3} of 1.15, 1.82, and 12.4. At pH 0, both the sulfate groups are negatively charged and all three nitrogens are positively charged giving the dye +1 net charge (the red form of the dye). Around pH 1.5, the neutral green form of the dye predominates. At neutral pH, the dye has a net charge of +1 (the blue form of the

dye). The red, green, and blue forms of the dye absorb visible radiation with absorption maxima at 470, 650, and 590 nm, respectively. It is the anionic form of the dye that binds to the protein. Binding of the blue form of Coomassie Blue G250 with proteins causes red-shift in its absorption spectrum; the absorption maximum shifts from 590 to 620 nm. It, therefore, looks sensible to record the absorption at 620 nm. The absorbance, however, is recorded at 595 nm to avoid any contribution from the green form of the dye. The dye binds more readily to the cationic residues, lysine and arginine. This implies that the response of the assay would depend on the amino acid composition of protein, the major drawback of the assay. The original assay developed by Bradford shows such variation between different proteins. Several modifications have been introduced into the assay to overcome this drawback; the modified assays, however, are more susceptible to interference by other chemicals than the original assay. The original Bradford assay, therefore, remains the most convenient and widely used method.

In this experiment, we shall be using the standard Bradford assay which is suitable for measuring theprotein amount ranging from 10 – 100 μg. A microassay suitable for the protein ranging from 1 – 10 μg is also briefly discussed.

Materials:

Equipments:

8. A visible range spectrophotometer
9. Vortex mixer
10. Weighing balance

Reagents:

1. Bradford reagent
2. Ovalbumin (Protein standard)

Glassware and plasticware:

1. Pipettes
2. Pipette tips
3. A 5 *ml* glass pipette
4. Pipette aid

5. 100 *ml* measuring cylinder
6. Test tubes (for standard assay)
7. 1.5 *ml* microfuge tubes (for microassay)
8. Plastic cuvettes

Preparation of reagents:

Bradford reagent: Bradford reagent is prepared as follows:

1. Weigh 200 *mg* of Coomassie Blue G250 dye and dissolve it in 50 *ml* of 95% ethanol.
2. Mix this solution with 100 *ml* of concentrated (85%) phosphoric acid.
3. Make the final volume of the solution to 1 *litre* by adding distilled water.
4. Filter the reagent through Whatman No. 1 filter paper.
5. Transfer the filtrate in an amber colored bottle and store at room temperature.

Protein standard: Ovalbumin; the standard solution is prepared as follows:

1. Weigh accurately 5*mg*ovalbumin.
2. Dissolve it in 5 *ml* distilled water; this gives a protein stock solution of 1 *mg/ml* concentration.
3. Store the protein standard at –20 °C.

Procedure of standard Bradford assay:

1. Take out the frozen protein standard and allow it to come to room temperature.
2. As the concentration of the unknown protein sample can be anything, the assay will be performed with a range of dilutions (1, 1:10, 1:100, and 1:1000). Prepare 100 μl of each of the dilutions.
3. Take 15 test tubes and label them from 1 to 15.
4. Pipette out 10 μl, 20 μl, 30 μl, …….., 100 μlovalbumin standard in the glass tubes labeled 1 – 10; leave blank the tube no. 11.
5. Add distilled water to make the final volume 100μl in each of the tubes (including blank).
6. Take 100μl of each of the unknown protein dilutions in the tubes labeled 12 – 15.

7. Add 5 *ml* of Bradford reagent in each of the tubes and mix well by inversion or gentle vortex mixing (avoid frothing).
8. Within 5 – 60 *min*, measure the absorbance of tubes 1 – 10 and 12 – 15 at 595 nm in the quartz/glass cuvette against the reagent blank (tube 11).
9. Record the readings in the suggested observation table below:

Observation Table:

Table 5.1: Observation table for the Bradford assay

Tube No.	Volume (μl)	Mass (μg)	Distilled water (μl)	Bradford reagent (ml)	A_{595}
Standard DNA					
1	10	10	90	5	
2	20	20	80	5	
3	30	30	70	5	
4	40	40	60	5	
5	50	50	50	5	
6	60	60	40	5	
7	70	70	30	5	
8	80	80	20	5	
9	90	90	10	5	
10	100	100	0	5	
11	Blank (0)	0	100	5	
Unknown sample					
12	100 (1:1000 dil.)	Unknown	0	5	
13	100 (1:100 dil.)	Unknown	0	5	
14	100 (1:10 dil.)	Unknown	0	5	
15	100 (Undiluted)	Unknown	0	5	

Microassay:

1. In the Bradford microassay, the standard protein stock solution is prepared with a concentration of 100 $\mu g/ml$.
2. Make the dilutions of the unknown protein sample exactly as prepared in standard assay.
3. Follow steps 3 – 6 of the standard Bradford assay.
4. Add 1*ml* of Bradford reagent in each of the tubes and mix well by inversion or gentle vortex mixing (avoid frothing).

5. Within 5 – 60 *min*, measure the absorbance of tubes 1 – 10 and 12 – 15 at 595 nm in the quartz/glass cuvette against the reagent blank (tube 11).
6. Record the readings in the observation table.

Analysis: Let us take some hypothetical readings for carrying out the analysis:

Tube No.	Volume (μl)	Mass (μg)	Distilled water (μl)	Bradford reagent (ml)	A_{595}
Standard DNA					
1	10	10	90	5	0.04
2	20	20	80	5	0.078
3	30	30	70	5	0.122
4	40	40	60	5	0.164
5	50	50	50	5	0.202
6	60	60	40	5	0.240
7	70	70	30	5	0.278
8	80	80	20	5	0.322
9	90	90	10	5	0.361
10	100	100	0	5	0.403
11	Blank (0)	0	100	5	Reference (0)
Unknown sample					
12	100 (1:1000 dil.)	Unknown	0	5	0.004
13	100 (1:100 dil.)	Unknown	0	5	0.041
14	100 (1:10 dil.)	Unknown	0	5	0.426
15	100 (Undiluted)	Unknown	0	5	2.768

The absorbance values of the standard protein are plotted against the amount of the protein added for the assay. The curve is fit using linear regression with intercept (0,0) as shown in figure 5.2.

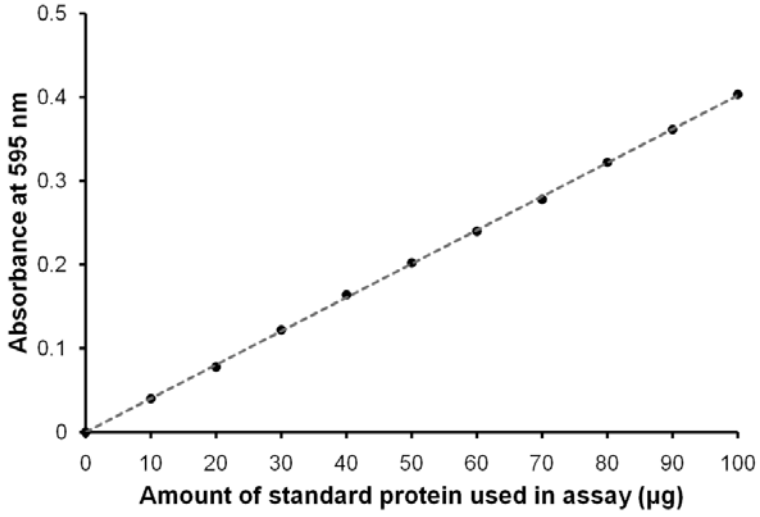

Figure 5.2: Plot of absorbance at 595 nm against the amount of protein

The equation of this regression line is:*Absorbance = 0.00402 ×amount of protein*

where, 0.00402 is the slope $\left(\frac{\Delta Absorbance}{\Delta Protein\ amount\ (\mu g)}\right)$.

Now, let us calculate the concentration of the unknown protein. We have got absorbance at different dilutions but which one should be used for determining the concentration. The absorbance values lying between 0.05 – 0.6 are most reliable. We shall, therefore, calculate the concentration for the 10-fold diluted sample that gave an absorbance of 0.426. The amount of protein is given by:

$$Protein\ (\mu g) = \frac{1}{slope} \times Absorbance$$

$$= \frac{1}{0.00402 \mu g^{-1}} \times 0.426 = 105.97\ \mu g$$

As this amount of the protein was present in the 100 μl of the 10-fold diluted protein sample, the concentration of the given protein sample $= \frac{105.97\ \mu g}{100\ \mu l} \times 10 =$ $10.597\ \mu g/\mu l \approx 10.6\ mg/ml$

The concentration of the unknown sample can directly be calculated as follows:

$$Protein\ concentration = \frac{Absorbance \times Dilution\ factor}{slope\left(\frac{1}{\mu g}\right) \times Volume\ of\ diluted\ protein\ used\ for\ the\ assay\ (\mu l)}$$

$$Protein\ concentration\ (\mu g/\mu l) = \frac{0.426 \times 10}{0.00402 \times 0.1} = 10597$$

Therefore, the concentration of the protein in the given sample = *10.597 µg/µl = 10.6 mg/ml*

Notes:

1. The Coomassie Blue G250 precipitates out of the solution with time. Bradford reagent should therefore be filtered through Whatman no. 1 filter paper before use.
2. The dye has preferential binding to lysines and arginines in the proteins but does not bind to free lysine and arginine.
3. The dye does not bind to the peptides smaller than about 3 kDa; Bradford assay, therefore, is not suitable for small peptides.
4. Quartz (silica) cuvettes should not be used for Bradford assay as Coomassie Blue G250 binds to silica. Disposable polystyrene cuvettes should be used for the assay.
5. UV/Visible spectrophotometer should be switched on at least 30 *min* before use.

Lecture 6 Estimation of DNA using diphenylamine method

Aim:

To determine the concentration of a given DNA sample using diphenylamine method

Introduction:

The principle underlying estimation of DNA using diphenylamine is the reaction of diphenylamine with deoxyribose sugar producing blue-coloured complex. The DNA sample is boiled under extremely acidic conditions; this causes depurination of the DNA followed by dehydration of deoxyribose sugar into a highly reactive ω-hydroxylevulinylaldehyde. The reaction is not specific for DNA and is given by 2-deoxypentoses, in general. The ω-hydroxylevulinylaldehyde, under acidic conditions, reacts with diphenylamine to produce a blue-coloured complex that absorbs at 595 nm. The mechanism of reaction of deoxyribose sugar with diphenylamine is shown in figure 6.1. As the sugar linked to only purine residues participates in the reaction, the readout is only from 50% of the total number of nucleotides. As this holds true for both the known standard and the given unknown sample, the concentration of the unknown sample can be directly calculated from the standard graph.

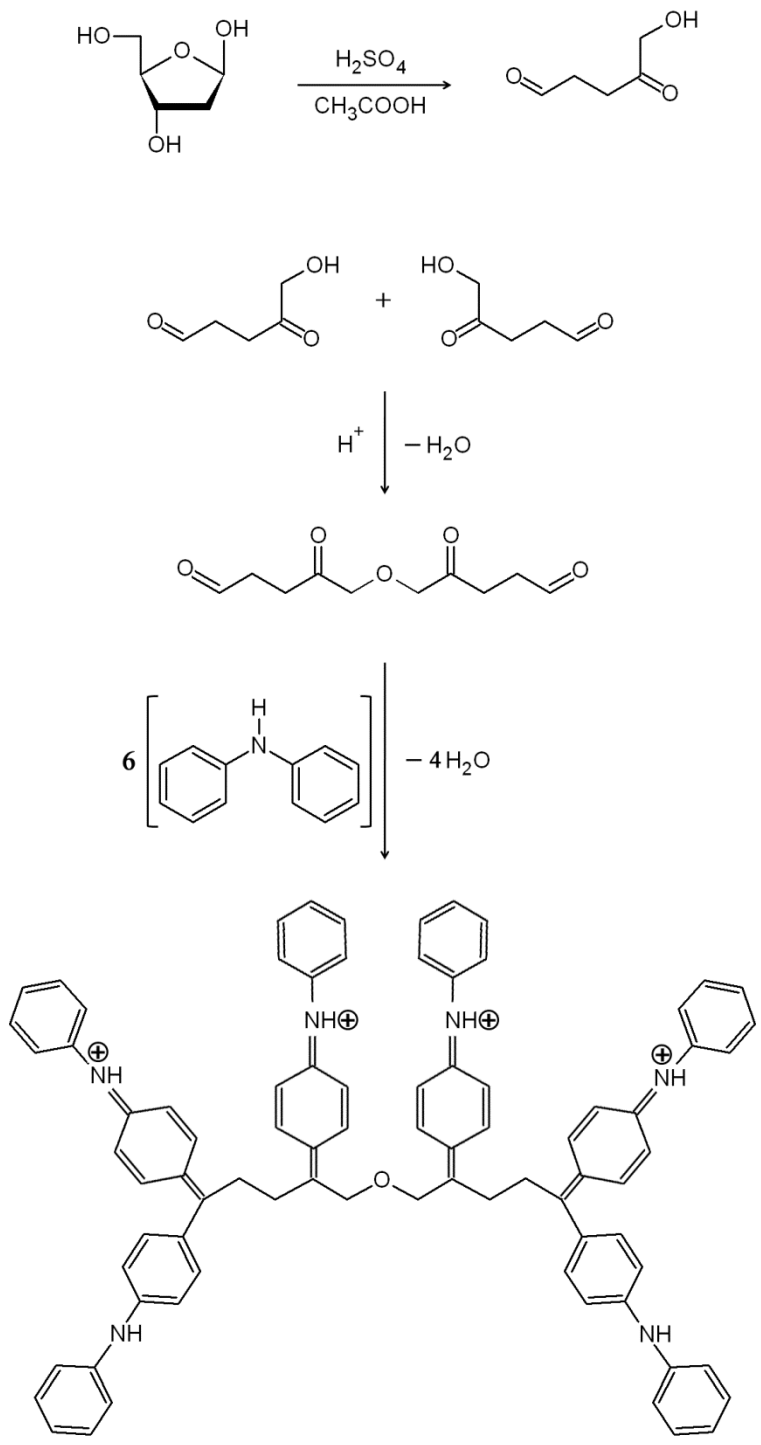

Figure 6.1 The reaction mechanism of diphenylamine reagent with deoxyribose sugar

Materials:

Equipments:

11. A UV/Visible spectrophotometer
12. Vortex mixer
13. Weighing balance
14. Water bath

Reagents:

3. Diphenylamine reagent
4. Calf thymus DNA
5. Glacial acetic acid
6. Concentrated sulfuric acid

Glassware and plasticware:

9. Pipettes
10. Pipette tips
11. A 5 *ml* glass pipette
12. Pipette aid
13. A 100 *ml* measuring cylinder
14. A 250 *ml* amber coloured glass bottle
15. Test tubes
16. Caps for glass tubes
17. Distilled water
18. Quartz or glass cuvettes

Preparation of reagents:

Diphenylamine (DPA) reagent:

1. Weigh 1 *g* of diphenylamine and transfer it into a 250 *ml* amber coloured glass bottle.
2. Add 100 *ml* glacial acetic acid and shake well to achieve complete dissolution.
3. Add 2.5 *ml* of concentrated sulfuric acid.
4. Store the reagent in dark at 2 – 8 °C.

Calf thymus DNA (100 μg/ml)

Prepare 100 *μg/ml* of calf thymus DNA solution in distilled water.

Procedure:

1. As the concentration of the unknown DNA sample can be anything, the assay will be performed with a range of dilutions (1, 1:10, 1:100, and 1:1000). Prepare 1 *ml* of each of the dilutions.
2. Take 15 test tubes and label them from 1 to 15.
3. Pipette out 100 *μl*, 200 *μl*, 300 *μl*, …….., 1000 *μl* calf thymus DNA standard in the glass tubes labeled 1 – 10; leave blank the tube no. 11.
4. Add distilled water to make the final volume 1 *ml* in each of the tubes (including blank).
5. Take 1 *ml* of each of the unknown DNA dilutions in the tubes labeled 12 – 15.
6. Add 3 *ml* of DPA reagent in each of the 15 tubes and mix well by vortexing.
7. Cover each of the tubes with the caps and place them in boiling water bath for 10 minutes.
8. Take out all the tubes from water bath and allow them to return to room temperature.
9. Measure the absorbance of tubes 1 – 10 and 12 – 15 at 595 nm in the quartz/glass cuvette against the reagent blank (tube 11).
10. Record the readings in the suggested observation table below:

Observation Table:

Table 6.1: Observation table for the diphenylamine assay

Tube No.	Volume (*μl*)	Mass (*μg*)	Distilled water (*μl*)	Diphenylamine reagent (*ml*)	A_{595}
Standard DNA					
1	100	10	900	3	
2	200	20	800	3	
3	300	30	700	3	
4	400	40	600	3	
5	500	50	500	3	
6	600	60	400	3	
7	700	70	300	3	

8	800	80	200	3	
9	900	90	100	3	
10	1000	100	0	3	
11	Blank (0)	0	1000	3	
Unknown sample					
12	1000 (1:1000 dil.)	Unknown	0	3	
13	1000 (1:100 dil.)	Unknown	0	3	
14	1000 (1:10 dil.)	Unknown	0	3	
15	1000 (Undiluted)	Unknown	0	3	

Calculations:

1. Plot the absorbance values obtained for tubes 1 – 10 against the amount of standard DNA added to these tubes.
2. Fit the data points using linear regression [with intercept (0,0)].
3. Determine the slope $\left(\frac{\Delta Absorbance}{\Delta DNA\ amount}\right)$ of the regression line.
4. The concentration of the unknown DNA is given by:

$$DNA\ concentration\ (\mu g/ml) = \frac{Absorbance \times Dilution\ factor}{slope\left(\frac{1}{\mu g}\right) \times Volume\ of\ diluted\ DNA\ sample\ used\ for\ the\ assay\ (ml)}$$

Notes:

6. It is recommended to use freshly prepared diphenylamine reagent. The solution, however, can be prepared in advance and stored in dark at 2 – 8 °C.

7. Prepare all the samples in triplicate and the tubes should be labeled properly. To follow the numbering used in the procedure and table given above, the tubes can be labeled as *a*, *b*, and *c*; for example the three samples for tube 1 can be labeled as 1*a*, 1*b*, and 1*c*. The calculations can then be performed taking the average absorbance of the three tubes.

8. Concentrated sulfuric acid should be carefully pipetted out using a 5 *ml* glass pipette with the help of a pipette aid in a chemical fume hood.

9. The dilution(s) of unknown sample that show absorbance between 0.05 – 1.0 should be used for calculations.

10. UV/Visible spectrophotometer should be switched on at least 30 *min* before use.

Lecture 7 Melting temperature of DNA

Aim:

To determine the melting temperature (T_m) of a given DNA sample using ultraviolet absorption

Introduction:

The structure of DNA is briefly reviewed in lecture 1. A double-helical DNA is made up of two strands that run antiparallel to each other. Each adenine (A) in one strand is paired with a thymine (T) on the other; similarly, each guanine (G) on one strand is paired with a cytosine (C) on the other. A–T and G–C are said to constitute the complementary base pairs. This pairing is achieved through stacking interactions and hydrogen bonding between the bases and is the basis of the double stranded DNA structure and its stability. Heating disrupts these non-covalent interactions between the bases; this could unwind the two strands separating the two strands apart. Separation of the two DNA strands is termed as denaturation or melting of DNA. In the double-helical structure, guanine forms three hydrogen bonds with cytosine while adenine forms two hydrogen bonds with thymine. It is therefore evident that the amount of heat required for denaturing the DNA would depend on its nucleotide composition. The temperature at which 50% of the DNA gets denatured is termed as its melting temperature (T_m).

Nucleic acids absorb very strongly in the near UV region. The absorbance is attributed to the heterocyclic rings present in the nucleotides. At neutral pH, DNA would typically absorb with an absorption maximum around 260 nm. Denaturation of DNA leads to higher absorption of ultraviolet radiation (hyperchromicity) (Figure 7.1). The melting temperature of DNA can therefore be determined simply by monitoring its absorbance at 260 nm while heating it.

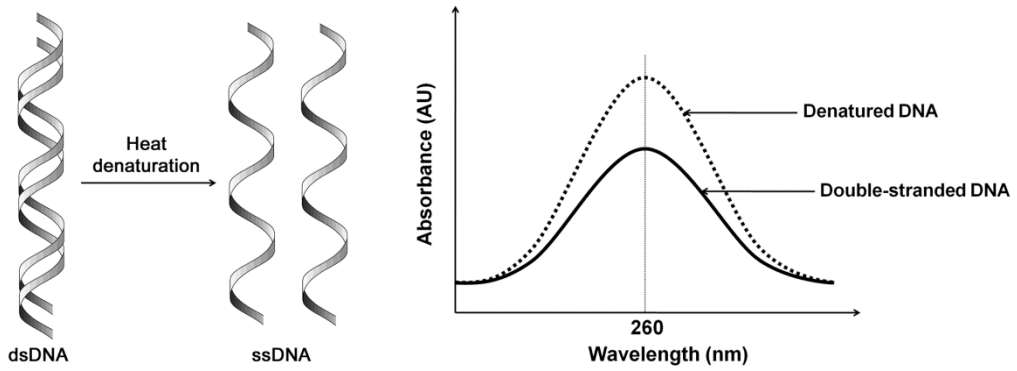

Figure 7.1: The hyperchromic effect in DNA; denaturation leads to higher absorption

Experimentally, the absorbance of the DNA molecule remains fairly constant at lower temperatures giving a plateau. As the temperature increases, the AT rich regions start melting thereby causing an increase in absorbance. Further increase in temperature causes steep rise in the absorbance followed by another plateau as the DNA gets completely denatured at these temperatures (Figure 7.2).

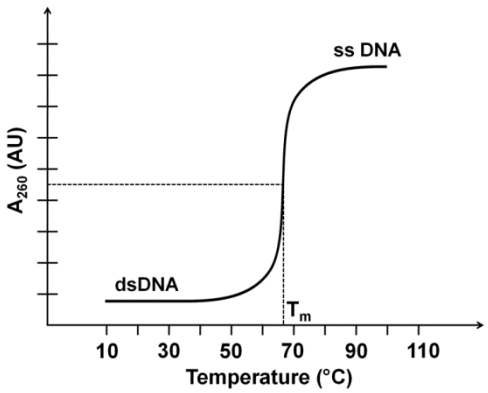

Figure 7.2: A typical DNA melting curve; the temperature at which half of the DNA is denatured is termed as the melting temperature (T_m).

Materials:

Equipments:

15. A UV/Visible spectrophotometer equipped with Peltier temperature cell:

    Peltier accessory is used for achieving very high temperature accuracies. The temperature of the cells or cell holders can be monitored by placing temperature sensors. It is recommended to place the temperature sensors in the cells for achieving more accurate results.

16. Water baths (in case equipment is not equipped with Peltier temperature control)

Glassware and plasticware:

19. Pipettes
20. Pipette tips
21. Parafilm
22. 1.5 *ml* microfuge tubes
23. Distilled water
24. 1 *ml* Quartz cuvettes (Note 1)
25. The buffer the given DNA is dissolved in

Procedure:

1. Switch “ON” the spectrophotometer.
2. Set the measurement mode to ‘Absorbance’ and wavelength to 260 nm.
3. Set the temperature to 20 °C in the Peltier attachment.
4. Measure the absorbance of the given DNA sample at 260 nm against the buffer used for dissolving the DNA.
5. An absorbance between 0.1 – 0.4 is suitable for determining the melting temperature. If the absorbance of the DNA is above 0.4, dilute the sample in the given buffer so as to achieve 1 *ml* DNA solution with an absorbance between 0.2 – 0.3.
6. Measure the absorbance of the diluted sample at 25 °C.
7. Increase the temperature by 5 °C and measure the absorbance when the cell reaches the specified temperature.
8. Repeat step 7 until a temperature of 95 °C is achieved.
9. Record the measurements in the observation table shown below (Table 7.1).

Alternative procedure, in case Peltier accessory is not there

1. Prepare sufficient volume (at least 15 *ml*) of the DNA sample in the given buffer so as to obtain an absorbance between 0.1 – 0.4.
2. Take fourteen 1.5 *ml* microfuge tubes and label them 1 – 14.
3. Add 1 *ml* of DNA solution into each of the microfuge tubes.
4. Tightly seal all the tubes with parafilm.

5. Label the three water baths as I, II, and III.
6. We shall be measuring absorbance at 20, 30, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, and 95 degrees Celsius *i.e.* at 14 different temperatures.
7. Set water baths I, II, and III at 20 °C, 30 °C, and 40 °C temperatures, respectively.
8. Place the tubes 1, 2, and 3 in water baths I, II, and III, respectively and incubate at least for 10 minutes.
9. Take tube 1 out and immediately measure its absorbance at 260 nm against the buffer blank.
10. Set the water bath I to 45 °C and place tube 4 in it once the specified temperature is achieved.
11. Meanwhile, take out tube 2 and measure its absorbance.
12. Set the water bath II to 50 °C and place tube 5 in it once the specified temperature is achieved.
13. Meanwhile, take out tube 3 and measure its absorbance.
14. Set the water bath III to 55 °C and place tube 6 in it once the specified temperature is achieved.
15. This cycle is to be followed until the absorbance is recorded for all the 14 tubes.
16. Record the measurements in the observation table shown below.

Observation table:

Table 7.1 Observation table for DNA melting curve

Temperature	**A_{260}**
20 °C	
…	
…	
…	
…	
…	
95 °C	

Analysis:

1. Plot the A_{260} against the temperature.
2. Determine the mid-point of the curve *i.e.* $\frac{\Delta A_{260}}{2}$, where ΔA_{260} is the maximum change in absorbance during thermal denaturation.
3. The temperature corresponding to $\frac{\Delta A_{260}}{2}$ is the melting temperature.

Notes:

1. The cuvettes need to be covered with the PTFE lids while making measurements to avoid any evaporation. The temperature sensor that is used in the Peltier equipped spectrophotometer should fit in the PTFE lids.
2. In the methods described above, temperature steps of 5 °C have been used. In case it is difficult to accurately determine the melting temperature, smaller temperature steps can be used near the T_m.

Lecture 8 Equilibrium unfolding of protein

Aim:

Monitoring equilibrium unfolding of protein using tryptophan fluorescence

Introduction:

Folding of a protein into its unique 3-dimensional structure is central for its function. The tertiary structure of a protein is determined by various intramolecularnon-covalent interactions such as H-bonding, electrostatic interactions, and hydrophobic interactions. The conformational stability of the protein structure is an important parameter that defines and limits its utility. In this lecture, we shall see how the stability of a single domain globular protein is determined.

Folding/unfolding of small globular proteins closely approaches the two state folding/unfolding mechanisms:

$$Folding\ (F) \Leftrightarrow Unfolding\ (U) \quad \text{---------- (8.1)}$$

The conformational stability of a small globular protein can be determined by calculating the equilibrium constant and the free energy, $\Delta G$for the reaction shown in equation (8.1). The value of ΔG for the unfolding reaction shown in equation 8.1 in the absence of a denaturant is referred to as the conformational stability of a protein at a given temperature and is represented by $\Delta G(H_2O)$. Comparison of conformational stability of a protein with its variants allows determination of various forces and factors responsible for the protein's stability.

Methods of unfolding

The native structure of a protein is sensitive to its environment such as pH, temperature, ionic strength, cosolvents, and presence of denaturants. A change in any of these parameters can disrupt the non-covalent interactions thereby causing unfolding (denaturation) of protein. The conformational stability of a protein is most routinely determined by thermal denaturation or by denaturing the protein with urea or guanidinium chloride. Urea solutions have historically been used for determining the conformational stabilities of proteins. Although guanidinium chloride is a stronger denaturant and chemically more stable than urea, it is not preferred over urea because

it is a salt and causes changes in the ionic strength of the solutions that could result in less reliable $\Delta G(H_2O)$. We shall, in this lecture, therefore be discussing the equilibrium unfolding of a protein using urea.

Methods for following unfolding

Unfolding of a protein can be studied by a variety of methods. The techniques that are more routinely used include ultraviolet difference spectroscopy, fluorescence spectroscopy, and circular dichroism spectroscopy. Unfolding can also be monitored using NMR spectroscopy, measuring biological activity of the protein, viscosity, and optical rotatory dispersion. Fluorescence spectroscopy and circular dichroism spectroscopy are perhaps the two most commonly used methods for monitoring protein unfolding and we shall be discussing the unfolding experiment keeping these two techniques in mind. To decide upon the technique to be used, the spectra of both folded and unfolded proteins need to be recorded (Figure 8.1). Following spectral features are then considered for deciding upon the technique to be used:

1. The magnitude of the response: At a given concentration of a protein, fluorescence intensity is usually much larger than the ellipticity. The sample amount may therefore be criteria for determining the method for monitoring unfolding. Furthermore, if the given protein lacks tyrosine and tryptophan residues, the fluorescence spectroscopy can simply not be employed.
2. The difference in response for folded and unfolded protein: The fluorescence intensity of the folded protein in figure 8.1A, for example, is ~4-fold more than that of the unfolded protein at ~322 nm. In general, the wavelength where maximum difference is observed is used. The difference in magnitude may be largest at ~195 nm in far-UV circular dichroism spectra (Figure 8.1B), it is however convenient to monitor unfolding at 220 nm as oxygen absorbs very strongly below 200 nm.
3. Signal to noise ratio: Apart from the difference in magnitude in the response, signal to noise ratio is an additional factor in determining the wavelength.
4. Finally, fluorescence spectroscopy is not recommended for monitoring thermal denaturation as pre- and post-transition baselines are steep and sensitive to temperature.

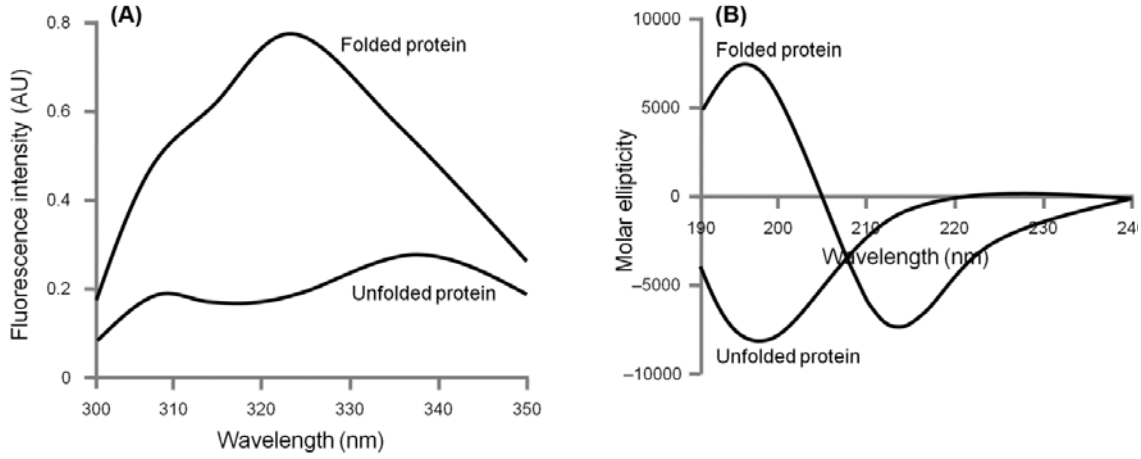

Figure 8.1: Fluorescence and circular dichroism spectra of a hypothetical protein in folded and unfolded state

Materials:

Equipments:

17. Spectrofluorometer
18. Weighing balance
19. pH meter

Reagents:

7. Urea
8. 3-(N-Morpholino)propanesulfonic acidsodium salt (MOPS sodium salt)
9. 1 *M* Hydrochloric acid
10. Given protein (RNase T1, commercially available)

Glassware and plasticware:

26. Pipettes
27. Pipette tips
28. 100 *ml* volumetric flasks
29. 100 *ml*beaker
30. Test tubes or 15 *ml* polypropylene tubes
31. Quartz cuvettes

Preparation of reagents:

Urea stock solution: Urea stock solution is prepared as follows:

6. Take a 100 *ml*volumetric flask, place it on the weighing balance, allow the reading to stabilize, and then tare it.
7. Weigh accurately 60*g* of urea and 0.694 *g* ofMOPS sodium salt and add them to a 100 *ml* beaker.For the sake of doing calculations is step 7, let us assume that the weight of the urea was 59.95 *g.*
8. Add 1.8 *ml* of 1 *M*HCl and 45*ml* of distilled water and allow the urea and MOPS salt to dissolve.
9. Measure the pH of the solution; if required, adjust the pH to 7.0 using 1 *M*HCl (note down the added mass).
10. Transfer the contents of the beaker into the 'tared' 100 *ml* volumetric flask.
11. Add distilled water to make the final volume to 100 *ml* and weigh the volumetric flask.Let us assume that the weight is 115.07*g*.
12. Calculate the urea concentration as follows:
    a. Calculate the ratio, $\frac{Weight\ of\ urea}{Weight\ of\ the\ solution}$, let us call this ratio, *W*.

       Here, $W = \frac{59.95}{115.07} = 0.521$

    b. If *d* is the density of the solution and d_0 is the density of water, then

$$\frac{d}{d_0} = 1 + 0.2658W + 0.0330W^2$$

$$= 1 + 0.2658 \times 0.521 + 0.0330 \times (0.521)^2$$

$$= 1.147$$

    c. The volume of the solution, $V = \frac{Weight\ of\ the\ solution}{d/d_0} = \frac{115.07}{1.147} = 100.32\ ml$

    d. Therefore, the molarity of urea $= \frac{Weight\ of\ the\ urea}{Molecular\ weight\ of\ the\ urea} \times \frac{1000}{V(ml)}$

$$= \frac{59.95}{60.056} \times \frac{1000}{100.32} = 9.95M$$

13. This gives a 9.95*M* urea solution in 30 *mM* MOPS buffer, pH 7.0.

MOPS buffer:

1. Weigh 0.694 *g* ofMOPS sodium salt and transfer to a 100 *ml* beaker.
2. Add 90 *ml* of distilled water and allow the salt to dissolve completely.
3. Adjust the pH to 7.0 using 1 *N*HCl.
4. Transfer the contents to a 100 *ml* volumetric flask and add distilled water to make the final volume 100 *ml*.

Protein stock solution:

1. Weigh accurately 100 *mg* of RNase T1 in a 15 *ml* polypropylene tube.
2. Add10*ml* of 30 *mM*MOPS buffer, pH 7.0.
3. This gives a 10 *mg/ml* solution of RNase T1 in 30 *mM*MOPS buffer, pH 7.0.
4. Store the protein stock solution at –20 °C.

Procedure:

1. Take out the frozen RNase T1 stock solution and allow it to come to room temperature.
2. Take 25 test tubes and label them from 1 to 25.
3. Add increasing volumes of urea stock solution, decreasing volumes of MOPS buffer and a fixed volume of protein stock solution as shown in table 8.1.
4. Allow the solutions to equilibrate (***Note 1***).
5. Switch ON the spectrofluorometer and allow it 30 *min* warm up.
6. Set the excitation wavelength to 280 nm and emission wavelength to320 nm (***Note 2***).
7. Measure the fluorescence emission for each of the samples at 90° for 30 seconds (this gives multiple readings, depending on the integration time used for each reading).
8. Calculate the average fluorescence reading for each of the samples from the multiple readings obtained in step 7 and record them in the observation table (Table 8.1)
9. Plot the fluorescence emission intensity against urea concentration as shown in figure 8.2.

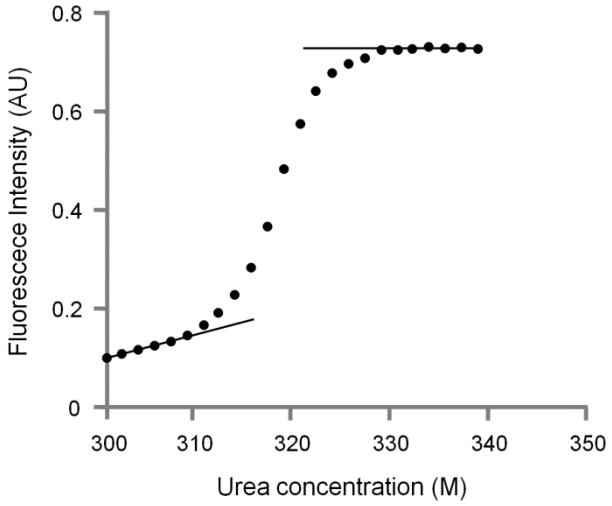

Figure 8.2: A plot between fluorescence intensity against urea concentration showing a typical two-state protein unfolding curve

Observation table:

Table 8.1: Observation table for the protein unfolding using fluorescence spectroscopy

Tube No.	MOPS buffer (*ml*)	Urea stock solution (*ml*)	Protein stock solution (*ml*)	Urea concentration (*M*)	Fluorescence intensity at 320 nm (*AU*)
1	2.8	0	0.2	0	-
2	2.7	0.1	0.2	0.33	-
3	2.6	0.2	0.2	0.67	-
4	2.5	0.3	0.2	1	-
⋮	⋮	⋮	⋮	⋮	-
⋮	⋮	⋮	⋮	⋮	-
⋮	⋮	⋮	⋮	⋮	-
25	0.4	2.4	0.2	8	-

Analysis of unfolding curve

For a two-state unfolding process, plotting the fluorescence intensity gives a curve as shown in figure 8.2. A least square fitting is performed on the data to obtain a continuous curve.

For a two-state folding/unfolding mechanism, only folded and unfolded protein states are populated at significant concentrations at any of the urea concentration. Therefore,

$$f_F + f_U = 1 \quad \text{---------- (8.1)}$$

where, f_F and f_U represent the fractions of the folded and unfolded proteins, respectively.

Thus the observed value of *y* (fluorescence intensity, in this case) at any point in the graph is given by:

$$y = y_F f_F + y_U f_U \quad \text{---------- (8.2)}$$

where, y_F and y_U represent the values of *y*characteristic of the folded and unfolded protein states, respectively and can be calculated from the unfolding curve (figure 8.2).

Combining equations 8.1 and 8.2:

$$f_U = \frac{(y_F - y)}{(y_F - y_U)} \quad \text{---------- (8.3)}$$

The unfolding curve can be divided into three regions:

i. *Pre-transition region*: it shows how *y* for the folded protein *i.e.*y_F responds to the denaturant.
ii. *Transition region*: it shows how *y* varies as the unfolding takes place.
iii. *Post-transition region*: it shows how *y* for the unfolded protein *i.e.*y_U responds to the denaturant.

The equilibrium constant,K_{eq} for the reaction can be calculated as follows:

$$K_{eq} = \frac{f_U}{(1 - f_U)} = \frac{f_U}{f_F} = \frac{(y_F - y)}{(y - y_U)} \quad \text{---------- (8.4)}$$

The free energy change, ΔG for the reaction can be calculated from equation 8.5:

$$\Delta G = -RTlnK = -RTln\left[\frac{(y_F - y)}{(y - y_U)}\right] \quad \text{---------- (8.5)}$$

The values of K_{eq} are most accurately measured near the midpoint of the denaturation curve and the errors become substantial for values outside the range 0.1 – 10. This corresponds to the ΔG values between –5.7 to +5.7 *kJ/mole* (~ ±1.36 *kcal/mol*). The data for this range can be tabulated as shown in table 8.2.

Table 8.2: Analysis of the urea denaturation curve

Urea concentration (*M*)	*y*	f_U	K_{eq}	ΔG
			>0.1	
			<10.0	

In the limited region where ΔG is most accurately measured, it varies linearly with the concentration of denaturant (Figure 8.3).

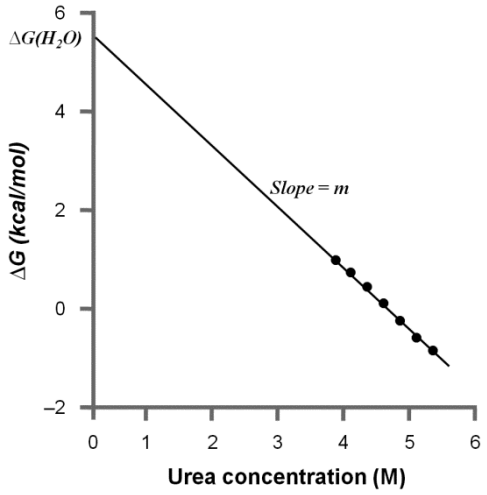

Figure 8.3: ΔG as a function of denaturant (urea) concentration. Intercept at ΔG gives the stability of the protein, $\Delta G(H_2O)$.

The equation of line is obtained from the least square analysis. ΔG in the absence of denaturant *i.e.* $\Delta G(H_2O)$ is calculated by extrapolating the line to zero denaturant concentration. The linear equation therefore is given by:

$$\Delta G = \Delta G(H_2O) - m\ [denaturant]$$

Notes:

1. As we are interested in determining the thermodynamic parameters in the unfolding reaction, it is important to ensure that the unfolding reactions have reached the equilibria before measurements are made. The equilibration time varies from protein to protein and depends on the temperature at which the reaction is being carried out; it can lie anywhere between seconds to days. Equilibrium for RNase T1, for instance, is achieved in minutes at 30 °C but takes hours at 20 °C. The solutions in the pre- and post-transition regions equilibrate faster than those in the transition region. For an unknown protein, it is necessary to carry out a pilot study for determining the urea concentration corresponding to the transition region and the equilibration times.
2. The optimum emission wavelength may vary from protein to protein and should be determined as discussed in the 'Introduction' section. For RNase T1, 320 nm is the optimum wavelength.

Lecture 9 Circular dichroism of proteins – I

Aim:

To record the far-UV circular dichroism spectrum of a protein

Introduction:

Circular dichroism, abbreviated as CD, is a chiropticalspectroscopic tool that is routinely employed to study the secondary structural elements of proteins and peptides. The technique is also used to determine the conformational stability of the proteins as discussed in the previous lecture, study the kinetics of folding/unfolding, binding with ligands, and to determine if an expressed, purified protein is correctly folded. Unlike X-ray crystallography and NMR spectroscopy, that can provide residue specific structural information, circular dichroism provides the overall secondary structural components with no residue-specific information. The advantages of CD include small sample requirement, rapid measurements, and measurements under physiological conditions. Protein/peptide concentration of $\leq$ 20 $\mu g/ml$ is usually sufficient for recording spectra in far-UV region; furthermore, recording is usually complete within 5-10 minutes.

Let us now see what actually circular dichroism is? Dichroism literally means “two colours”. In chiroptical spectroscopy, dichroism refers to the differential absorption of lights with different polarizations. Circular dichroism therefore refers to the differential absorption of lights with different circular polarizations. You may already be familiar with plane or linearly polarized light. Let us see what circular polarization is and how it is achieved. Consider two plane polarized electromagnetic waves of same wavelength, polarized in two perpendicular planes and out of phase by 90°. The 90° phase difference implies that when one of the waves has maximum amplitude, the other one has zero amplitude (Figure 9.1).

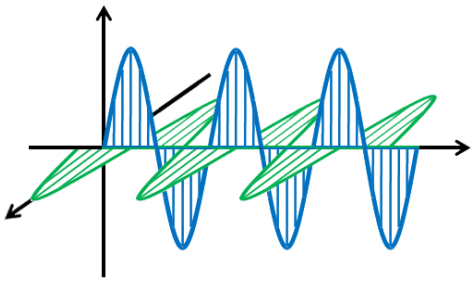

Figure 9.1 Two plane polarized waves (blue and green), polarized in two perpendicular planes and out of phase by 90º. Only electric field vectors are shown here for clarity.

The superposition of these two plane waves is shown in figure 9.2.

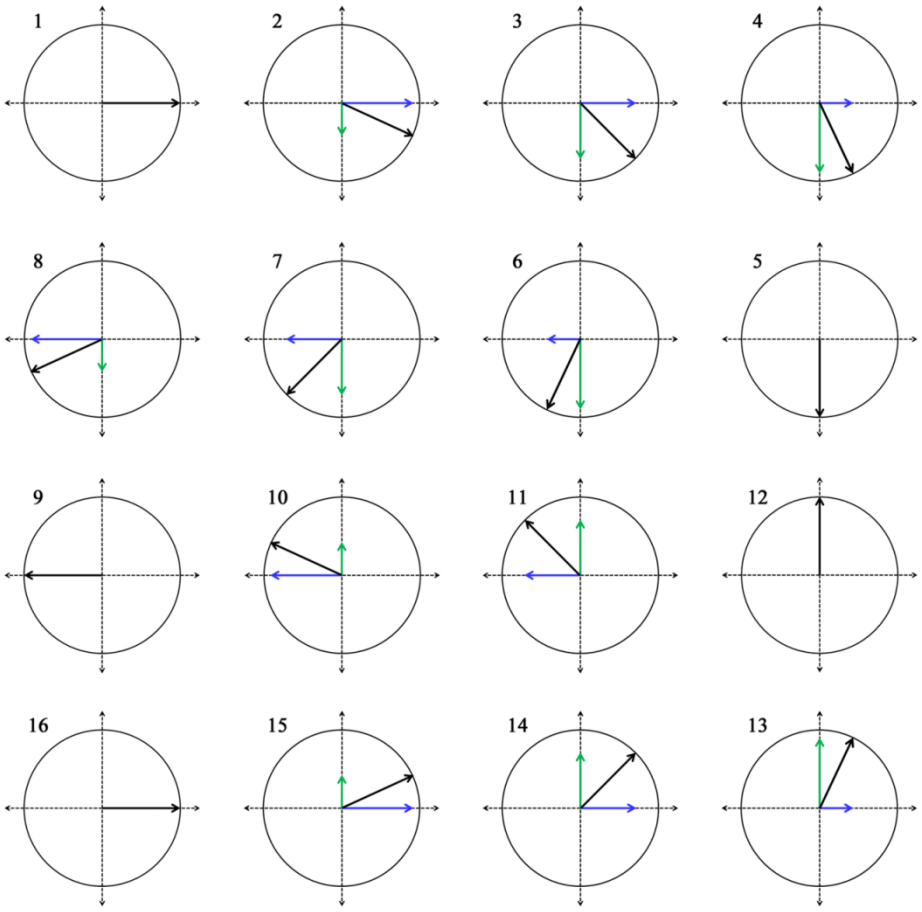

Figure 9.1 Generation of circularly polarized light through superposition of two plane polarized waves (blue and green), polarized in two perpendicular planes and out of phase by 90º. Only electric field vectors are shown here for clarity.

It is clear that the superposition results in a wave wherein the electric field vector traverses a circular path; this is termed as the circularly polarized light.The direction of rotation is determined by the phase difference; a –90° phase difference would result in a circularly polarized light with opposite rotation of the electric field vector.Chiral or asymmetric molecules can absorb the left and right circularly polarized lights to different extents; this differential absorption is termed as circular dichroism or CD:

$$CD = \Delta A = A_L - A_R \quad \cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots \quad (9.1)$$

where, A_L and A_R are the absorbances for the left and right circularly polarized lights, respectively. Equation 9.1 can be rewritten in terms of the molar absorption coefficients of the molecule for the left and right circularly polarized lights:

$$CD = (\varepsilon_L - \varepsilon_R)cl \quad \cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots (9.2)$$

where; $\varepsilon_L, \varepsilon_R, c,$ and l represent the molar absorption coefficient for left circularly polarized light, molar absorption for right circularly polarized light, molar concentration of the molecule, and the path length of the cell, respectively.

$$CD = \Delta\varepsilon cl \quad \cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots (9.3)$$

Differential absorption of the two circularly polarized lights results in elliptically polarized light and CD is historically represented in terms of ellipticity (θ) which is the angle whose tangent is the ratio of the minor to major axis of the ellipse (Figure 9.3).

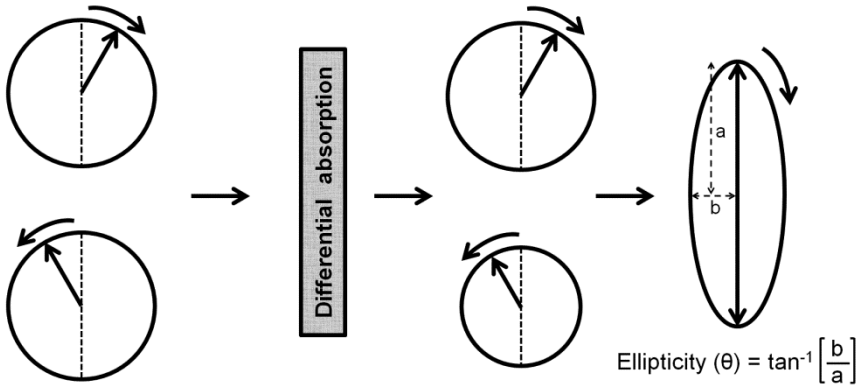

Figure 9.3:Differential absorption of left and right circular polarized light results in elliptically polarized light. Ellipticity is the arc tangent of the ratio of minor to major axis.

CD of proteins and peptides

Proteins are the linear polymers made up of 20 amino acids, 19 out of which (except glycine) are chiral. This chirality is also reflected in the secondary structures the proteins/peptides adopt. Far-UV CD spectra of proteins are typically recorded from 190 – 250 nm. Peptide bond is the major chromophore in this region and the relative orientations of the peptide bonds with respect to each other lead to characteristic CD signals thereby allowing identification of the secondary structural elements.

Materials:

Equipments:

20. Circular dichroismspectropolarimeter
21. Weighing balance
22. pH meter

Reagents:

11. 50 *mM* phosphate buffer, pH 7.0
12. Given protein (Hen egg white lysozyme)
13. 0.1 *M*KCl (***Note 1***)

Glassware and plasticware:

32. Pipettes
33. Pipette tips
34. 100 *ml* volumetric flasks
35. 100 *ml*beaker
36. Test tubes or 15 *ml* polypropylene tubes
37. 1 mm path length Quartz cuvettes

Preparation of reagents:

*Phosphate buffer (**Note 1**)*:

1. Prepare 50 mM sodium phosphate buffer, pH 7.0 as described in lecture 3.
2. Filter the buffer through a 0.22 μm filter.

*Protein solution (**Note 2**):*

1. Switch on the UV/visible spectrophotometer and allow it 30 minutes warm up.
2. Meanwhile, weigh 5*mg* lysozyme and dissolve it in 500μl phosphate buffer.
3. Filter the solution through 0.22 *micron* filter.
4. Take 10μl of the filtered lysozyme solution and add 990μl of 0.1 *M*KCl.
5. Measure the absorbance of the 100-fold diluted lysozyme solution against 0.1 M KCl at 281.5 nm $\left(E^{1\%}_{281.5\ nm} = 26.4\ in\ 0.1M\ KCl\right)$.
6. Estimate the concentration of the lysozyme stock solution using the formula:
$$A = E^{1\%}_{281.5\ nm} \times C(wt\%) \times l(cm) \times Dilution factor$$
7. Dilute the lysozyme stock solution to prepare a 20 $\mu g/ml$ solution in 50 *mM* phosphate buffer, pH 7.0. This is the working protein solution.

Procedure:

10. Purge the CD spectropolarimeteroptics compartment with ultrapure nitrogen gasat ~10 *litres/minute* for 15 minutes (As long as the instrument is on, there should be uninterrupted N_2 supply).
11. Turn ON the lamp of the spectropolarimeter.
12. TurnON the other parts of the spectropolarimeter and the computer; allow 30 minutes warm up.
13. Open the spectra collecting software.
14. Set the half bandwidth between 1 – 1.5 nm.
15. Set the wavelength range: wavelength range from 260 – 185 nm is suitable for the 0.1 – 0.2 *mg/ml* protein solutions in the buffers that don't absorb in this range.
16. Set the number of scans to 8 (This means that the final CD spectrum will be an average of 8 different scans).
17. Define the path in the software for storing the data.

18. Set the wavelength interval: for samples with signal to noise ratio > 20:1, 0.5 nm is an optimum interval; if the signal to noise ratio is low, the interval can be set at 0.1 or 0.2 nm.
19. Set the data collection time at each wavelength to 1 *second*.
20. Set the instrument time constant to 100 *ms* (***Note 3***).
21. Set the instrument to record the ellipticity and the PMT voltage (***Note 4***).
22. Take 200 μl of filtered phosphate buffer in the 1 *mm* path length quartz cuvette.
23. Record the CD while monitoring the PMT voltage (PMT voltage increases as the instrument scans lower wavelengths) (***Note 4***).If the PMT goes above 500 V, the buffer may not be suitable for the lower wavelengths.
24. Remove the buffer from the cuvette and add 200 μl of 20 $\mu g/ml$ lysozyme solution.
25. If PMT voltage goes above 500 V, the spectrum becomes noisy and less reliable. In that case, the protein solution needs to be diluted or the spectra be recorded to a higher wavelength, say 190 or 195 nm.
26. Record the CD spectrum for the protein solution.
27. Subtract theblank spectrum from the recorded protein spectrum to obtain the corrected protein spectrum.
28. Save the corrected spectrum as a separate text file.

Results and analysis:

1. Most CD instruments will generate data in ellipticity (*millidegrees*).
2. CD values of proteins and peptides are generally reported as mean residue ellipticity values [θ] in $deg \cdot cm^2 \cdot dmol^{-1}$. This is achieved as discussed in the subsequent steps.
3. Convert the corrected spectrum into text (ASCII) file using the CD software.
4. Open the text file using a computing and graphing software such as Microscoft Excel or Origin.
5. Calculate the mean residue ellipticity, [θ] in $deg \cdot cm^2 \cdot dmol^{-1}$ at each wavelength using following formulae:

$$[\theta(deg \cdot cm^2 \cdot dmol^{-1})] = \frac{\theta(millidegrees) \times Mean\ residue\ weight}{Pathlength\ (mm) \times Concentration\left(\frac{mg}{ml}\right)} \quad \cdots\cdots\cdots\cdots\cdots\cdots (9.4)$$

$$[\theta(deg \cdot cm^2 \cdot dmol^{-1})] = \frac{\theta(millidegrees)}{Pathlength\ (mm) \times Concentration(M) \times No.of\ residues\ in\ protein} \quad \cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots (9.5)$$

6. Plot mean residue ellipticity [θ] as a function of wavelength (λ) to obtain the far UV CD spectrum.

Notes:

1. Buffers to be used for CD spectroscopy have to be free of any optically active component. The buffer has to be as transparent in the far-UV region as possible. Water alone is the most transparent solvent but absence of salts may result in denaturation of certain proteins.
2. It is necessary to determine the concentration of the protein very accurately for obtaining high quality CD data. As the response of Bradford and Lowry methods vary from protein to protein, these methods are not suitable for determining very accurate concentrations. The protein concentration should therefore be calculated either through quantitative amino acid analysis or using the published molar absorption coefficients of the proteins. In this experiment, we have used 0.1 *M*KCl for estimating the hen egg white lysozyme concentration as $E^{1\%}$ for this protein is reported in 0.1 *M*KCl; the method for preparing the protein stock solution will differ from that discussed in this experiment.
3. Instrument time constant is the measure of how quickly an instrument responds to an input. An instrument constant of 100 *ms* is usually sufficient for routine CD spectroscopy. The instrument response time should not be greater than the one-tenth of the data collection time at each point (1 *second* in this experiment).
4. The PMT detectors will produce currents in response to the incoming photons. Most CD spectropolarimeters work in the constant current mode. As the wavelength decreases, the absorbance increases thereby causing lesser number of photons reaching the detector. This results in increase in the PMT voltage so as to maintain the constant current. As the PMT voltage crosses the 500 V,

the spectra become noisy and less reliable. In such cases, the sample is diluted so that the absorbance of the sample decreases. If the PMT is still high, the spectra should be recorded up to relatively higher wavelengths *i.e.* up to ~190 or 195 nm than 185 nm.

Lecture 10 Circular dichroism of proteins – II

Aim:

To estimate the secondary structural components of a protein from its far-UV circular dichroism spectrum using CDPro software suite

Introduction:

As discussed in the previous lecture, peptide bond constitutes the major protein chromophore in the far-UV region. The absorbance in the far UV region is the manifestation of$n \rightarrow \pi^*$ transitions around 220 nm and $\pi \rightarrow \pi^*$ transitions below 210 nm. The orientation of the peptide bonds in the secondary structural elements greatly influences their CD signals. Let us have a look at the characteristic spectral features of the major secondary structural components present in proteins (Figure 10.1).

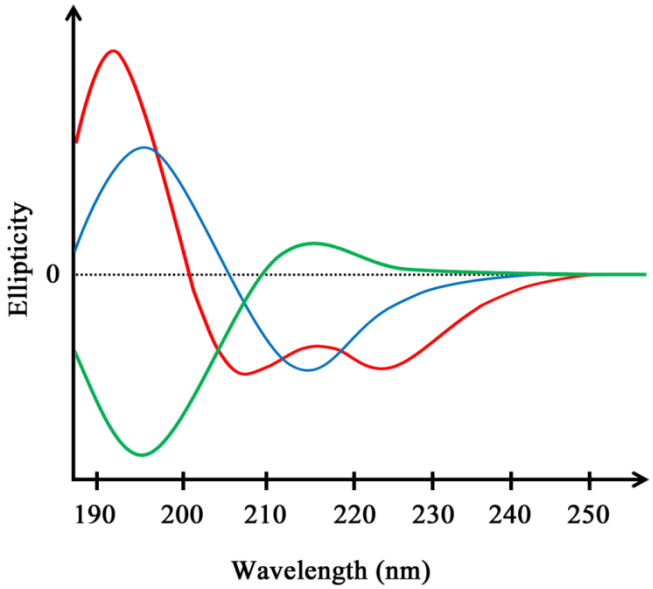

Figure 10.1 Far UV CD spectra of α-helix (red), β-sheet (blue), and unordered conformations (green)

- *α-helix:* CD spectrum of right-handed α-helix is characterized by two negative absorption bands of nearly same intensity centered around 222 nm (arises due to $n \rightarrow \pi^*$ transition) and 208 nm (a fraction of the $\pi \rightarrow \pi^*$ transition) and a relatively more intense positive band around 192 nm (a fraction of the $\pi \rightarrow \pi^*$ transition).

- *β-sheet:* β-sheets display a negative band centered around 216-218 nm (arises due to $n \rightarrow \pi^*$ transition) and a positive band of comparable intensity at ~195 nm (arises due to $\pi \rightarrow \pi^*$ transition).

- *β-turn:* β-turn is a four residue protein motif that causes the polypeptide backbone to take a turn of approximately 180°. β-turns do not have a well defined spectral signature. A typical β-turn, however, shows a weak negative band around 225 nm (arises due to $n \rightarrow \pi^*$ transition), a strong positive band between 200 – 205 nm (arises due to $\pi \rightarrow \pi^*$ transition), and a strong negative band (arises due to $\pi \rightarrow \pi^*$ transition) between 180 – 190 nm.

- *Random coil:* Random coil or the unordered conformation displays a weak positive band around 218 nm (arises due to $n \rightarrow \pi^*$ transition) and a strong negative band (arises due to $\pi \rightarrow \pi^*$ transition) below 200 nm.

It is due to these characteristic signatures for the secondary structures that the secondary structural components in the proteins can be identified and estimated.

Many different methods are available for analyzing the circular dichroism spectra of proteins. All these methods work on the assumption that the CD spectrum of the protein is a linear combination of the spectra of its secondary structural elements, plus noise. The ellipticity of the protein at any wavelength can therefore be represented by equation 10.1

$$\theta_\lambda = \sum f_i S_{i\lambda} + noise \quad \cdots\cdots\cdots\cdots\cdots\cdots (10.1)$$

whereθ_λ represents the ellipticity of the protein at wavelength, λ; f_i, represents the fraction of the i^{th} secondary structural element; and $S_{i\lambda}$ represents the ellipticity of the secondary structural element, S_i at wavelength, λ.

The methods that are in practice utilize the CD spectra derived from the proteins whose crystal structures have been determined as the reference. A number of algorithms have been developed that utilize the reference spectra database to evaluate the secondary structural components in a protein from its CD spectrum. We shall not be discussing these algorithms but details can be found elsewhere [1–4].

The initial attempts in deconvoluting the protein CD spectra utilized poly-L-lysine CD spectra as reference. Poly-L-lysine can adopt, depending on the conditions, three different conformations in dilute aqueous solutions. It adopts random coil in aqueous solutions at acidic and neutral pH. At pH 11.2, it adopts a predominantly α-helical conformation. Heating the poly-L-lysine solution at pH 11.2, for 20 minutes at 51 °C, results in antiparallel β-sheets. The CD spectra of poly-L-lysine can therefore be, to a very good approximation, be treatedas those arising for pure conformations and used for analyzing the structures of unknown proteins. Unlike poly-L-lyine, however, proteins are heteropolymers and the CD spectra of a homopolymer may not represent a good basis for estimating their secondary structural components. Modern methods therefore utilize the CD spectra of the proteins whose structures have been determined by X-ray crystallography as the reference database.

CDPro software suite

CDPro is a suite of programs developed by Sreerama and Woody for analyzing the far-UV CD data [2]. The suite contains three programs, SELCON3, CONTINLL, and CDSSTR and is freely available at http://lamar.colostate.edu/~sreeram/CDPro. The information about the algorithms these programs use can be found elsewhere [1, 2, 4].

Materials:

1. CDPro software suite: CDProcan be downloaded asCDPro.zip filefrom http://lamar.colostate.edu/~sreeram/CDPro.
2. The far UV CD spectrum to be analyzed.

Procedure:

29. Extract the files from CDPro.zip in a new directory, CDPro.
30. Most CD instruments will generate data in ellipticity(*millidegrees*).
31. Correct the protein/peptide spectrum by subtracting the blank spectrum.
32. Save the corrected spectrum as a text (ASCII) file.
33. Covert the ellipticity (*mdeg*) into mean residue ellipticity, [θ] as discussed in previous lecture.
34. Convert the mean residue ellipticitydata into per residue Δε units as follows:

$$\Delta\varepsilon = \frac{[\theta]}{3298}$$

35. Save the file having wavelength in the first column and Δε values in second as a text (ASCII) file with the protein name "protein.txt".*Important*: There should not be any other text or numbers except the data values and the file name should not be longer than 12 characters (including the file extension, .txt).
36. Copy this file (protein.txt) in the 'CDPro' directory.
37. Open the directory, 'CDPro'.
38. Double click the 'CRDATA' application file; a window appears.
39. The window will have a set of questions/instructions; follow them as discussed in the subsequent steps.
40. Question/Instruction 1: Do you want to create a new INPUT file?
    a. Type 0 for creating a new INPUT file and press ENTER.
41. Question/Instruction 2:Enter TITLE for your data –40 characters
    a. Type the name of the protein you are studying (not more than 40 characters)and press ENTER.
42. Question/Instruction 3:The number of lines to be skipped in CD file
    Enter the number of CD values per nm
    a. Enter the correct number as suggested (this depends on the wavelength interval the data is recorded with) and press ENTER.
43. Question/Instruction 4: INPUT INITIAL wavelength
    a. Enter the maximum wavelength the CD data is recorded at (do not type *nm*), and press ENTER.
44. Question/Instruction 5: INPUT INITIAL wavelength
    a. Enter the minimum wavelength the CD data is recorded at (do not type *nm*), and press ENTER.
45. Question/Instruction 6: Is the data in Molar Ellipticity units?
    a. Type the appropriate number and press ENTER (Note that the data we are using as input is in Δε form and therefore does not require any conversion for analysis by CDPro programs. Therefore, type 0 and press ENTER).
46. Question/Instruction 7:ASCII-file name (CD data)-MAX of 12 letters

a. Type the name of the file (with file extension) containing CD data(protein.txt in this case) and press ENTER (***Note 1***).

47. Question/Instruction 8: Hit ENTER to continue
    Ctrl-C to ABORT
    a. If all the details you have entered are correct, press ENTER. The basis sets available for analysis are displayed.
48. Question/Instruction 9: Select a IBasis value
    a. Select the reference set with maximum number of proteins but encompassing the smallest wavelength the CD data is recorded at.
    b. The software suggests an option which basis set should be used. It is recommended to use the suggested option.
    c. Type the suggested IBasis number and press ENTER.
49. The above steps generate a file, INPUT.SMP; this file is used as the input file for CD spectrum deconvolution. *Important*: If there is an INPUT.SMP file already present in the CDPro directory, it gets overwritten when steps 12 – 19 are followed.
50. Execute the CDSSTR.exe application by double clicking it. The application calculates the fractions of the secondary structure components using the INPUT file.
51. Press ENTER.
52. Execute the CONTINLL.exe application by double clicking it. The application calculates the fractions of the secondary structure components using the INPUT file.
53. Press ENTER.
54. Execute the SELCON3.exe application by double clicking it. The application calculates the fractions of the secondary structure components using the INPUT file.
55. Press ENTER.
56. Open the CDSSTR.out, CONTIN.out, and SELCON3.out to see the output of CDSSTR, CONTINLL, and SELCON3 algorithms, respectively.
57. Open ProtSS.out file using Notepad. The file displays the CD deconvolution results obtained from all the three algorithms.
58. Choose the appropriate results based on the RMSD (root mean squared deviation) and NRMSD (normalized root mean square deviation).

Notes:

1. It is a common mistake not to include '.txt' after the file name. Make sure the file extension is included.

References:

1. Sreerema, N. and Woody, R.W. (1993) A self-consistent method for the analysis of protein secondary structure from circular dichroism. Analytical Biochemistry, 209, 32-44.
2. Sreerama, N. and Woody, R. W. (2000) Estimation of Protein Secondary Structure from Circular Dichroism Spectra: Comparison of CONTIN, SELCON, and CDSSTR Methods with an Expanded Reference Set. Analytical Biochemistry,287, 252-260.
3. Whitmore, L. and Wallace, B. A. (2004) DICHROWEB: an online server for protein secondary structure analyses from circular dichroism spectroscopic data. Nucleic Acids Research, 32, W668-W673.
4. Greenfield, N. J. (2006) Using circular dichroism spectra to estimate protein secondary structure. Nature Protocols, 1, 2876-2890.

Lecture 11 Fourier transform infrared spectroscopy of proteins

Aim:

To identify the secondary structural elements of a protein using infrared spectroscopy

Introduction:

The region of electromagnetic spectrum ranging from ~780 nm to 250000 nm is defined as the infrared (IR) spectrum. As writing such big numbers is inconvenient, wavelengths of infrared region are often represented in micrometers. Spectroscopists prefer to use wavenumbers ($\bar{\nu}$) for representing IR spectra. Wavenumber in cm^{-1} is given by:

$$\bar{\nu}\,(cm^{-1}) = \frac{1}{\lambda\,(\mu m)} \times 10^4$$

The energies of infrared region correspond to the energies associated with molecular vibrations. Infrared spectroscopy is therefore also known as vibrational spectroscopy. The infrared spectrum can be divided into three different regions: near IR (λ ~0.8 – 2.5 μm), mid-IR (λ ~2.5 – 25 μm), and far IR (λ ~25 – 250 μm). Mid-region of IR is the one that is used for studying molecular vibrations. More details about infrared spectroscopy can be found here: http://nptel.ac.in/courses/102103044/10.

Like CD, infrared spectroscopy also utilizes the peptide bonds for secondary structure determination. The peptide groups result in nine distinct absorption bands labeled as amide A, B, and I-VII. Amide I is the most useful IR band in analyzing the polypeptide backbone conformation. It arises largely due to the carbonyl stretching vibration with small contributions from C–N stretching and N–H bending vibrations, and appears between 1700 – 1600 cm^{-1}. The precise frequency of vibration is determined by the nature of the hydrogen bonds the C=O and N–H groups are involved in. The nature of the H-bonding the backbone amide groups are involved in depends on the conformation of the polypeptide backbone. It should therefore be possible to determine the secondary structural elements of the proteins from the frequencies present in the amide I band. The absorption bands for different polypeptide conformations are shown in table 11.1

Table 11.1 Absorption bands of protein secondary structural elements in H_2O

Polypeptide conformation	Wavenumber (cm^{-1})
α-helix	1657 – 1648
β-sheet	1641 – 1623
Unordered	1657 – 1642
Antiparallel β-sheet	1695 – 1675

There is large overlap between the bands for α-helical and unordered conformations in H_2O. It is therefore difficult to unambiguously assign the bands in this region. These two secondary structures, however, can be distinguished if the spectrum is recorded in D_2O. In D_2O, the exchangeable protons of the proteins are exchanged with deuterium. Hydrogens of the backbone amides in unordered conformation are more readily exchanged as compared to those involved in the secondary structures. Therefore, the bands from unordered and α-helical conformations are observed at ~1644 cm^{-1} and ~1648 – 1657 cm^{-1}, respectively.

Infrared optical materials:

The sample to be analyzed is placed in front of an infrared beam in the infrared spectrometer. We have seen that glass, quartz, or polypropylene cells are good for visible spectroscopy while quartz cells are required for ultraviolet spectroscopy. What kind of sample cells do we use for infrared spectroscopy? The fact is that all materials have some sort of vibration associated with them that could lead to infrared absorption. If the material chosen for IR spectroscopy absorbs the frequencies close to those absorbed by our samples, the sample signal may simply not be distinguishable from the signal of sample cell. Materials have their characteristic IR absorption spectra; we need to select the material that does not strongly absorb in the region where our sample absorbs. This region is termed as the optical window and the material is said to be transparent in this region. Table 11.2 shows the optical window (transparent region) of some of the materials routinely used for infrared spectroscopy.

Table 11.2 Characteristics of materials used in infrared spectroscopy

Material	Transparent Region (cm^{-1})	Solubility	Notes
Silica glass	55000 – 3000	HF	–
Quartz	40,000 – 2,500	HF	–
Sapphire	20,000 – 1,780	–	Strong
Diamond	40,000 – 2,500 1,800 – 200	–	Very strong, expensive
NaCl	40,000 – 625	H_2O	Easy to polish, hygroscopic
CaF_2	70,000 – 1,100	Acids	Not suitable for acidic pH, avoid ammonium salts
BaF_2	65,000 – 700	–	Avoid ammonium salts
ZnSe	10,000 – 550	Acids	Brittle, not suitable for acidic pH
AgCl	25,000 – 400	–	Soft, sensitive to light
KCl	40,000 – 500	H_2O, diethylether, acetone	–
KBr	40,000 – 400	H_2O, ethanol	Hygroscopic, soft, easily polished, commonly used in making pellets
CsBr	10,000 – 250	H_2O, ethanol, acetone	Hygroscopic, soft
CsI	10,000 – 200	H_2O, ethanol, methanol, acetone	Hygroscopic, soft

KBr is inexpensive and transparent for the entire mid-IR region. It is one of the most routinely used materials for infrared spectroscopy including biomolecular infrared spectroscopy.

Materials:

Equipments:

23. Fourier Transform Infrared Spectrometer
24. Hydraulic press
25. Weighing balance
26. Hot air oven

Reagents:

14. Given protein (Hen egg white lysozyme)
15. KBr (Infrared spectroscopy grade)
16. D_2O
17. DCl
18. NaOD

Glassware, plasticware, etc.:

38. Pipettes
39. Pipette tips
40. 1.5 *ml* microfuge tubes
41. Pestle and mortar

Procedure:

59. Dry the KBr in hot air oven to remove any moisture present in it.
60. Take out the dried KBr powder and weight ~60 mg of it.
61. Grind it in a pestle mortar to obtain a very fine powder.
62. Prepare the KBr pellet using a die and hydraulic press as discussed below:
    a. The different parts of the dye are shown in figure 11.1.

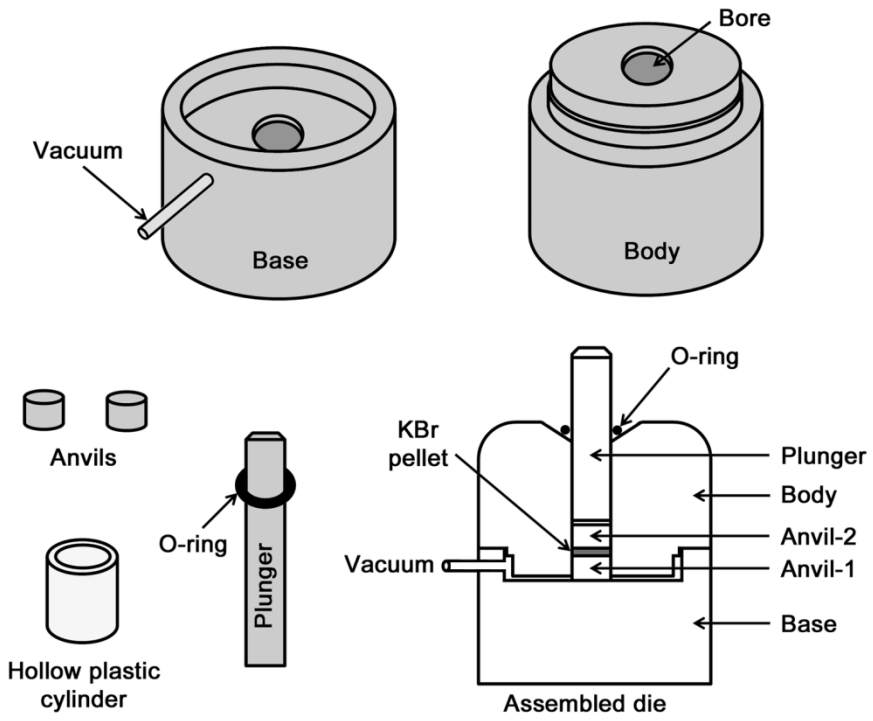

Figure 11.1: Different parts of a die for preparing KBr (and other) pellets

b. Place the base of the dye on a horizontal surface (as shown in figure 11.1).
c. Place the top part of the dye (called body) on the base and press it down using your fingers so that the two pieces get properly snapped.
d. Place one of the anvils into the bore of the die assembly using tweezers (one of the circular surfaces of the anvil is more shiny, keep this shiny side up).
e. Weigh exactly 20 *mg* of the grounded KBr and pour it into the bore using a funnel. If the amount does not cover the entire surface of the anvil, add more KBr in 5 *mg* increments.
f. Place the second anvil in the die bore (shiny surface down).
g. Place the plunger on the second anvil (in the die bore) with the beveled side towards outside.
h. Push the plunger O-ring down.
i. Twist the plunger by hand a few times for flattering the KBr powder.
j. Position the entire set up (fully assembled die having the sample) between the plates of the hydraulic press.

k. Evacuate the dye assembly by applying vacuum for 2-5 minutes (this is done for drying the pellet)
l. While continuing evacuation, apply 8 metric tonnes pressure and maintain it for 2-4 minutes (never exceed 9 metric tonnes pressure).
m. Release the pressure and vacuum gently while securing the die with your hand.
n. Remove the die from the hydraulic press and place it on a horizontal surface.
o. Secure the base of the die and try to push up the body at any point along the joint using a screw driver.
p. Separate the base and the body of the die apart by pushing the body up at various points spanning the entire circumference of the joint.
q. Remove the body from the base and place it upside down between the plates of the hydraulic press upside down (*i.e.* the body rests on the beveled end of the plunger).
r. Place the plastic hollow cylinder on the center of the body opposite to the plunger end and apply pressure slowly so that the first anvil and the KBr pellet come out of the bore.
s. Carefully remove the anvil and take out the pellet using a tweezer and place it in the sample holder.

63. Prepare 10 *ml* of 50 *mM* NaCl solution in D_2O.
64. If required, adjust the pD to 7.0 using deuterium chloride (DCl) or sodium deuteroxide (NaOD).
65. Weight accurately 5 *mg* of protein and dissolve it in 1 *ml* of above prepared 10 *mM* NaCl solution; this is the working protein sample.
66. Switch ON the infrared spectrometer and allow it 30 minutes warm up.
67. Place 20 μl of '10 *mM* NaCl solution in D_2O' in the centre of the KBr pellet.
68. Dry the pellet in a vacuum desiccator for 10 minutes.
69. Set the spectrometer in the 'Absorbance' mode and the data acquisition range from 2000 – 1000 cm^{-1}.
70. Record the spectrum with 64 scans at 4 cm^{-1} resolution and save the file as 'blank'.
71. Prepare another KBr pellet using same amount of KBr as used for the blank (buffer).

72. Place 20 μl of the protein sample in the centre of the KBr pellet.
73. Dry the pellet in a vacuum desiccator for 10 minutes.
74. Record the spectrum with 64 scans at 4 cm^{-1} resolution and save the file as 'protein'.

Results

1. Open the blank and protein spectra using graphing software such as Microsoft Excel or Origin.
2. Subtract the absorbance values in the 'blank' spectrum from those in the 'protein' spectrum.
3. Plot the subtracted absorbance against wavenumber (cm^{-1}); this gives an infrared spectrum of the protein as shown in figure 11.2.
4. The peak that appears in the 1700 – 1600 cm^{-1} region corresponds to the amide I band of the protein.

Analysis

1. An FTIR spectrum for a polypeptide is shown in figure 11.2 for analysis.

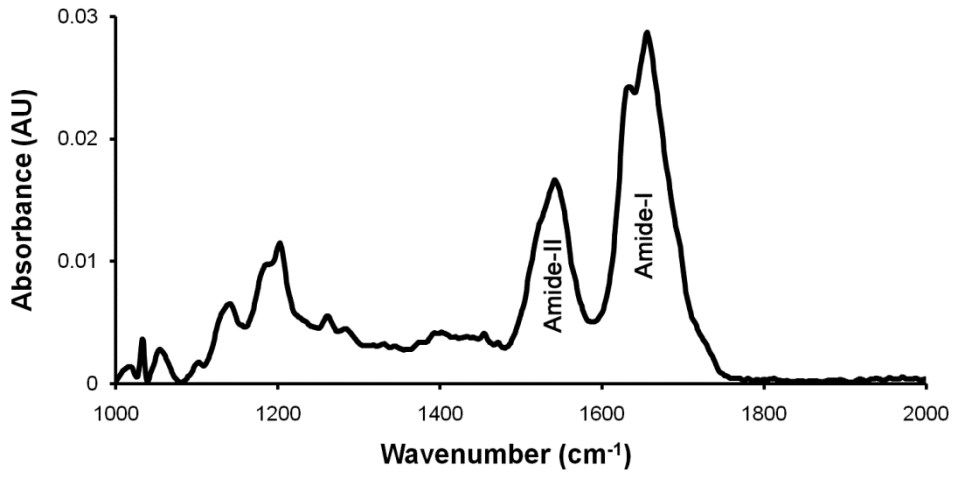

Figure 11.2: An infrared spectrum of a polypeptide

2. The amide I band shows two overlapping bands with maxima ~1634 and ~1655 cm^{-1}. The spectrum indicates that the polypeptide has both α-helical and β-sheet conformations.
3. It is useful to obtain a double derivative of the spectrum for resolving the overlapping bands.

Notes:

5. Only infrared spectroscopy grade KBr should be used for making pellets.
6. Synthetic peptides usually have trifluoroacetic acid (TFA) as the ion pair. TFA absorbs at ~1674 cm^{-1} and should therefore be exchanged by an IR inactive anion. This is usually achieved by dissolving the peptide in 5 *mM* HCl followed by freezing in liquid nitrogen and finally lyophilization. Repeating this process provides peptides sufficiently good for infrared spectroscopy.

Lecture 12 Enzyme activity

Aim:

To determine the activity of the enzyme alkaline phosphatase

Introduction:

Enzymes play essential roles by carrying out a plethora of biological reactions. Just because a reaction has very large negative free energy change does not imply that reaction will take place at rapid rate. What it implies is that the $\frac{[product]}{[substrate]}$ concentration ratio is smaller than that at equilibrium. Oxidation of glucose into CO_2 and H_2O, for example, is a reaction with $\Delta G'$ of –686 *kcal/mol*. The glucose, therefore, is thermodynamically unstable. But we know, by experience, that a glucose solution does not break down into CO_2 and H_2O at a measurable rate. We can say that glucose is kinetically stable. The kinetic stability is provided by the large energy barrier between the reactant and the product (Figure 12.1)

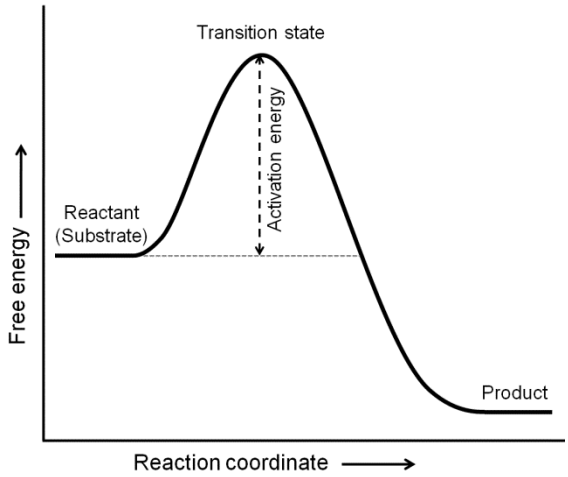

Figure 12.1: A diagrammatic representation of free energies of reactant, transition state, and the product

As is clear from figure 12.1, the reactants need excess energy, the activation energy (E_a) to cross the energy barrier between reactants and the products. The rate of the reaction is determined by the number of molecules that enter the transition state per unit time. The number of molecules populating the transition state can be increased either by increasing the temperature or by somehow decreasing the activation energy. As biological organisms survive and function within a narrow temperature window,

they can't increase the rate of reaction by increasing the temperature. They manage to carry out a plethora of chemical reactions by means of enzymes that function as biological catalysts by decreasing the activation energy. The enzymes can enhance the reaction rates by up to 15 orders of magnitude. It is important to note that the enzymes do not change the equilibrium constant (K_{eq}) or free energy change (ΔG) of the reaction. Each enzyme present in a cell has its characteristic enzyme parameters. The plot of initial reaction velocity, V_0 against the substrate concentration [S] has same general shape (rectangular hyperbolic shape) which is given by Michaelis-Menten equation:

$$V_0 = \frac{V_{max}[S]}{K_m + [S]} \qquad \text{---------- (12.1)}$$

where, V_0 is the initial reaction rate, V_{max} is the maximum rate, [S] is the molar substrate concentration, and K_m is a constant called Michaelis constant.

V_{max} and K_m are the characteristic properties of an enzyme. As is clear from equation 12.1, K_m can be defined as the substrate concentration at which initial reaction rate, V_0 equals $\frac{V_{max}}{2}$.

The response of enzymes to the concentrations of substrates and products plays important role in the reaction control. This behavior of enzymes to the substrate/product concentration is studied under enzyme kinetics and is used to determine the important enzyme parameters such as K_m and V_{max}. We have chosen to study the kinetics of the enzyme alkaline phosphatase. The enzyme catalyses the hydrolysis of a phosphoester bond, producing inorganic phosphate (P_i) and an alcohol. We have chosen p-nitrophenylphosphate as the substrate for the hydrolysis reaction. Para-nitrophenylphosphate is a colourless compound; the enzyme, alkaline phosphatase hydrolyses the phosphoester bond to produce the coloured product, p-nitrophenol which can be detected colorimetrically (Figure 12.2).

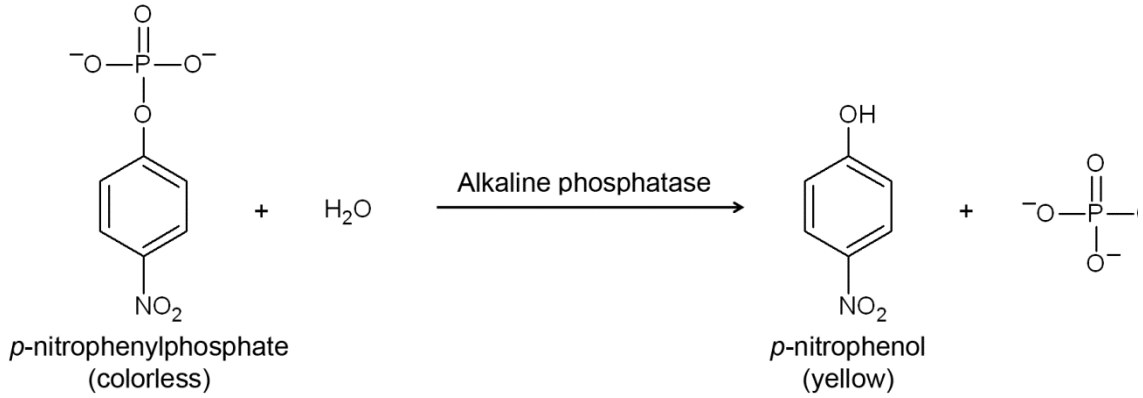

Figure 12.2: Hydrolysis of *p*-nitrophenylphosphate into *p*-nitrophenol and phosphate

Materials:

Equipments:

27. UV/Visible spectrophotometer
28. Weighing balance

Reagents:

19. 100 *mM* Tris-HCl buffer, pH 8.0
20. Para-nitrophenol (PNP)
21. Para-nitrophenylphosphate (PNPP)
22. Alkaline phosphatase from *E. coli*

Glassware, plasticware, etc.:

42. 1.5 *ml* microfuge tubes
43. Pipettes
44. Pipette tips
45. A pair of matched glass or quartz cuvettes (volume: 3 *ml*)

Procedure:

Standard curve of PNP

75. Switch ON the spectrophotometer and allow it 30 *min* warm up.
76. Meanwhile, prepare 0.1 *mM* PNP solution in 100 *mM* Tris-HCl buffer, pH 8.0
77. Take 11 microfuge tubes and label them from 1 – 11.

78. Prepare the PNP dilutions as shown in table 12.1.

Table 12.1: Observation table for the enzyme assay

Tube No.	Tris-HCl buffer (*ml*)	PNP solution (*ml*)	PNP concentration (μM)	A_{410}
1	2.7	0.3	10	
2	2.4	0.6	20	
3	2.1	0.9	30	
4	1.8	1.2	50	
5	1.5	1.5	50	
6	1.2	1.8	60	
7	0.9	2.1	70	
8	0.6	2.4	80	
9	0.3	2.7	90	
10	0	3.0	100	
11	6.0	Blank (0)	Zero	

79. Set the spectrophotometer to 410 nm and select the “Absorbance mode”.
80. Use the blank (Tube No. 11) in both the cuvettes to set the spectrophotometer readings to ZERO.
81. Measure the absorbance of tubes 1 – 10 against the blank and record the readings in the table 12.1.
82. Plot the absorbance values against the PNP concentration.
83. Fit the data points using linear regression to obtain the standard curve.

Enzyme kinetics and determination of K_m and V_{max}

1. Prepare 100 μM solution of the enzyme (200 μl) in the Tris-HCl buffer, pH 8.0.
2. Prepare 5, 10, 15, 20, 25, 50, 75, and 100 *mM* PNPP solutions in Tris-HCl buffer, pH 8.0.
3. Record the absorbance at 410 nm using each of the PNPP solutions as follows:
    a. Add 2.97 *ml* Tris-HCl buffer in each of the 3 *ml* cuvettes.
    b. Add 30 μl of PNPP solution in both the cuvettes and mix well.
    c. ZERO the reading at 410 nm.

   d. Add 20 μl of the enzyme solution to the cuvette kept in sample cell, start the stop watch, cover the cuvette with a piece of parafilm, and quickly mix the contents by 4-5 inversions (***Note 1***).
   e. Immediately record the absorbance and then after 10 seconds interval for 2 *min*.
4. Repeat the assay for each of the samples at least once and take the average readings for analysis.
5. Plot the average absorbance value against time for each of the samples. This gives the time course of the enzymatic reaction.
6. Calculate the initial velocity, V_0 for each of the substrate (PNPP) concentration.
   a. The plot between absorbance against time is linear for the initial part of the plot and V_0 is simply the slope of this line
   b. Fit the initial region of the curve (first 3 or 4 points) linearly and determine the slope of the line.
7. Plot V_0 against substrate concentration to obtain the Michaelis-Menten curve.
8. The Michaelis-Meneten equation shown in equation 12.1 can be rewritten as:

$$\frac{1}{V_0} = \frac{K_m}{V_{max}}\frac{1}{[S]} + \frac{1}{V_{max}} \qquad \text{---------- (12.2)}$$

   A plot between $\frac{1}{V_0}$ and $\frac{1}{[S]}$ gives a straight line with a slope $\frac{K_m}{V_{max}}$ and an intercept of $\frac{1}{V_{max}}$ on $\frac{1}{V_0}$ axis. This plot is known as Lineweaver-Burk plot or double-reciprocal plot and allows easy determination of the K_m and V_{max} of the enzyme.
9. Calculate the $\frac{1}{V_0}$ and $\frac{1}{[S]}$ for each of the substrate concentration, obtain the Lineweaver-Burk plot and calculate the K_m and V_{max} from the plot.

Notes:

7. All the samples need to be mixed with the same number of inversions and absorbance reading recorded after same time. Mixing and the first reading should be completed within few seconds (preferably ≤ 10 *seconds*).