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I. Dye Characterization

INTRODUCTION & METHODS

In chemical analysis, spectroscopy is an important method to determine the presence and amount of a particular substance as well as the structure of atoms and molecules. Spectra can be obtained by either measuring emission or absorption. Both methods consist of a light source, a disperser and a detector.

For absorption spectroscopy the loss of electromagnetic energy after illuminating a sample is detected, resulting in a spectrum where the absorbed wavelengths are missing. Since energy levels of many molecules are unique to them, the characteristic absorption spectra allow their identification. In emission spectroscopy, atoms or molecules are excited with a particular wavelength, resulting in a distinctive emitting radiation when photons fall back to the original or lower level.[1,2]

In this experiment we want to analyze the physical attributes of a fluorescent dye solution by determining its absorption and emission spectrum. Measurements for absorption spectroscopy were taken from a two-fold dilution series with 70% ethanol. Emission spectroscopy was conducted with dilutions that retained an absorption value below 0.7 [Table 1]. Results were analyzed and plotted using RStudio.

Table 1. Spectroscopy measurements of the dye with respective dilutions and concentrations

Dilution	c [µM]	A_{max}	Fluorescence Intensity [cps]
1:2	4.1	2.94	-
1:4	2.05	1.81	-
1:8	1.025	1.07	-
1:16	0.5	0.65	456.072
1:32	0.25	0.45	278.492

RESULTS AND DISCUSSION

Absorption Spectroscopy

To determine the absorption spectrum UV-Vis spectroscopy was conducted in the wavelength range of 250 nm to 600 nm, using 2-fold dilutions of the dye. Independent of concentration, maximum absorption is depicted at 520 nm, brought about by the chemical composition of the dye [Figure 1].

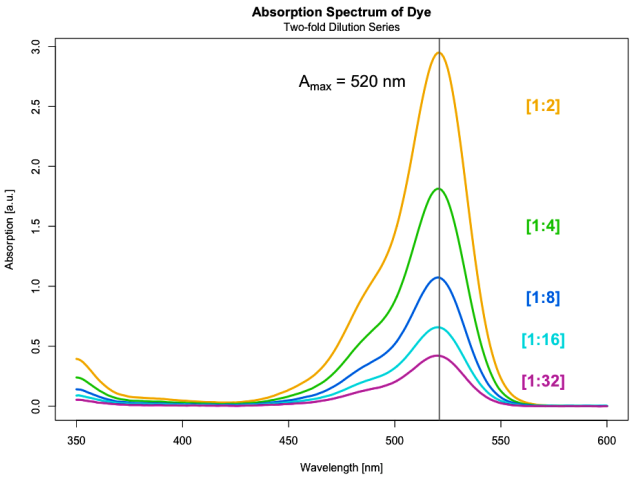

Figure 1. Absorption spectrum of the dye in various concentrations. Independent of dilution the maximum absorption is measured at a wavelength of 520 nm.

However, as values over 1.5 resemble an absorption of 95% of the light source and thus can not be accurately detected by the spectrometer, these measurements are deemed unreliable.

The linear regression model derived from the maximum absorption values, estimates an extinction coefficient ε of 0.65 $\mu M^{-1}cm^{-1}$ [Figure 2]. The molar extinction coefficient is an intrinsic property, referring to the ability of a chemical substance to attenuate light at a given wavelength.

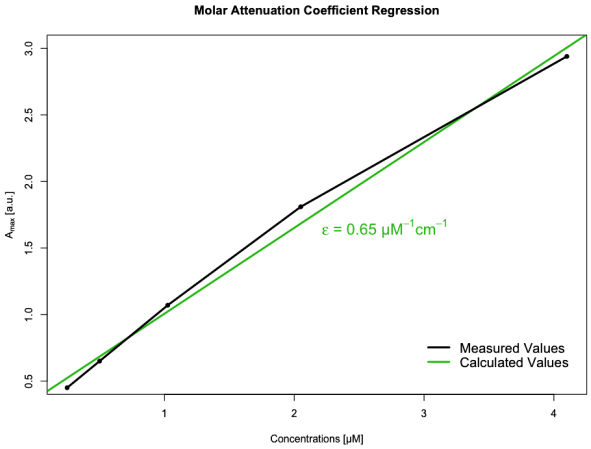

Figure 2. Calibration curve of absorbance vs. concentration for the fluorescent dye at A_{max} (520 nm). The linear regression model determines a molar extinction coefficient of approximately 0.65 $\mu M^{-1}cm^{-1}$.

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Emission Spectroscopy

For emission spectroscopy, dilutions with the lowest absorption saturation (1:16, & 1:32) were excited with a wavelength of 525 nm. For comparability, averaged values for both absorption and emission spectroscopy were normalized and plotted against wavelength (Figure 3). Analysis of the graph clearly depicts a deviation of about 25 nm in the positions of the absorption and emission maxima along the band.

This phenomenon, called Stoke's Shift, represents the energy difference

$$E = \frac{hc}{\lambda} \quad (1)$$

between absorbed and emitted photons and is commonly illustrated in a Perrin-Jablonski diagram [Figure 4]. The extend of such a shift depends on the fluorophore, particularly the solvent polarity and local environment.[3]

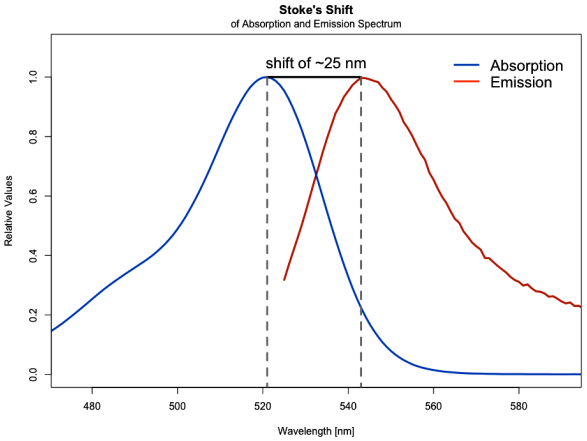

Figure 3. Due to the loss of energy between absorption and emission, the wavelength maximum shifts approximately 25 nm, characteristic for the dye. This difference is called Stoke's Shift and it's underlying mechanism can be illustrated in a Perrin-Jablonski diagram (Figure 4).

CONCLUSION

Absorption and emission spectroscopy are important tools in order to detect, identify and quantify atoms and molecules. To study a given dye solution, absorption spectroscopy was conducted in the ultraviolet as well as the fully visible region of the electromagnetic spectrum. Due to electrons absorbing energy at distinct frequencies, a characteristic spectrum can be derived, representing the atomic and molecular composition of a probe. For the experimental dye solution, the absorption maximum was assessed at approximately 520 nm.

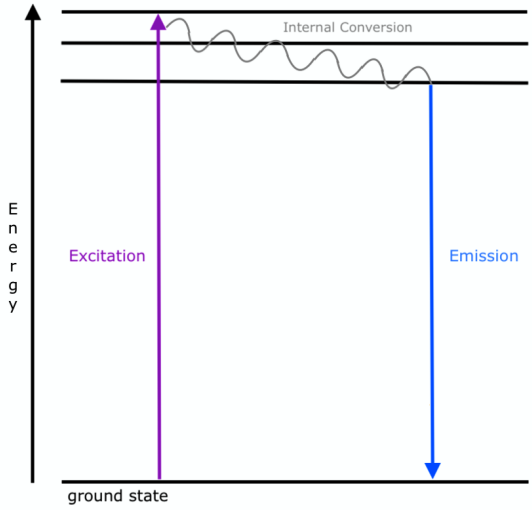

Figure 4. Schematics of a Perrin-Jablonski Diagram illustrating the electronic states of a molecule and the transitions between them. Internal conversion describes a transition from a higher to a lower electronic state without emitting photons.

In the subsequent emission spectroscopy assay, the fluorescent dye was excited at a frequency close to its maximum absorption wavelength. When excited electrons transition back to the ground state, a set of particular wavelengths is emitted, characteristic for the elemental composition of a sample. In case of the dye solution, emission measurements peaked at around 545 nm. Comparison of both spectra with literature suggests that the dye could belong to a rhodamine or similar family (e.g. 6-JOE, TET).[4]

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2. Lysozyme Spectroscopy

INTRODUCTION & METHODS

Circular dichroism spectroscopy is a form of absorption spectroscopy measuring the difference in speed, wavelength and absorbance of circularly polarized light, passing optically active substances. The broadest field of CD spectroscopy application is protein chemistry. Especially the ultraviolet region of the spectrum is sensitive to peptide bonds and amino acid chains, making UV-CD an eligible tool to analyze different secondary structure types. Similar to other absorption spectroscopy methods, CD is particularly powerful in determining conformational changes. The fraction of alpha-helix, beta-sheet, beta-turn, or random coil conformations can be estimated by signature CD spectra [Figure 5].[5,6]

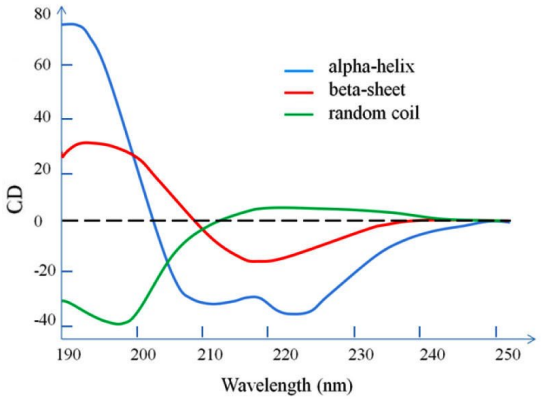

Figure 5. Standard CD spectra showing each of the three basic secondary structures and their distinctly different spectral signatures.

In this experiment, absorption and CD spectroscopy were conducted to examine the characteristics of the protein lysozyme, extracted from hen eggs. Lysozyme is an antimicrobial enzyme, forming part of the innate immune system and present abundantly in secretions like tears, salvia, human milk and mucus. With hen egg lysozyme being thermally stable and maintaining its activity in a large range of pH, it is particularly suited for research.[7] Measurements were taken from samples with various lysozyme concentrations and buffers (Table 2). Calculations and analysis was done using RStudio.

Table 2. Lysozyme samples used in the experiment

Sample	Buffer	Dilution
Stock Solution	—	—
Native Buffer	20 mM Glycine, pH 2.5	1:3
Denaturation Buffer	20 mM Glycine, pH 2.5 8 M GdnHCl/GdmCl	1:3

RESULTS & DISCUSSION

Lysozyme Concentrations

For an initial analysis of the lysozyme solutions, absorption spectroscopy was conducted in a range of 190 nm to 350 nm wavelength [Figure 6A]. The stock solution shows a clear local maximum at around 280 nm, illustrating the absorption caused by amino acid chains. However, the peak is barely visible for either of the diluted solutions [Figure 6B]. An additional peak can be deduced at around 220 nm, for all three concentrations, most likely representing the absorption of peptide backbone and side chains respectively. With HT detector voltage saturating at around 211 nm, signal detection in higher frequencies can be deemed unreliable.[8]

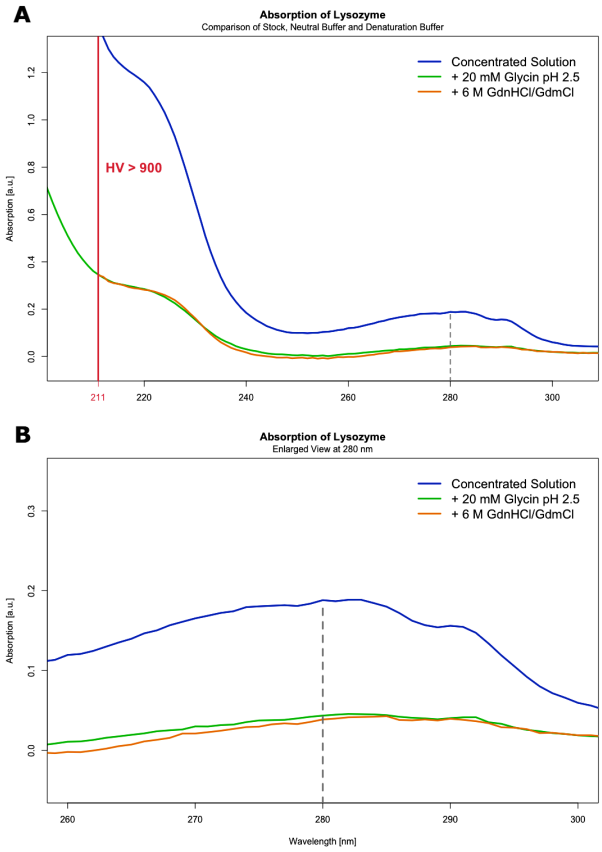

Figure 6. Absorption spectroscopy comparing the native stock with diluted solution and denaturation solution. **A** Maxima can be deduced at 280 nm and 225 nm. Voltage saturation leaves values below 211 nm unreliable. **B** Enhancement of the peak around 280 nm shows that diluted and denatured lysozyme can not be distinguished via absorption spectroscopy.

To determine the concentration of the dilutions, calculations for absorption values at 205 nm and 280 nm were performed based on the Beer-Lambert-Law:

$$A = \varepsilon c d \quad (2)$$

A - absorbance
ε - molar decadic attenuation coefficient
c - concentration
d - layer thickness

Extinction coefficients for both wavelengths (e_{205} = 560000, e_{280} = 37500) were obtained from an online calculator.[9] While calculated concentrations at 280 nm closely resemble experimental values, ambiguous results were acquired for all three samples at 205 nm. Inconsistencies are most likely caused by increasing noise in the lower wavelength range. Also, the Beer-Lambert-Law only holds true for absorbance maxima and is not applicable for high concentrations (> 10 mM).[10,11]

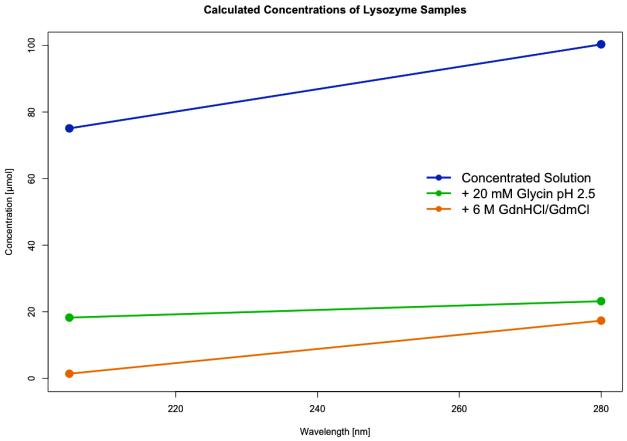

Figure 7. Calculated concentrations at the absorption maximum (280 nm) represent values close to the ones used experimentally. At 205 nm the values deviate due to limitations of the measurement method as well as the Beer-Lambert-Law.

Protein Unfolding

In order to assess the protein structure of lysozyme in the different solutions, circular dichroism spectroscopy was performed [Figure 8]. Spectra of the stock solution as well as the sample diluted with neutral buffer reveal a signature curve most similar to an alpha-helical structure. Comparison with the 3D structure found in literature veritably discloses lysozyme to mainly consist of alpha-helices.[12]

Measurements of the lysozyme in denaturation buffer clearly depicts a loss of its spectral signature, demonstrating that the protein is present in its unfolded state. Although, strong absorption of the concentrated solution and the denaturation agent led to early signal saturation in lower wavelengths (~190-210 nm), the CD measurements sufficiently depicted the lysozyme's predominant protein structure as well as its conformational change in the unfolding buffer.

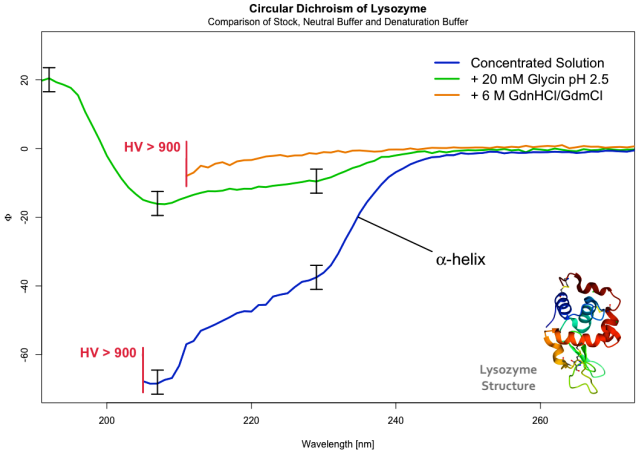

Figure 8. CD spectroscopy of the three different lysozyme solutions. The concentrated and diluted solution (blue, green) show a spectral signature comparable to an alpha-helix spectrum [Figure 5] congruent with its 3D structure. The unfolded lysozyme (orange) clearly lost its typical spectral shape.

CONCLUSION

Spectroscopy is one of the mayor tools in order to determine protein concentration and structure. In this experiment, hen egg lysozyme was analyzed using absorption and circular dichroism spectroscopy. While the absorption spectrum displayed weakness in differentiating between folded and unfolded proteins when comparing similar dilutions, CD spectroscopy proved to be a suitable method, not only to distinguish protein states, but also in denotation of the proteins structural composition. Although CD provides less structural information compared to other analysis methods (e.g. X-ray crystallography, protein NMR) and is only applicable to proteins in solution, it stands out by attaining quick results, requiring only small amounts of protein and little data processing.[6]

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3. BSA Spectroscopy

INTRODUCTION & METHODS

In this experiment, the denaturation of Bovine serum albumin (BSA) is traced by changes in fluorescence intensity and circular dichroism spectroscopy. BSA is a small, stable and moderately non-reactive protein derived from cow's blood. Due to its properties, it is widely used in various biochemical applications (e.g. ELISA, Western Blot, Immunohistochemistry).[13]

In order to trace the unfolding of BSA, the denaturation agents Urea and Guanidium Chloride were added in increasing concentrations and measurements conducted with Fluorometer and CD Spectrometer. The experimental data was provided by the instructors and analyzed using RStudio.

RESULTS AND DISCUSSION

Spectroscopy Measurements

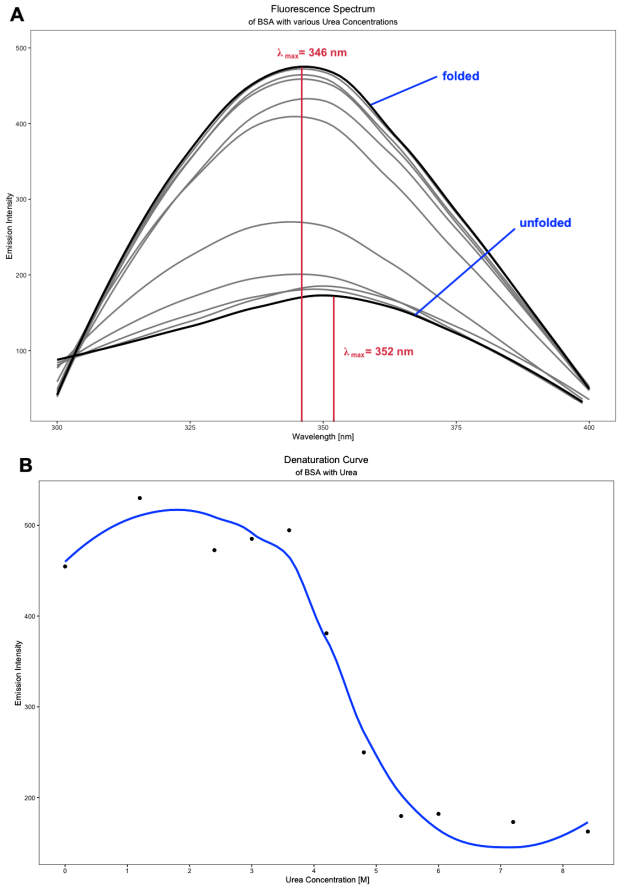

Figure 9. Fluorescence intensity measurements of BSA with increasing urea concentrations. **A** Emission intensity decreases the more the BSA unfolds. Although the maximum emission wavelength is shifting, the slight difference is not a reliable determinant for structural changes. **B** Denaturation curve depicts slow unfolding, with only high concentrations of urea achieving denaturation.

To investigate the gradual denaturation of BSA via fluorescence and CD spectroscopy, the protein was incubated with various concentrations (0 M - 7.2 M) of the denaturation agent urea. Fluorescence spectroscopy clearly shows a loss of emission intensity with progressing denaturation [Figure 9A]. Maximum intensity was measured at 346 nm and barely shifted in the course of unfolding (352 nm). Plotting against the concentrations shows a long lagging phase followed by a steep slope, outlining a high denaturation concentration threshold for urea [Figure 9B].

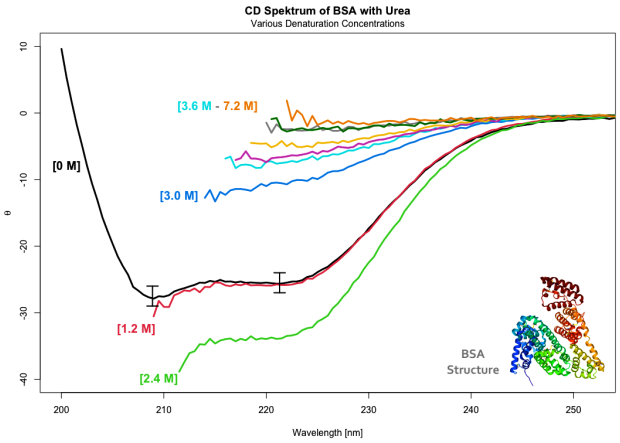

Figure 10. CD spectrum of BSA incubated in urea concentrations from 0 M - 7.2 M. While the spectra of the folded protein resemble an alpha-helical structure, loss of the characteristic shape is observable with increasing amount of denaturation agent.

Similarly, the CD spectrum depicts little alteration in the various concentrations with the exception of a definite structural change between 2.4 M and 3.6 M [Figure 10]. Furthermore, CD measurements illustrate the predominant structural composition of BSA, i.e. alpha helices, congruent with structural analysis found in the literature.[14]

Comparison of Methods

In a subsequent analysis, the two measurement methods are further examined. Figure 11 represents a direct comparison of the normalized fluorescence and CD measurements against the experimental urea concentrations. Values for the fluorescence spectrum were derived from the intensity maximum (346 nm), CD values were taken from a local minimum (223 nm).

The graph depicts CD measurements to be insufficient to accurately determine the denaturation concentrations, while fluorescence spectroscopy represents the unfolding of the protein conclusively.

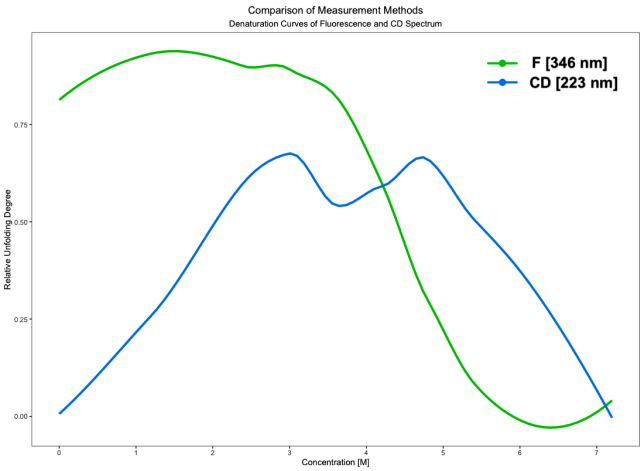

Figure 11. Comparing normalized fluorescence and circular dichroism values plotted against concentration reveals CD to be insufficient to distinctly display the unfolding process.

With the curve sloping at concentrations of 3 - 4 M, it can be concluded that urea is a quite weak denaturation agent. This becomes even more apparent when compared to guanidium chloride [Figure 12], which attains rapid protein unfolding at concentrations as low as 1 - 2 M.

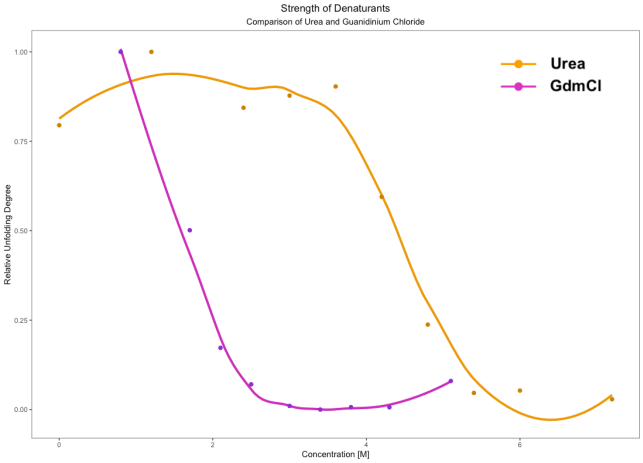

Figure 12. Comparison of relative unfolding degrees using either urea or guanidium chloride as denaturation agent. While greater amounts of urea are needed to attain the unfolding process of BSA, guanidium chloride almost instantly denatures the protein.

CONCLUSION

Protein denaturation involves structural or conformational changes from the native state without alteration of the amino acid sequence. These changes can be induced by pH, detergents, urea and guanidine hydrochloride, as well as by heat.[15]

As there are several biological consequences to denaturation, usually an effort is made to avoid this destructive process. However, some laboratory techniques rely on unfolded nucleic acid strands, such as PCR, Northern/Southern Plot and Sequencing. Furthermore, determination of a proteins stability is essential in drug discovery, medical diagnostics, drug formulation, biosensing and synthetic biology.[16,17]

To investigate the denaturation of the protein Bovine serum albumin, fluorescence and CD spectroscopy were conducted. Fluorescence intensity measurements showed that emission decreased in the course of protein unfolding, but were insufficient to precisely determine subtle conformation changes. In contrast, CD spectroscopy not only provided information about the secondary structure of BSA but also adequately depicted the unfolding process. Nonetheless, comparison of both methods revealed that a conclusive denaturation curve can be derived from fluorescence measurements, although not from CD values.

Lastly, the relation of denaturation agent and protein unfolding was tested by incubating BSA in various guanidium chloride concentrations respectively. Results indicate that, while high concentrations of urea were required to successfully unfold the protein, guanidium chloride proved to be a very strong denaturation agent.[18]

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4. Enzyme Kinetics

INTRODUCTION & METHODS

Not only is spectroscopy useful to detect proteins, determine their structure and trace their conformational changes, but it is also a suitable tool to analyze enzyme kinetics. By conducting a time scan in UV-Vis spectroscopy, generated data can ascertain a proteins enzymatic activity along with types of inhibition.

In this experiment, the enzyme Tyrosinase is characterized by determining its kinetic parameter (V_{max}) and Michaelis-Menten constant (K_M). Tyrosinase is a rate-limiting enzyme carrying out the oxidation of phenols (e.g. tyrosine, dopamine) using dioxygen (O_2). For the analysis, conversion of the substrate L-DOPA, a precursor of neurotransmitter, to Dopachrome and CO_2 was measured by the increase in absorption over time. Furthermore, the effect of the inhibitor Thiourea on the enzymatic reaction is tested. Data was provided by the instructors and analyzed using RStudio.

RESULTS AND DISCUSSION

Enzymatic Activity

To investigate the enzymatic activity of Tyrosinase, absorption spectroscopy was conducted with varying substrate concentrations between 80 µM and 1280 µM as well as a constant enzyme concentration of 2 µM [Figure 13].

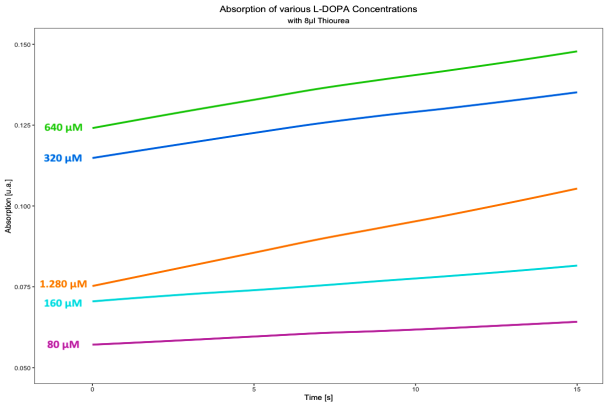

Figure 13. Absorption values of varying substrate concentrations to determine the conversion rate ΔA/min within the linear range (= first 15 sec) of the curve.

Applying the Beer-Lambert Law, substrate conversion rates were determined (Table 3) within the linear range and visualized in a fitted Michaelis-Menten curve (Figure 14A). From the graph a K_M value of approximately 110 µM can be derived at the ½ v_{max} reaction rate.

Table 3. Kinetic parameters of 2 µl Tyrosinase with increasing L-DOPA concentrations, measured and calculated from absorption at 475 nm

#	[S] µM	1/[S] 10^3	ΔA/min	v 10^{-4}	1/v 10^3
1	80	12.5	0.063	3.41	2.94
2	160	6.25	0.069	3.73	2.68
3	320	3.13	0.093	5.03	1.99
4	640	1.56	0.120	6.49	1.54
5	1280	0.78	0.126	6.81	1.47

This parameter, the so-called Michaelis constant, is numerically equal to the substrate concentration at which half of the substrate is bound to the enzyme. With a value as low as 110 µM, high affinity between Tyrosinase and L-DOPA can be concluded.[19,20]

The corresponding Lineweaver-Burk Plot (Figure 14B), or double reciprocal plot, is a graphical representation of the Lineweaver-Burk equation

$$\frac{1}{v} = \frac{K_M}{v_{max}} \frac{1}{[S]} + \frac{1}{v_{max}} \quad (3)$$

from which K_M and v_{max} could be determined respectively. The graph represents a straight line with an intercept on the y-axis equal to $1/v_{max}$, a x-interception representing $-1/K_M$ and a slope illustrating K_M/v_{max}.

The turnover number (k_{cat}) was calculated using the formula

$$k_{cat} = \frac{v_{max}}{[E_0]} \quad (4)$$

which is defined as the maximum number of substrate molecules converted to product per second.[21] Thus, a catalytic rate constant of 2.04 s^{-1} was established with a catalytic efficiency (k_{cat}/K_M) of 18.5 $mM^{-1}s^{-1}$.

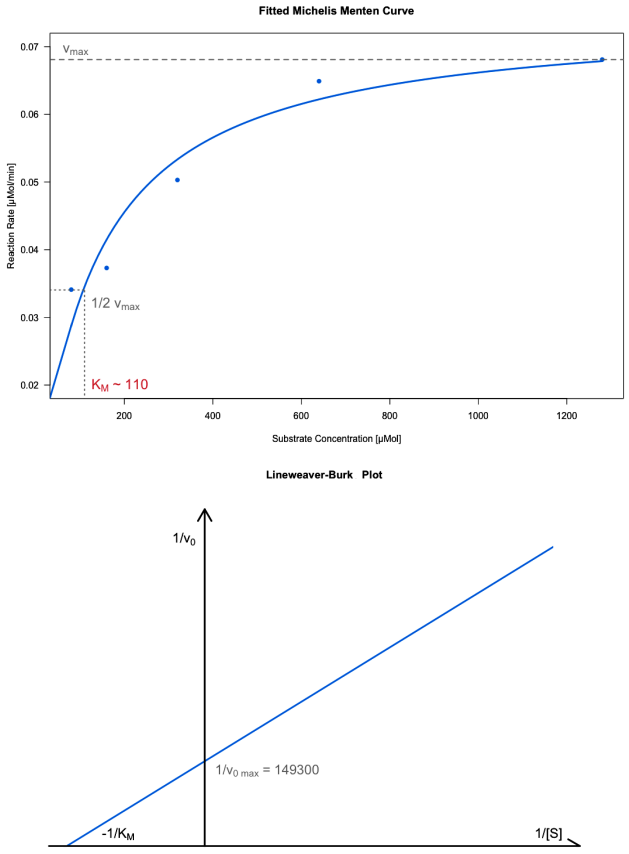

Figure 14 A. Fitted Michelis-Menten saturation curve with the kinetic parameters v_{max} (0.0681) and K_M (110 µMol) graphically depicted. **B.** Corresponding Lineweaver-Burk Plot representing the enzyme kinetics of Tyrosinase and L-DOPA. The y-intercept depicts a $1/v_{max}$ value of approximately 149300.

Enzyme Inhibition

Enzyme inhibition refers to a major control mechanism of biological systems, aiming to decrease enzyme activity. Inhibition is either reversible, characterized by a rapid dissociation of the enzyme–inhibitor complex, or irreversible, occurring when the inhibitor binds covalently to the enzyme and is only dissociating very slowly.

Depending on wether the inhibitor binds to the active or allosteric site, a reversible inhibition is further classified as competitive or non-competitive. In competitive inhibition, the inhibitor resembles the substrate, leaving both to compete for the binding site. A non-competitive inhibitor acts by decreasing the turnover number by preventing the substrate-enzyme complex to perform a chemical reaction (Figure 15).[22,23]

In this experiment, absorption spectroscopy was conducted to investigate the inhibition effect of thiourea on the enzymatic activity of tyrosinase. To do so, measurements at 475 nm were taken with constant concentrations of enzyme (2 µl) and inhibitor (8 µl) while adding substrate in increasing volumes (80 µM - 1280 µM). Kinetic parameters were calculated analogous to the Enzymatic Activity experiment (Table 4) and graphically depicted in a Lineweaver-Burk plot (Figure 16).

Comparison of measurement series of the inhibited enzymatic activity to the proteins native substrate conversion rate allows a qualitative and mechanistic interpretation for the type of inhibition. For competitive inhibitors, the v_{max} would remain constant, whereas the K_M would increase, resulting in the double-reciprocal line to raise its slope while keeping the y-intercept unchanged. In the case of a non-competitive inhibitor, v_{max} would decrease without changing the K_M value. Thus, the y-intercept and slope would shift, while the x-intercept would remain unaffected.[24] Compliant to literature, thiourea resembles a graphical form typical for a competitive inhibition type ($K_M \uparrow, \overline{v_{max}}$).

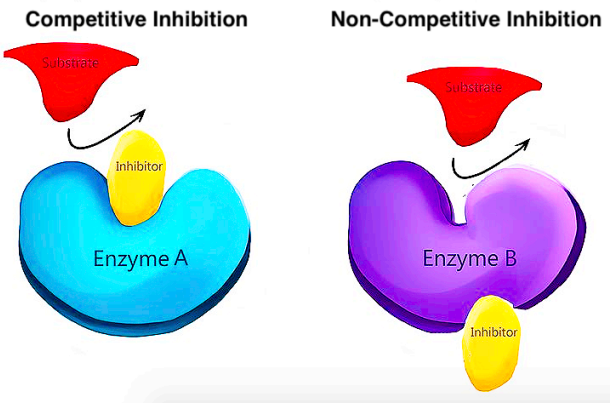

Figure 15. Schematics of competitive versus non-competitive inhibition. Competitive inhibitors occupy the active site, thus hindering substrate binding. Non-competitive inhibitors bind to an allosteric site, resulting in a conformational change of the enzyme, consequently preventing substrate conversion.

Table 4. Kinetic parameters of 2 µl Tyrosinase with 8 µl Thiourea at increasing L-DOPA concentrations

#	[S] µM	1/[S] 10^3	ΔA/min	v 10^{-4}	1/v 10^3
1	80	12.5	0.022	1.19	8.41
2	160	6.25	0.036	1.95	5.14
3	320	3.13	0.059	3.19	3.14
4	640	1.56	0.075	4.05	2.47
5	1280	0.78	0.099	5.35	1.87

CONCLUSION

Enzyme kinetics is an important research area, aiming to explain enzyme function, as well as predict how they behave in living organisms. The kinetic constants K_M and v_{max} are critical parameters in understanding how enzymes work together in order to control metabolism, useful for medical purposes and patient health.[25,26]

The enzyme Tyrosinase and the corresponding inhibitor Thiourea were characterized by conducting absorption spectroscopy. Thus, a quite high enzyme-substrate affinity for L-DOPA was identified (K_M = 110 µM), with a maximum reaction rate (v_{max}) of 0.0681 and a low turnover rate (k_{cat}) of 2.04s^{-1}. With tyrosinase regulating melanin biosynthesis, substrate conversion should be low, as excessive production or abnormal distribution could cause irregular hyperpigmentation.

In order to develop therapies or prophylactics for hyperpigmentary disorders, disruption of tyrosinase activity has been targeted.[27] Respectively, inhibition of thiourea was investigated in this experiment. Lineweaver-Burk plot analysis showed that thiourea can be classified as competitive inhibitor. In addition, the K_M value increased drastically when thiourea was added to the samples, showing a rapid decrease in enzyme-substrate affinity, concluding thiourea to be an efficient inhibitor.

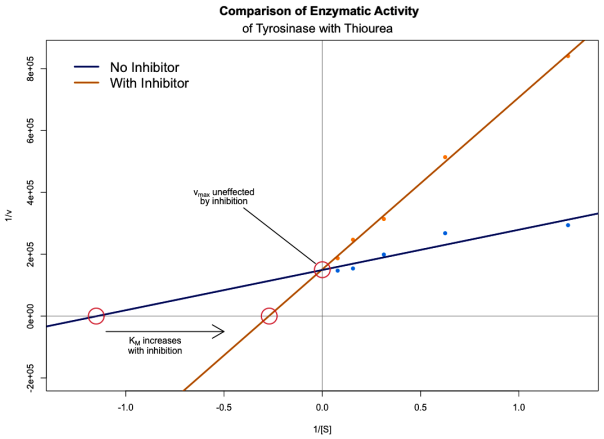

Figure 16. Lineweaver-Burk plot comparing enzyme kinetics with and without the inhibitor Thiourea. While K_M significantly increases when thiourea is added, v_{max} is uneffected by the inhibitor, concluding a competitive inhibition type.

LITERATURE

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Supplementary

Figure 1. Absorption spectrum of the dye in various concentrations

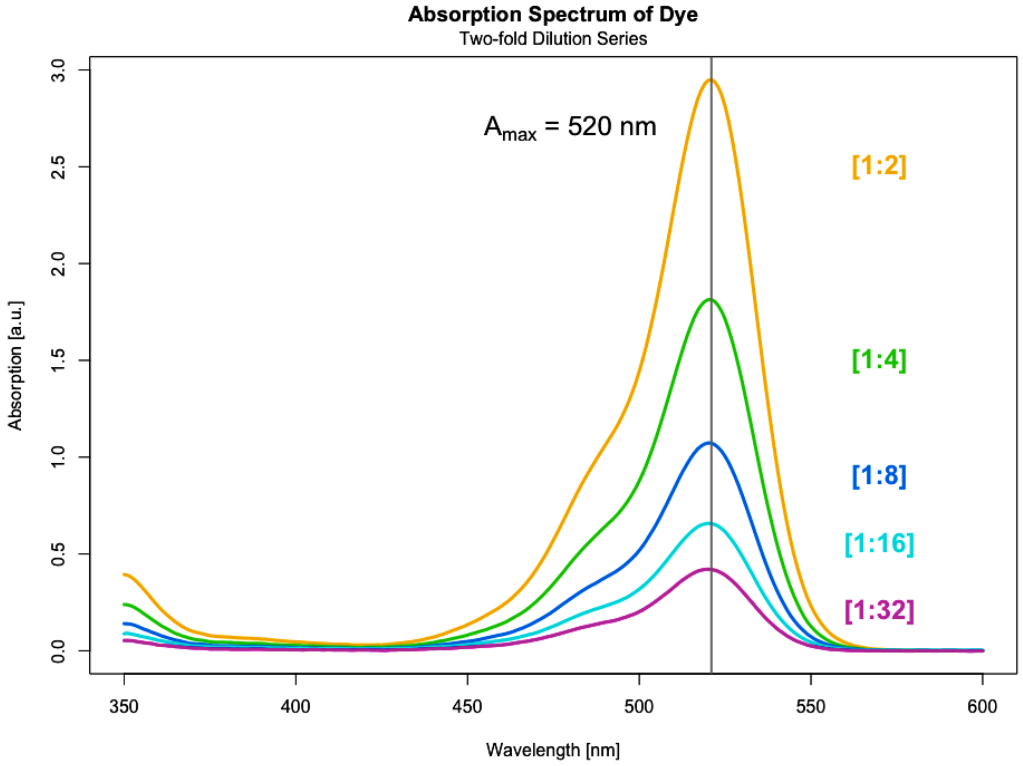


Figure 2. Calibration curve for the fluorescent dye at 520 nm

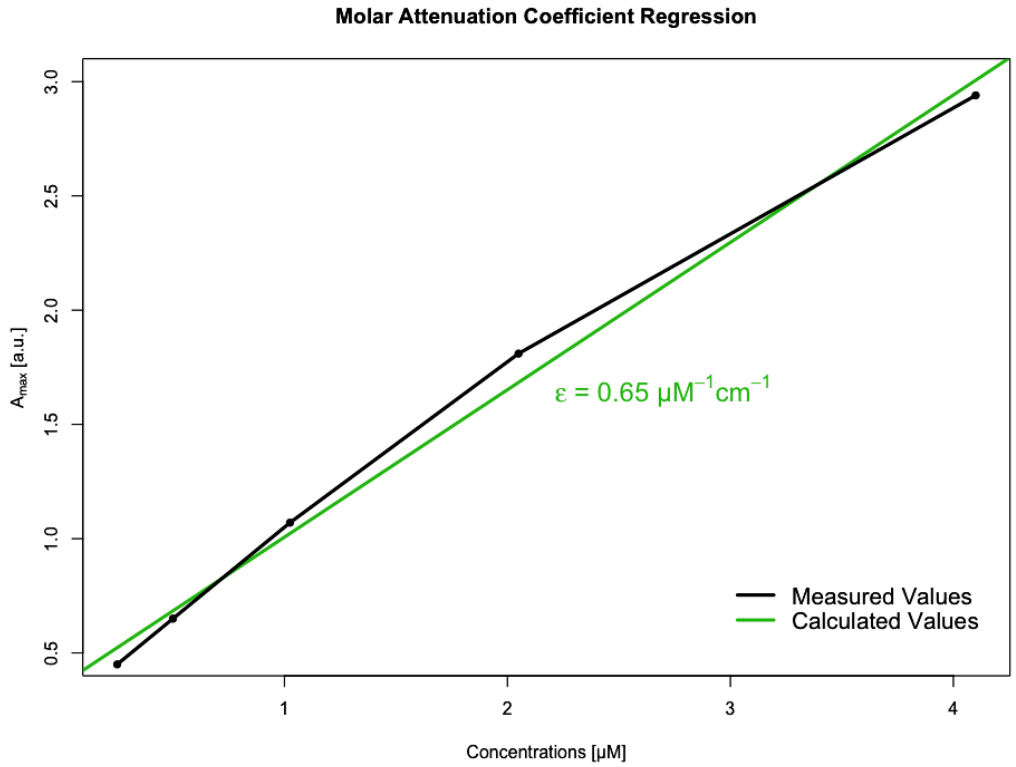


Figure 3. Stoke's shift between absorption and emission

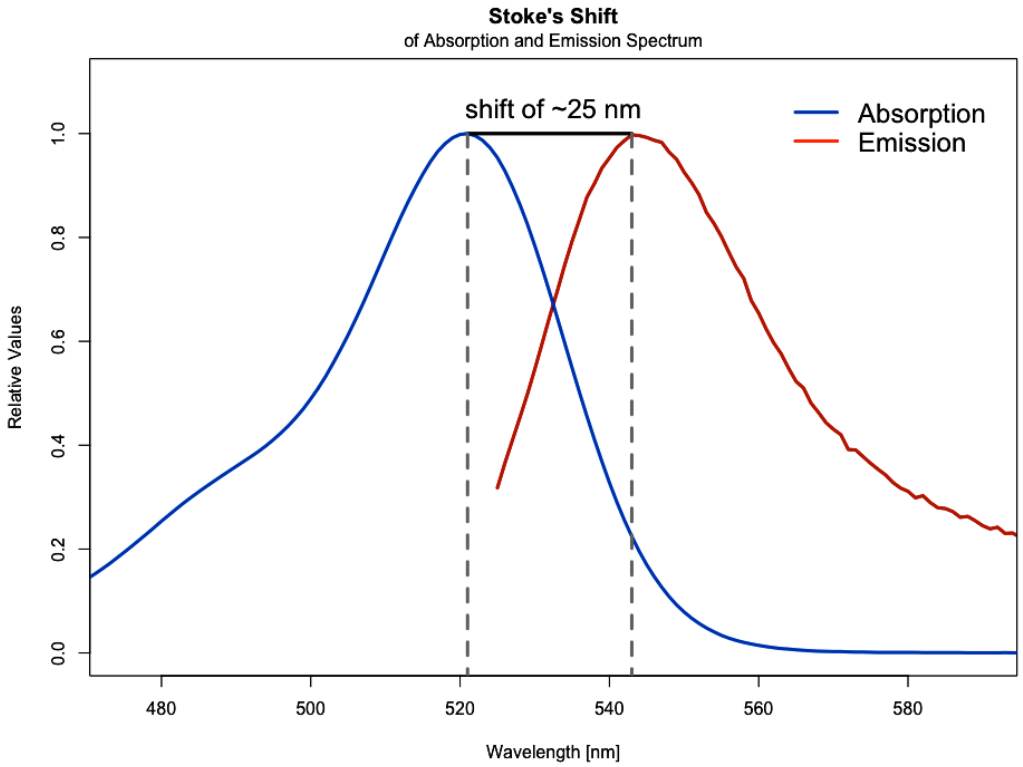


Figure 4. Schematics of a Perrin-Jablonski diagram

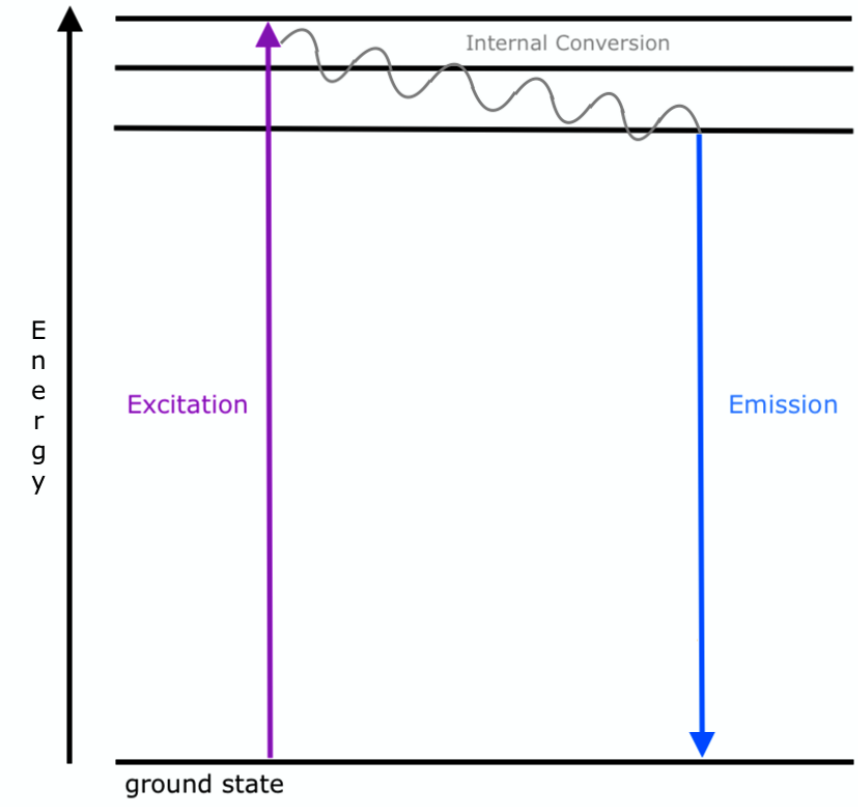


Figure 5. Standard CD spectra with basic secondary structures

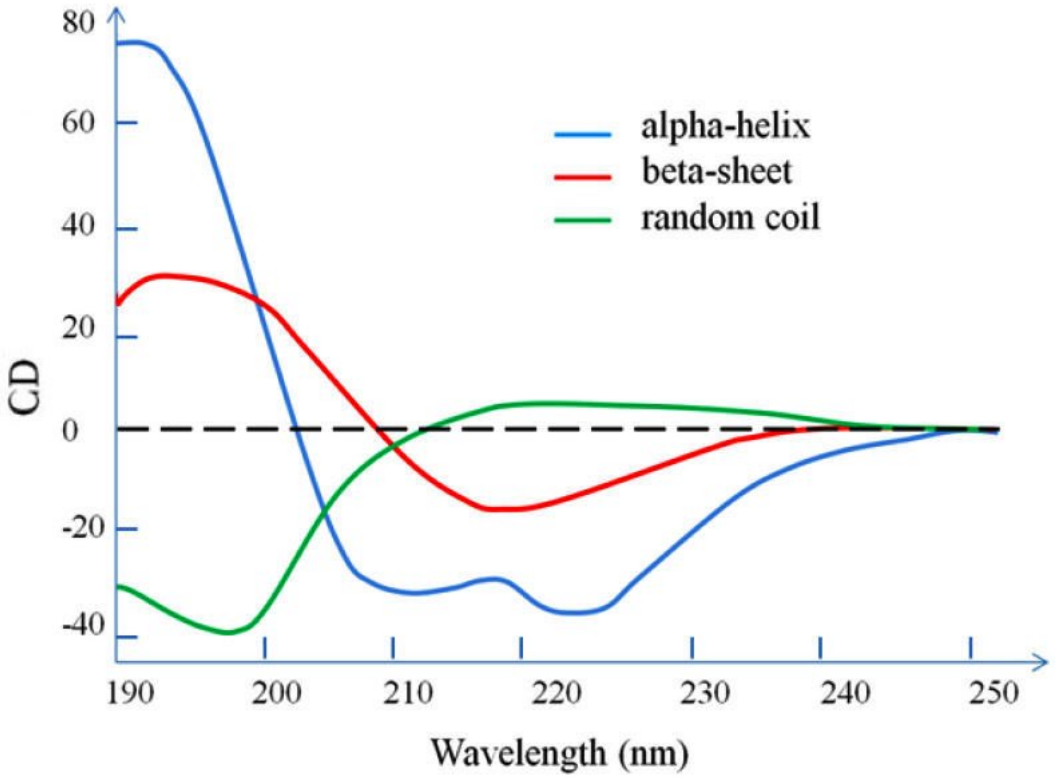
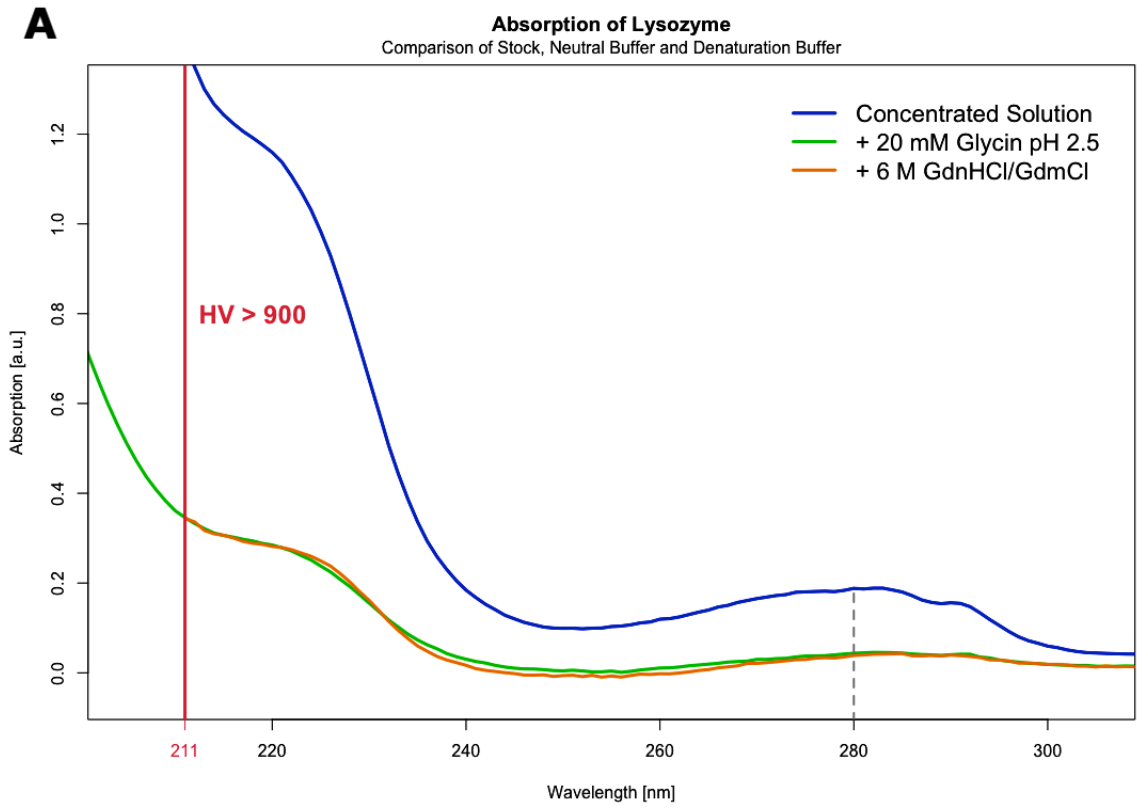


Figure 6. Absorption spectroscopy comparing all three samples



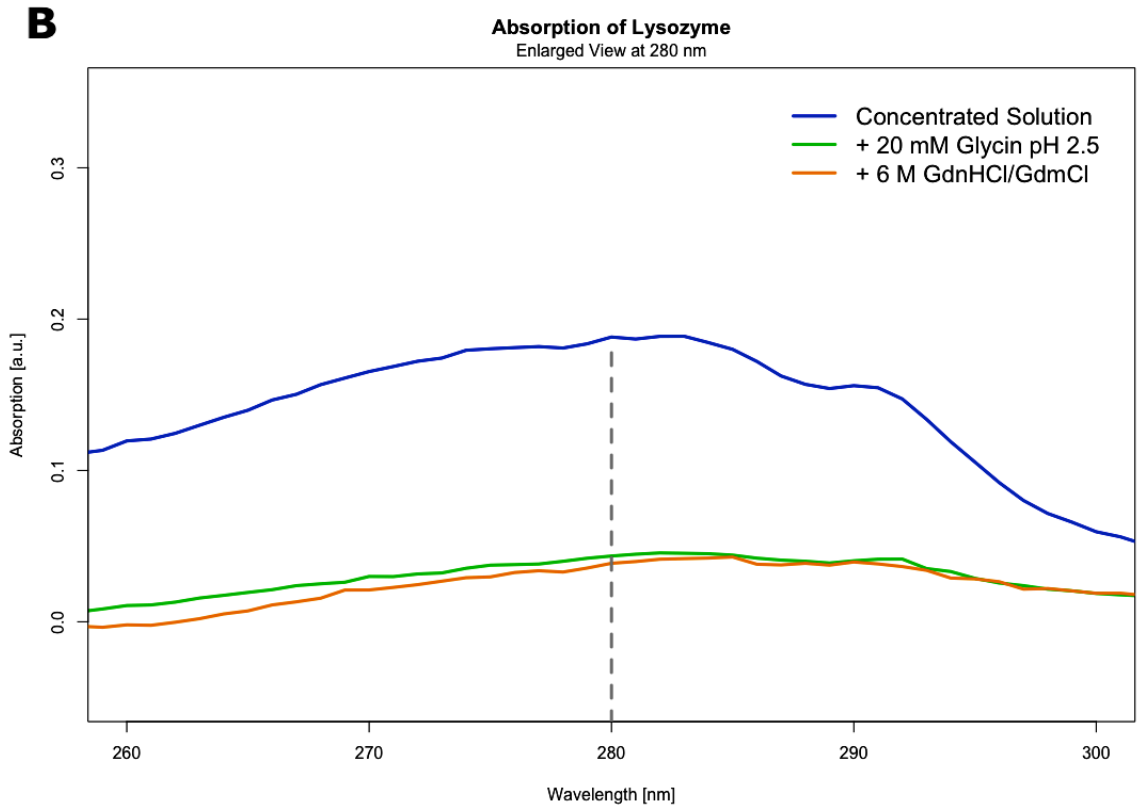


Figure 7. Calculated concentrations at 205 nm & 280 nm

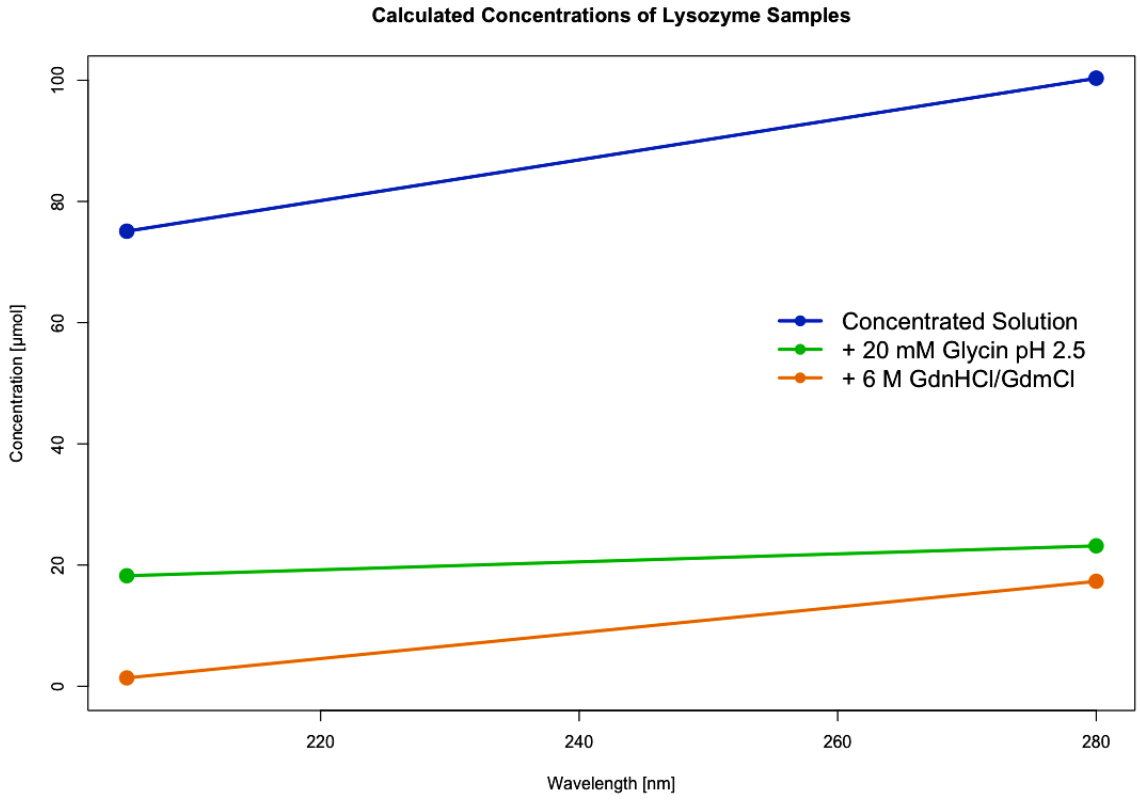


Figure 8. CD Spectroscopy of the different lysozyme solutions

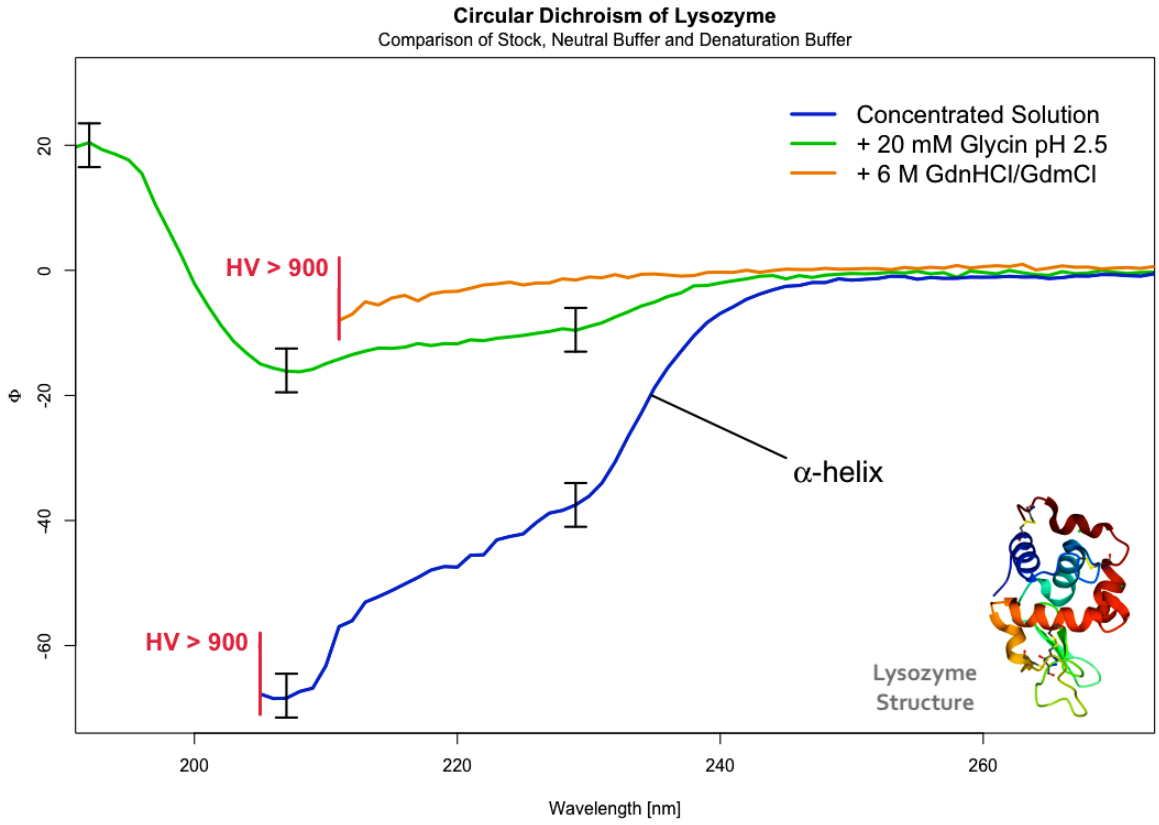


Figure 9A. Fluorescence spectrum of BSA

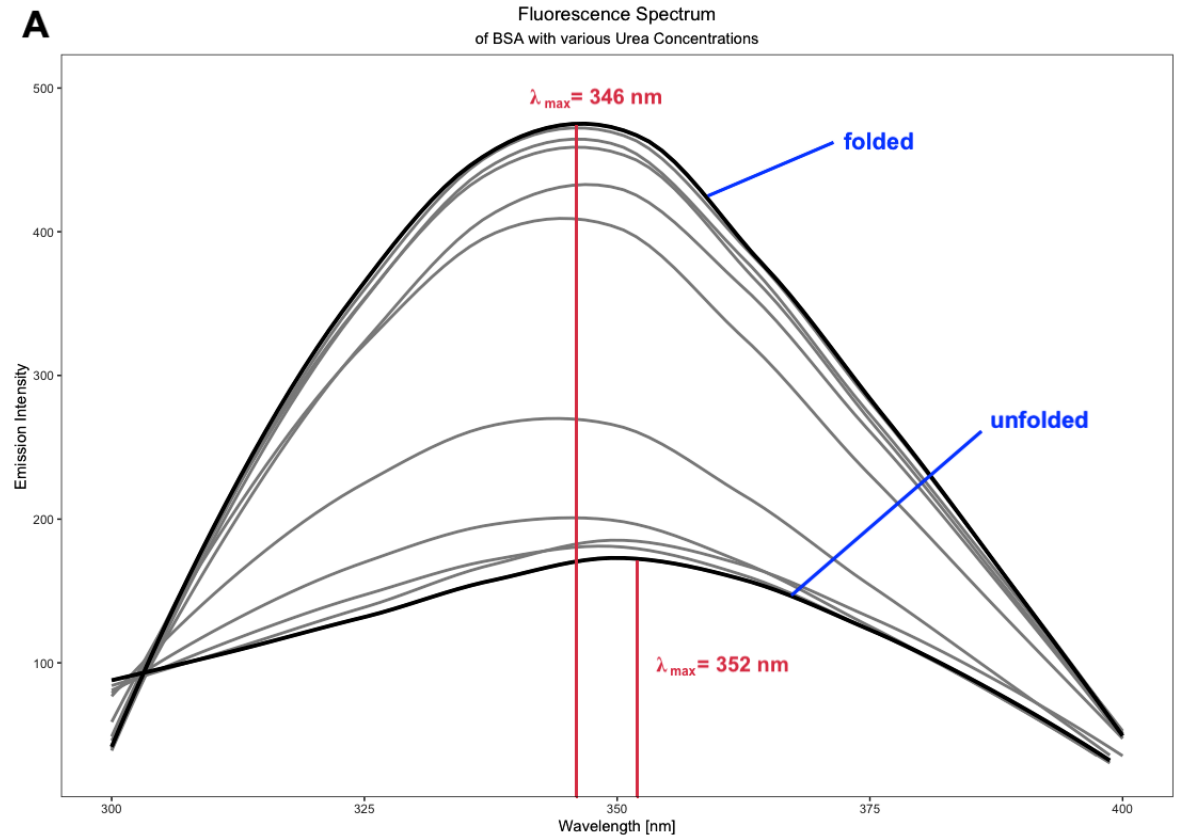


Figure 9B. Denaturation curve of BSA with urea

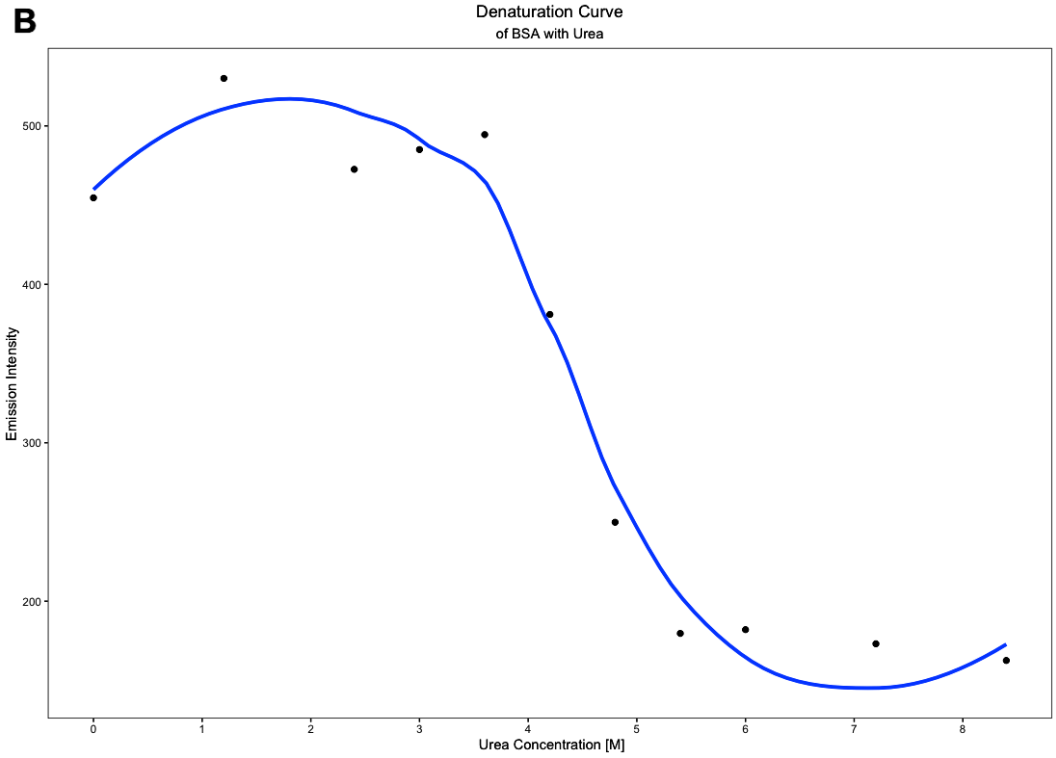


Figure 10. CD Spectra of BSA with varying urea concentrations

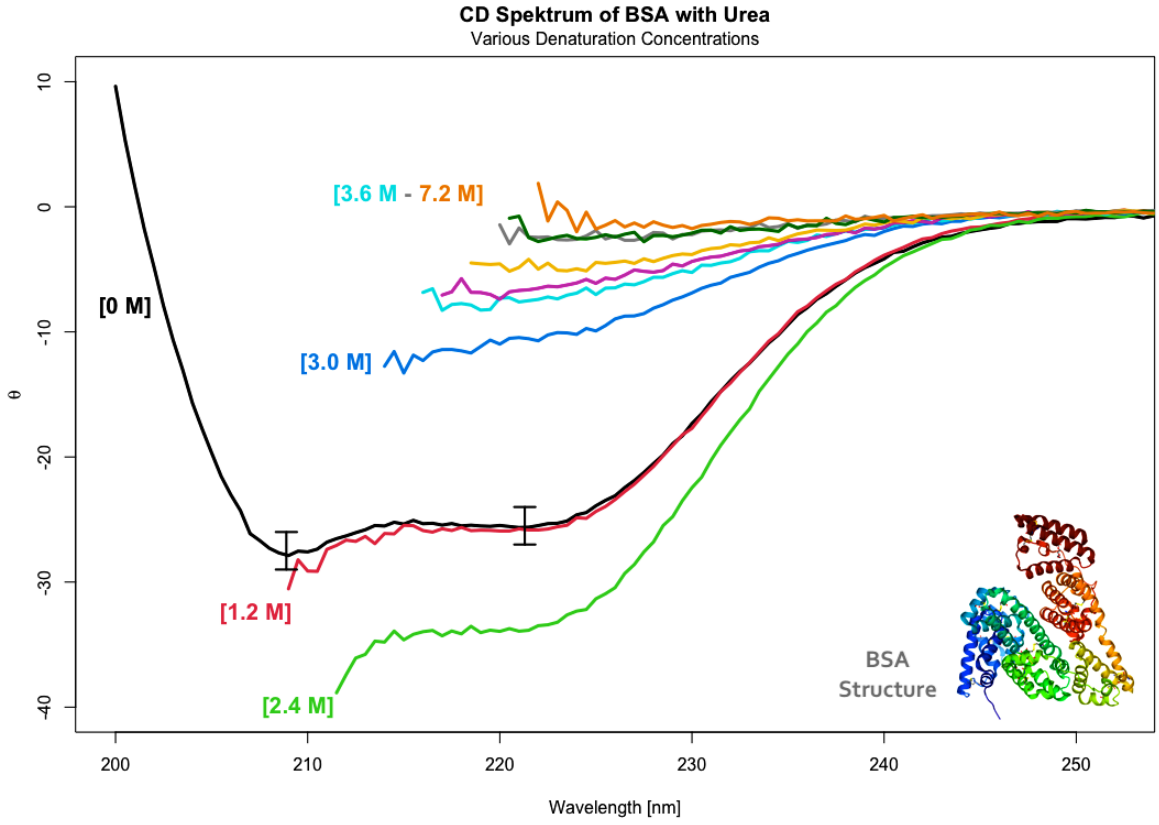


Figure 11. Comparison of denaturation curves

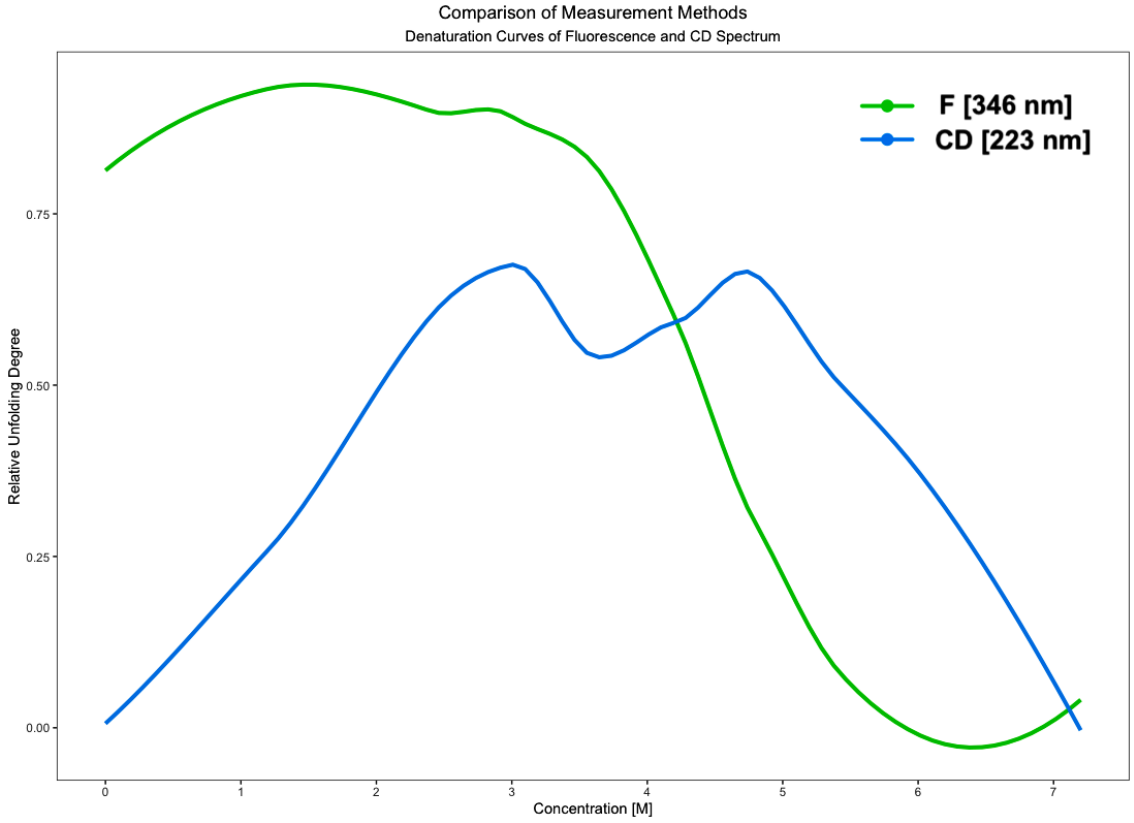


Figure 12. Comparison of denaturation agents

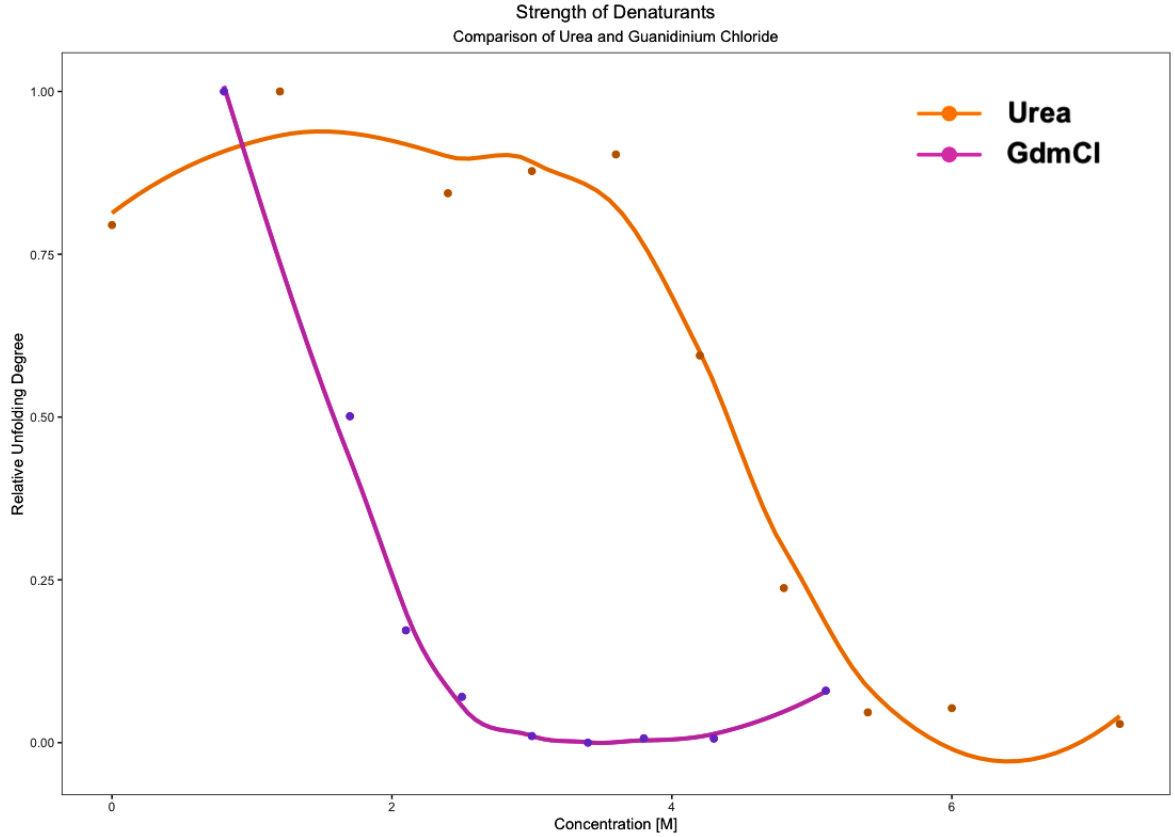


Figure 13. Linear range of Uv-Vis time scan with varying substrate concentrations

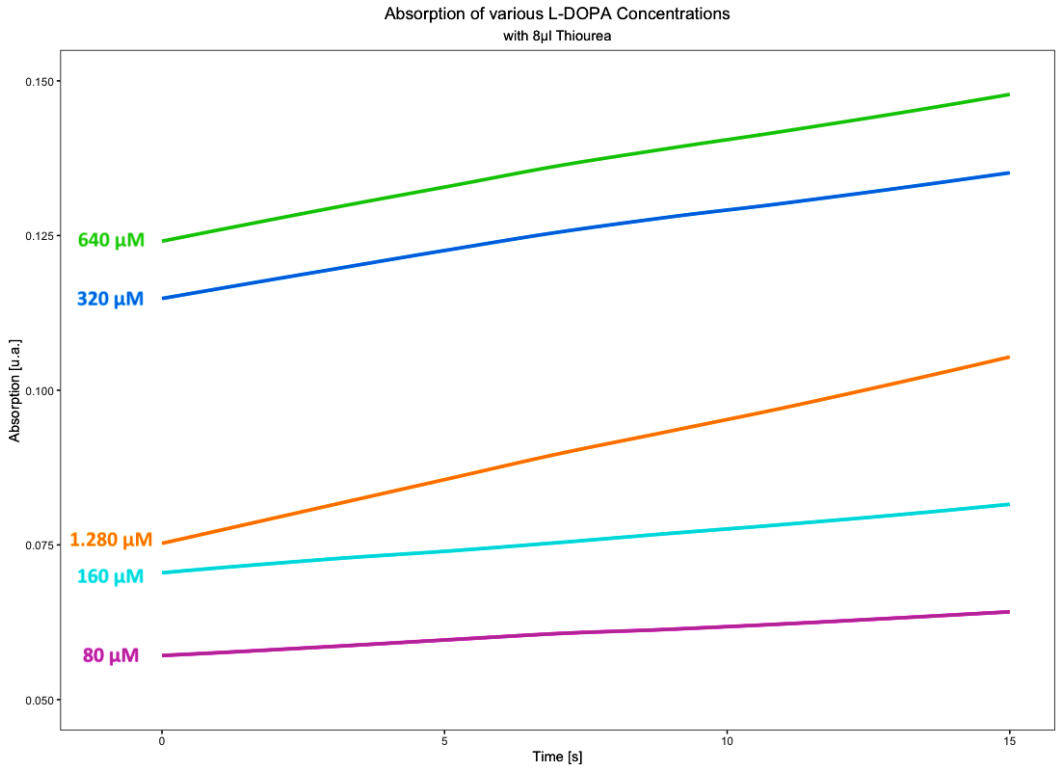


Figure 14A. Michaelis-Menten curve depicting the enzymatic activity of Tyrosinase

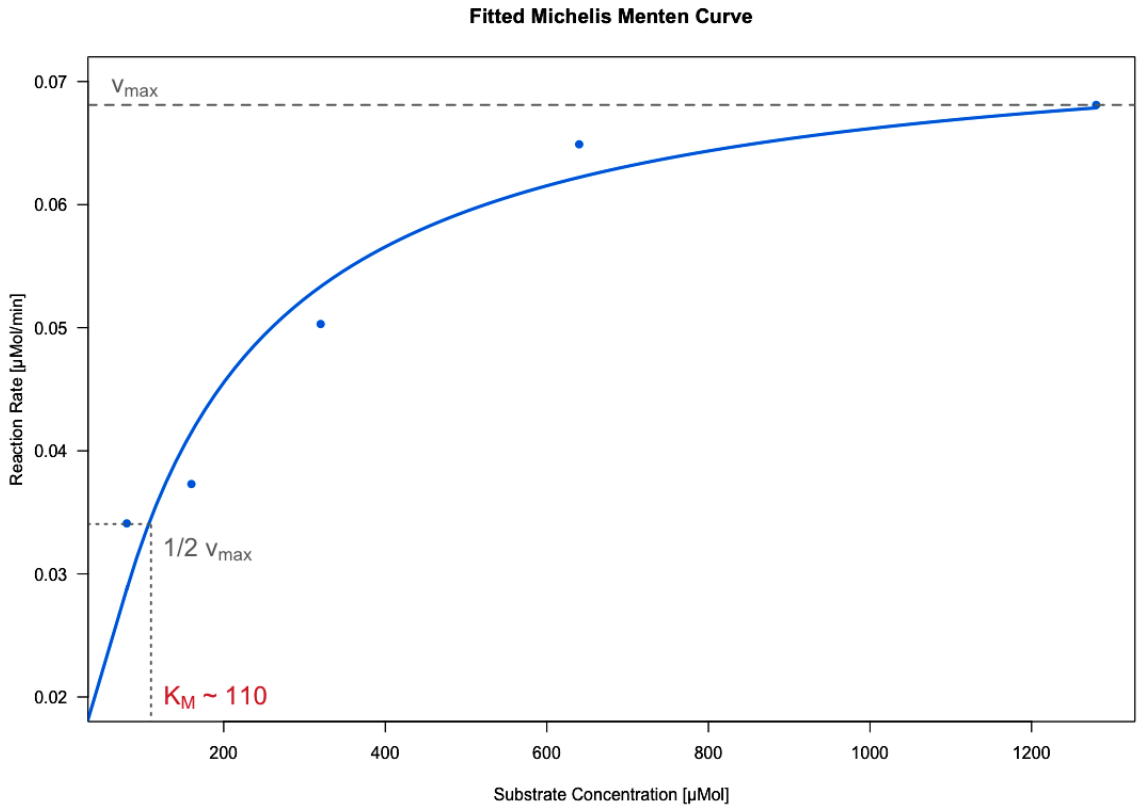


Figure 14B. Lineweaver-Burk plot of Tyrosinase and L-DOPA

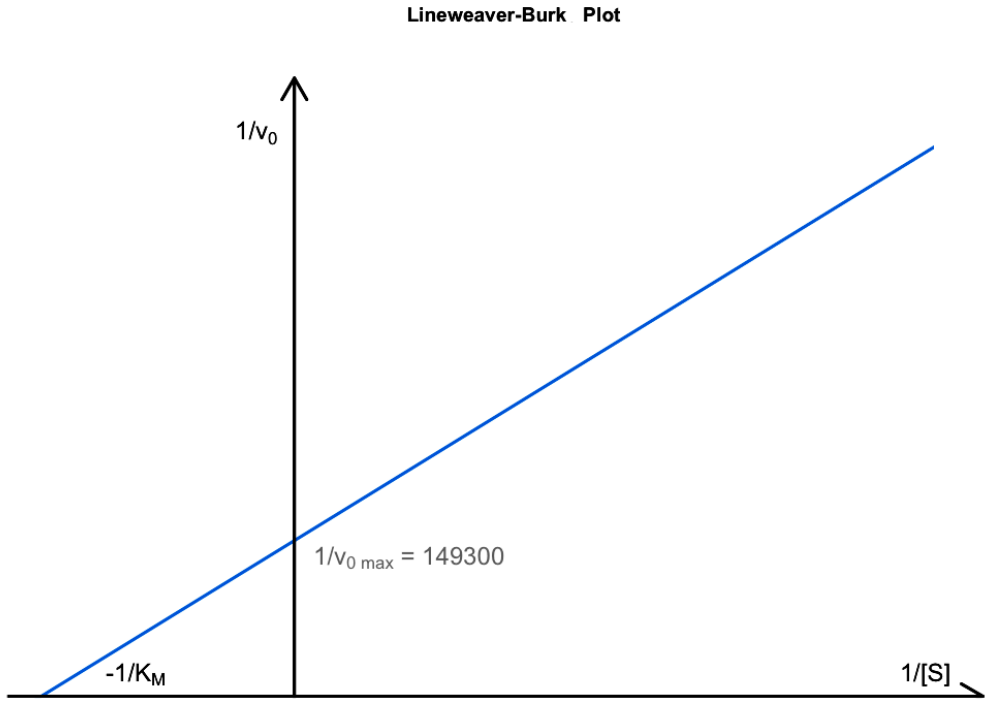


Figure 16. Lineweaver-Burk plot of Tyrosinase inhibited by Thiourea

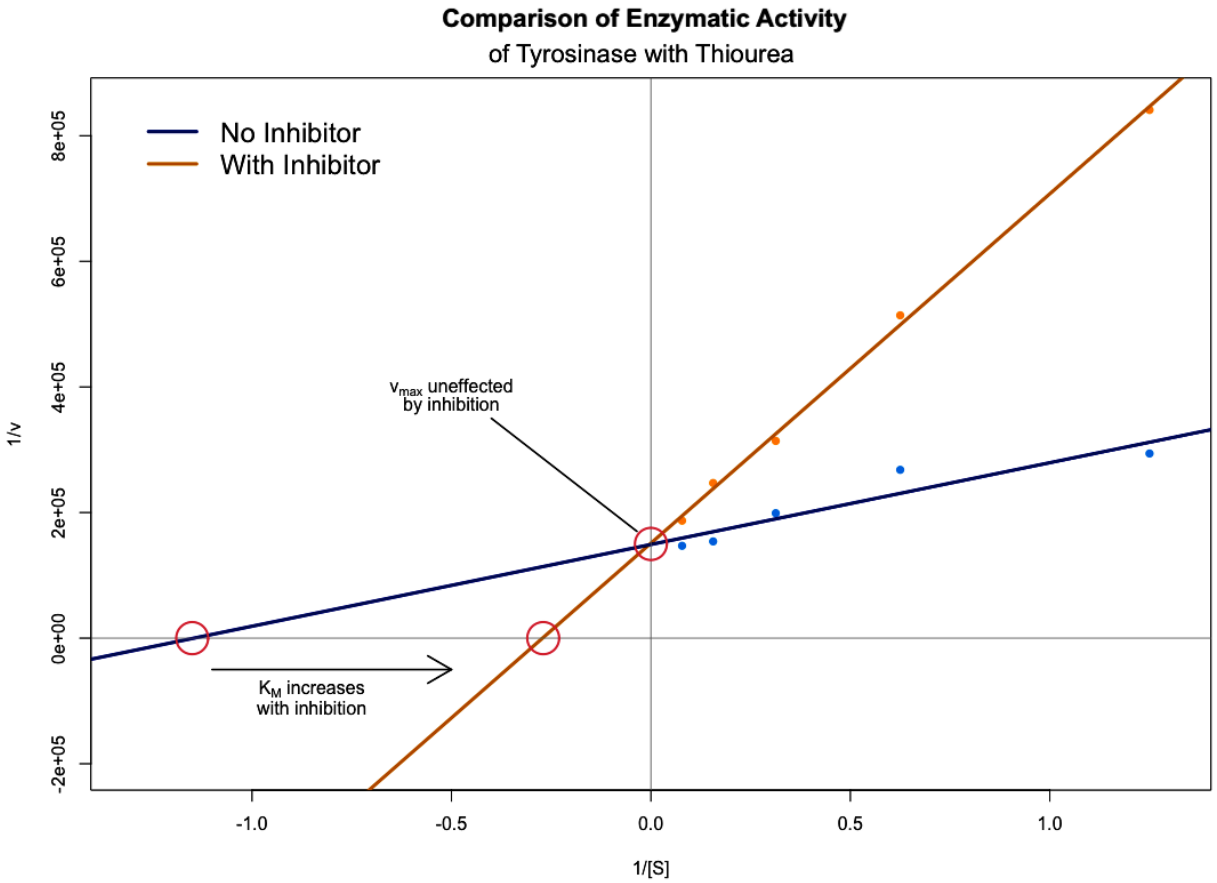


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Table 1. Measurements of the dye with respective dilutions and concentrations

Dilution	c [µM)	A_{max}	Fluorescence Intensity [cps]
1:2	4.1	2.94	-
1:4	2.05	1.81	-
1:8	1.025	1.07	-
1:16	0.5	0.65	456.072
1:32	0.25	0.45	278.492

Table 2. Lysozyme samples used in the experiment

Sample	Buffer	Dilution
Stock Solution	—	—
Native Buffer	20 mM Glycine, pH 2.5	1:3
Denaturation Buffer	20 mM Glycine, pH 2.5 8 M GdnHCl/GdmCl	1.3

Table 3. Kinetic parameters of Tyrosinase with increasing L-DOPA concentrations

#	[S] µM	1/[S] 10^3	ΔA/min	v 10^{-4}	1/v 10^3
1	80	12.5	0.063	3.41	2.94
2	160	6.25	0.069	3.73	2.68
3	320	3.13	0.093	5.03	1.99
4	640	1.56	0.120	6.49	1.54
5	1280	0.78	0.126	6.81	1.47

Table 4. Kinetic parameters of Tyrosinase with Thiourea at increasing L-DOPA

#	[S] µM	1/[S] 10^3	ΔA/min	v 10^{-4}	1/v 10^3
1	80	12.5	0.022	1.19	8.41
2	160	6.25	0.036	1.95	5.14
3	320	3.13	0.059	3.19	3.14
4	640	1.56	0.075	4.05	2.47
5	1280	0.78	0.099	5.35	1.87