応用化学実験第一

片山研究室

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題目： DNA の紫外光吸収による定量と融解曲線の作成

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1 Introduction

DNA and RNA spontaneously form double helical structures with complementary single strands. This double helix structure is formed by non-covalent bonds such as hydrogen bonds and stacking, and dissociates into single strands at high temperatures. In this study, experiments were conducted to investigate the melting temperature, which represents the stability of the double helical structure with respect to temperature, and to study the effect of salt on the melting temperature (Tm).

2 Materials

2.1 Reagents

- X g/mL DNA (from salmon sperm) solution (unknown concentration sample)
- 100 mM Tris-HCl buffer solution (pH=7.5)
- 100 mM NaCl

2.2 Glassware

- Eppendorf microtubes
- 1 UV cuvette ($l = 1.0\ cm$)

2.3 Equipment

- UV/Visible spectrophotometer
- One 10-100 ul adjustable micropipette
- One 100-1000 ul adjustable micropipette

3 Methodology

3.1 DNA quantification

1) Solution A and solution B were prepared with and without NaCl as follows.

	A (without NaCl)	B (NaCl 50 mM)
X ug/mL DNA	100 uL	100 uL
100 mM Tris-HCl (pH 7.5)	100 uL	100 uL
100 mM NaCl	-	500 uL
H_2O	800 uL	300 uL
Total	1 mL	1 mL

2) Dissolved oxygen in the DNA solution was removed under reduced pressure (degassing) to prevent the formation of bubbles at high temperatures.

3) The DNA solution was placed in a quartz cell (optical path length 1 cm), tightly stoppered, and placed in a spectrophotometer at 50°C. The absorption spectrum of the DNA solution in the wavelength range of 200-400 nm was measured at 50°C.

4) Similarly, absorption spectra were also measured at 90°C and compared with those at 50°C.

3.2 Melting curve measurement

1) The change in absorbance at the wavelength of maximum absorption (λ = 260 nm) was observed when the solution temperature was increased from 50°C to 90°C at a constant rate of 2°C/min.

2) Melting curves were constructed from the data in 1).

3) Two parallel tangent lines were added to the curve in 2), and a line parallel to the two tangent lines was drawn in the middle of the curve. The intersection of this line and the melting curve is the melting temperature (Tm).

4) The same procedure was performed for solutions A and B and compared.

4 Results

4.1 Results of DNA quantitation by UV light absorption

The following graphs represent the absorbance spectrum of solutions A and B at 50°C.

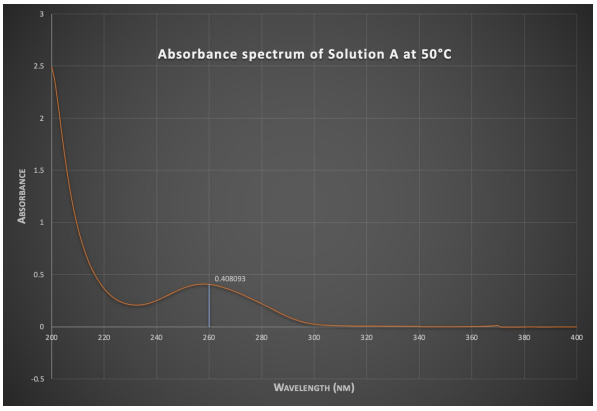

Figure 1: UV absorbance spectrum of solution A at 50°C

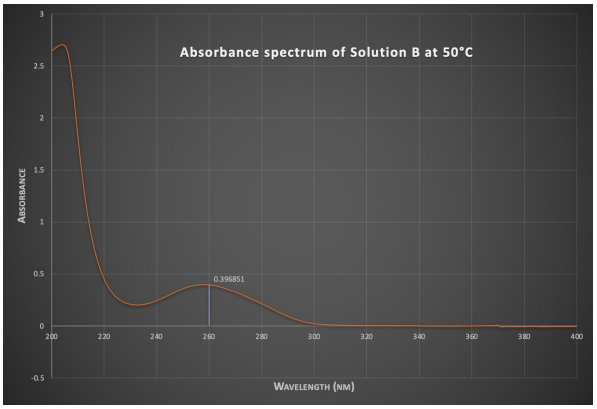

Figure 2: UV absorbance spectrum of solution B at 50°C

4.2 Calculation of DNA concentration

The DNA concetration in both solutions (A and B) are essentially the same. This is because both solutions used the same volume of the DNA sample and were disolved in untill 1 mL of each solution was obtained. The concentration of the DNA sample is calculated by using the Lambert-Beer law as shown in Fig. 3.

$$A = \varepsilon c l$$

A	Absorbance	
ε	Molar absorption coefficient	$M^{-1}cm^{-1}$
c	Molar concentration	M
l	optical path length	cm

Figure 3: Lambert-Beer law

The optical path length l is indicated as 1 cm on the experiment guide. Moreover, the absorbance of 50 ug/mL of DNA at 260 nm is indicated as 1.0. From this information, the concentration of the DNA sample will be calculated.

$$\begin{aligned} 1.0 &= \varepsilon \cdot 50ug/mL \cdot 1cm \\ \varepsilon &= 0.02mL/ug.cm \end{aligned} \tag{1}$$

From Eq. 1, the value of ε for DNA was obtained. Consequently, the concentration of the DNA sample can be calculated by using the average absorbance of solutions A and B.

$$\begin{aligned} A &= \frac{0.4081 + 0.3969}{2} \\ A &= 0.402 \end{aligned} \tag{2}$$

Subsequently, the Lambert-Beer law is used to calculate the DNA concentration.

$$\begin{aligned} 0.402 &= 0.02mL/ug.cm \cdot X \cdot 1cm \\ X &= 20.1ug/mL \end{aligned} \tag{3}$$

From Eq. 3, the value of the concentration of DNA in solutions A and B was obtained. However, the concentration of DNA in the original sample is 10 times this value, because the sample was diluted.

$$\text{DNA concentration} = 20.1ug/mL \cdot 10 = 201ug/mL \tag{4}$$

Therefore, the initially unknown concentration of DNA is 201 ug/mL.

4.3 Absorbance spectrum graphs at 50°C and 90°C

The absorbance spectrum graphs of solution A at 50°C and 90°C were overlapped in Fig. 4.

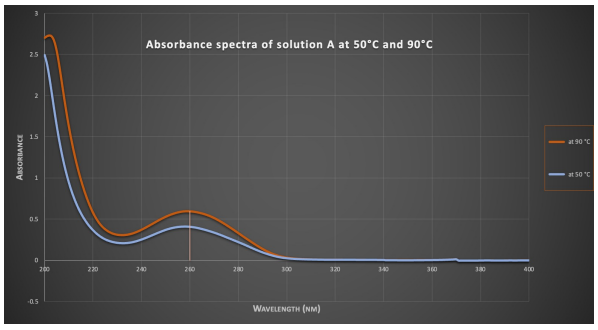

Figure 4: UV absorbance spectra of solution A at 50°C and 90°C

Similarly, the absorbance spectrum graphs of solution B at 50°C and 90°C were overlapped in Fig. 5.

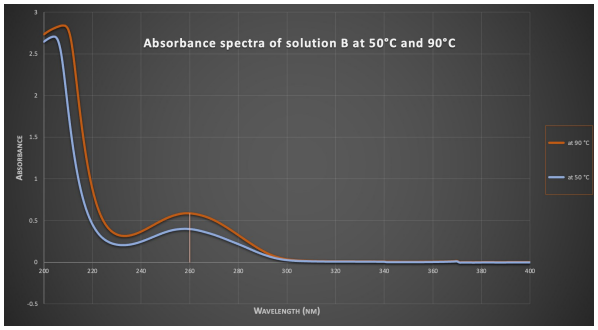

Figure 5: UV absorbance spectra of solution B at 50°C and 90°C

4.4 Drawing up of the melting curve

The DNA melting curves of both solutions were examined and drew in Fig. 6.

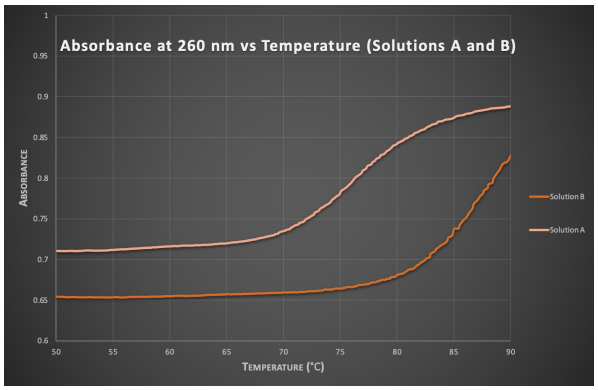

Figure 6: UV absorbance at 260 nm vs Temperature graphs (Solutions A and B)

The melting temperature of DNA was analyzed separately for each solution. The melting temperature of DNA in solution A is shown in Fig. 7.

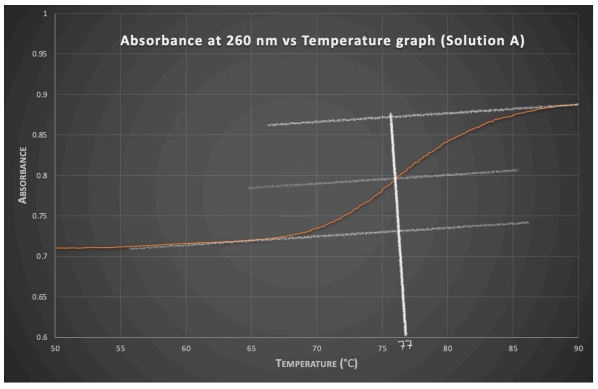

Figure 7: Melting temperature for solution A

Similarly, the melting temperature of DNA in solution B is shown in Fig. 8.

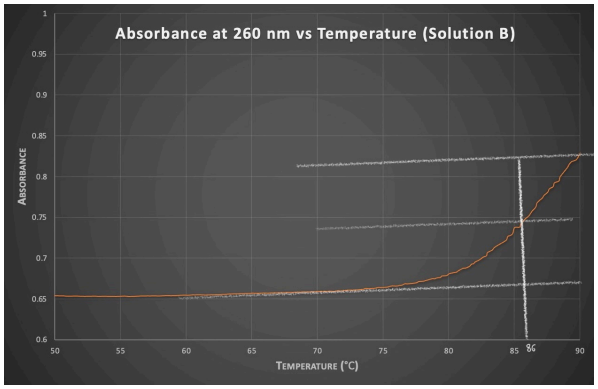

Figure 8: Melting temperature for solution B

Therefore, the melting temperature (Tm) in solution A is 77°C, whereas the Tm in solution B is 86°C.

5 Discussion

At the time of the reagents preparation, the DNA sample was observed to have a white color and a powder-like texture.

The double helix structure of DNA is broken to form a single-stranded structure, which allows the bases in DNA that absorb ultraviolet light to absorb ultraviolet light unimpeded, resulting in a higher absorbance. In other words, the higher the absorbance, the more the double helix structure is broken. For instance, when comparing absorbance at the same wavelength at 90°C for both Solution A (Fig. 4) and Solution B (Fig. 5), the absorbance is higher at 90°C compared to at 50°C. This is likely to be due to the destabilization of the double helical structure caused by the increased thermal motion of the atoms as the temperature rises.

Moreover, the melting temperature, which is the temperature at which 50% of the double helix structure of DNA is broken, increased with the addition of salt (NaCl). By adding sodium ions in solution the negative phosphate groups on the chains are stabilized (electric shielding by associating negative phosphate groups with positive sodium ions) meaning it takes more kinetic energy to unzip the two chains. Without the positive ions, the two strands each have large negative charges which create repulsion between the strands tending to separate them (destabilized). Therefore, a higher temperature is needed to provide the kinetic energy (collisions with water molecules) to knock the chains apart when they have this extra stability.

The present experiment was succesfully conducted with the anticipated results obtained.

6 Conclusion

The present experiment was conducted with the purpose of studying the effects of added salt in the melting temperature of DNA. As stated in discussion, the experiment was succesfully conducted. The increase in the absorbance of the DNA solution when the temperature was increased could be confirmed in Fig. 4 and Fig. 5. Moreover, the effect of salt (NaCl) in the DNA solution was confirmed in Fig. 6. The melting temperature of the DNA solution increased with the presence of salt in the solution (86°C compared to 77°C).

7 Assignment

1. Did you observe an increase in absorbance at 260 nm at 90°C compared to 50°C? Explain the physicochemical mechanism by which the hyperchromicity effect occurs.

Yes, there was an increase in the absorbance at 260 nm at 90°C compared to at 50°C. Hyperchromicity is the increase of absorbance (optical density) of a material. An example of hyperchromicity of DNA occurs when the DNA duplex is denatured. The UV absorption is increased when the two single DNA strands are being separated, either by heat or by addition of denaturant or by increasing the pH level. Heat denaturation of DNA, also called melting, causes the double helix structure to unwind to form single stranded DNA. When DNA in solution is heated above its melting temperature (usually more than 80 °C), the double-stranded DNA unwinds to form single- stranded DNA. The bases become unstacked and can thus absorb more light. In their native state, the bases of DNA absorb light in the 260-nm wavelength region. When the bases become unstacked, the wavelength of maximum absorbance does not change, but the amount absorbed increases. Double bonds absorb ultraviolet light, but in the double helical structure, the aromatic rings with double bonds are located inside the helical structure and are blocked by the structure outside the double helical structure, reducing the ability to absorb ultraviolet light. The hyperchromic effect is the striking increase in absorbance of DNA upon denaturation. The two strands of DNA are bound together mainly by the stacking interactions, hydrogen bonds and hydrophobic effect between the complementary bases. The hydrogen bond limits the resonance of the aromatic ring so the absorbance of the sample is limited as well. When the DNA double helix is treated with denatured agents, the interaction force holding the double helical structure is disrupted. The double helix then separates into two single strands which are in the random

coiled conformation. At this time, the base-base interaction will be reduced, increasing the UV absorbance of DNA solution because many bases are in free form and do not form hydrogen bonds with complementary bases. As a result, the absorbance for single- stranded DNA will be 37% higher than that for double stranded DNA at the same concentration as shown in the experiment conducted.

2. You want to calculate the concentration of DNA in the DNA solution A at 50°C from the molar absorption coefficient. Therefore, you decided to use the average molar absorption coefficient (10.7x10 3 L/mol/cm) of the four deoxynucleotide triphosphates (dNTP: dATP, dGTP, dCTP, dTTP) as the molar absorption coefficient. First, calculate the molar concentration. Next, convert this to a concentration by weight. Use the average molecular weight (660 g/mol) of the two base pairs (A-T, G-C) for the conversion.

$$A = \varepsilon \cdot C \cdot l$$

$$0.402 = \left(10.7 \times 10^{3}\ L/mol \cdot cm\right) \times C \times (1\ cm)$$

$$C = 0.0376\ mol/L$$

$$X = 3.757 \times 10^{-5}\ mol/L \times (660\ g/mol)$$

$$X = 0.0248\ g/L \times \frac{10^{6}\ \mu g}{1\ g} \times \frac{1\ L}{10^{3}\ mL}$$

$$X = 24.8\ \mu g/mL$$

By using the Lambert-Beer equation (Fig. 3), the concentration was calculated in Fig. 7. The concentration of DNA is, therefore, 24.8 ug/mL.

3. The concentration obtained in 2) is smaller than the concentration obtained in Eq. 1. Explain why. (Hint: the concentration effect in assignment (1) is relevant)

Yes, the concentration obtained in 2) is 24.8 ug/mL, whereas the concentration obtained in Eg. 4 is 201 ug/mL. When light strikes the base, the polarity of the electrons becomes polarized and the molecule becomes unstable. This polarity is affected by the surrounding environment, which determines whether the molecule is stable or unstable. Under the condition of 2) with an average mole of only bases, the molecule is surrounded by polar molecules such as water,

so the molecule is stable and the excited state is lowered. Therefore, the molecule shifts to longer wavelengths On the other hand, in the double-stranded case (Eq. 1), the bases are lined up around the base. Therefore, the excited state shifts to shorter wavelengths.

In conclusion, the absorption coefficients of the equation used in 2) and Eq. 1 are different, and the single difference equation with a shift to a higher wavelength has a larger absorption coefficient, resulting in a smaller concentration than that calculated by Eq. 1.

4. What should be added to the solution to lower the melting temperature of DNA? Explain, along with reasons.

The ph should be raised to protonate the nitrogen of the heterocyclic ring and prevent hydrogen bonding.Besides, an organic solution (methanol) weakens the stacking and, therfore, lowers the melting temperature of DNA.

5. You have found that the salmon sperm DNA melting temperature is much higher than the body temperature of salmon (1-15°C). Nevertheless, phenomena that would require DNA melting (DNA replication, DNA to RNA translation) occur at body temperature. Explain how these phenomena are possible.

In the case of DNA replication, the DNA helicases catalyze the disruption of the hydrogen bonds that hold the two strands of double-stranded DNA together. This energy-requiring unwinding reaction results in the formation of the single-stranded DNA required as a template or reaction intermediate in DNA replication, repair and recombination. Therefore, it is not necessary to heat up the DNA molecule to perform the DNA melting. This works smoothly because the DNA contains several fixed gene sequences called replication start sites, and specific proteins bind to these sites.

6. Explain the effect of base pair composition and DNA length on the melting temperature of DNA.

Melting temperature is the temperature at which 50% of the DNA molecules and solution are denatured. When we're talking about the denaturation for DNA, we're referring to the double-stranded DNA molecules separating into two individual single-stranded DNA molecules. So the melting temperature is the temperature at which 50% of the DNA molecules and solution are separated into a single-stranded DNA molecules. The melting temperature depends on two factors:

- The first factor is the GC-content. AT-base pairs are held together by two hydrogen bonds, whereas GC-base pairs are held together by three hydrogen bonds. What this means is the higher the percentage of GC-base pairs in the DNA sequence, the higher

the melting temperature because it has more hydrogen bonds.

- The second factor that the melting temperature depends on is the length of the DNA molecule. This is simply because the longer the DNA molecules, the more hydrogen bonds there are. So similarly, this means the longer the DNA molecule, the higher the melting temperature.

7. The property that complementary single-stranded DNA spontaneously forms a double helix structure is applied to the FISH method (fluorescence in situ hybridization). Explain the mechanism and application of the FISH method.

Fluorescence in situ hybridization (abbreviated FISH) is a laboratory technique used to detect and locate a specific DNA sequence on a chromosome. In this technique, the full set of chromosomes from an individual is affixed to a glass slide and then exposed to a "probe" - a small piece of purified DNA tagged with a fluorescent dye. The fluorescently labeled probe finds and then binds to its matching sequence within the set of chromosomes. With the use of a special microscope, the chromosome and sub-chromosomal location where the fluorescent probe bound can be seen. The mechanism is illustrated in Fig. 9.

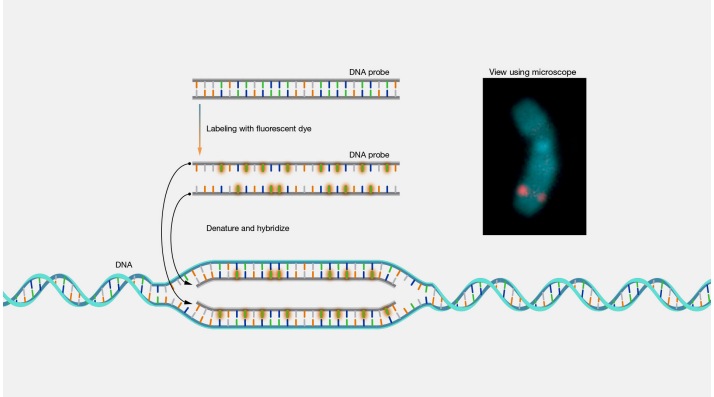

Figure 9: FISH technique

8. If there is anything in this experiment that you find difficult to understand or would like to see improved, please let us know.

Nothing in particular. It was well explained by the professor in charge.

References

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