Laboratory Protocol

Characterization of Protein Stability, Catalysis and Aggregation

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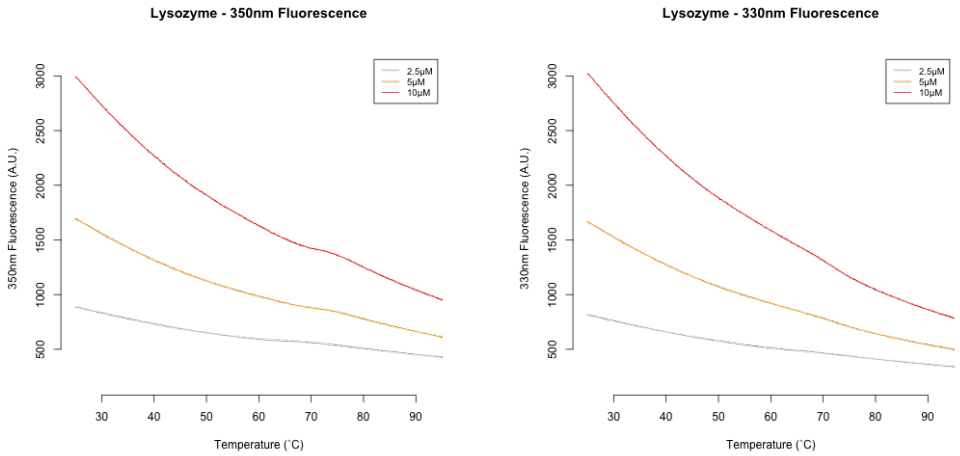
1 Temperature Induced Unfolding Transitions

The goal in this experiment is to determine the stability of the proteins Lysozyme and BSA (Bovine Serum Albumin) at different concentrations, 2.5, 5 and 10 µM, by inducing unfolding transitions through temperature changes. We detect unfolding using Fluorescence spectroscopy by measuring emission at two wavelengths, 330nm and 350nm, and we expect to see a sharp increase in the ratio of these emissions when the protein unfolds.

Measurements

The temperature was increased from 25 °C to 95 °C, at a rate of 2 °C per minute, and corresponding fluorescence at wavelengths 350nm and 330nm was measured.

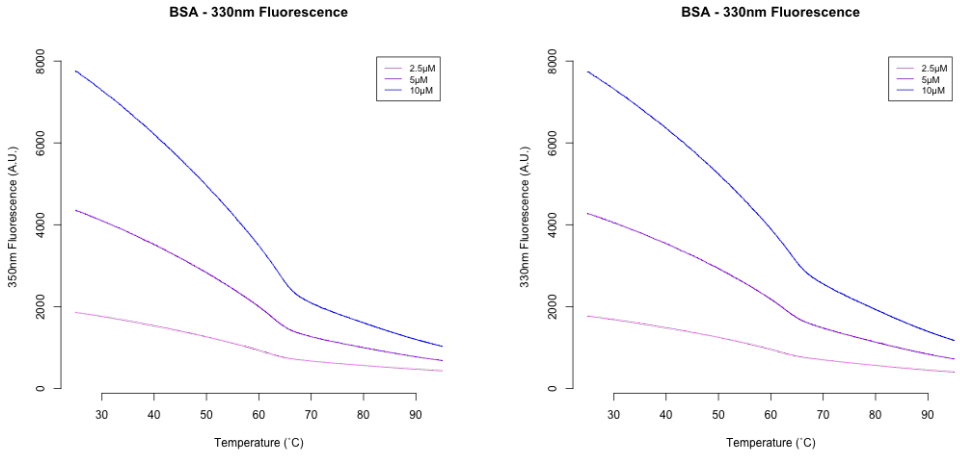
Lysozyme - Fluorescence vs. Temperature



(a) (b)

Figure 1: Lysozyme Fluorescence at wavelengths (a) 350nm and (b) 330nm

BSA - Fluorescence vs. Temperature



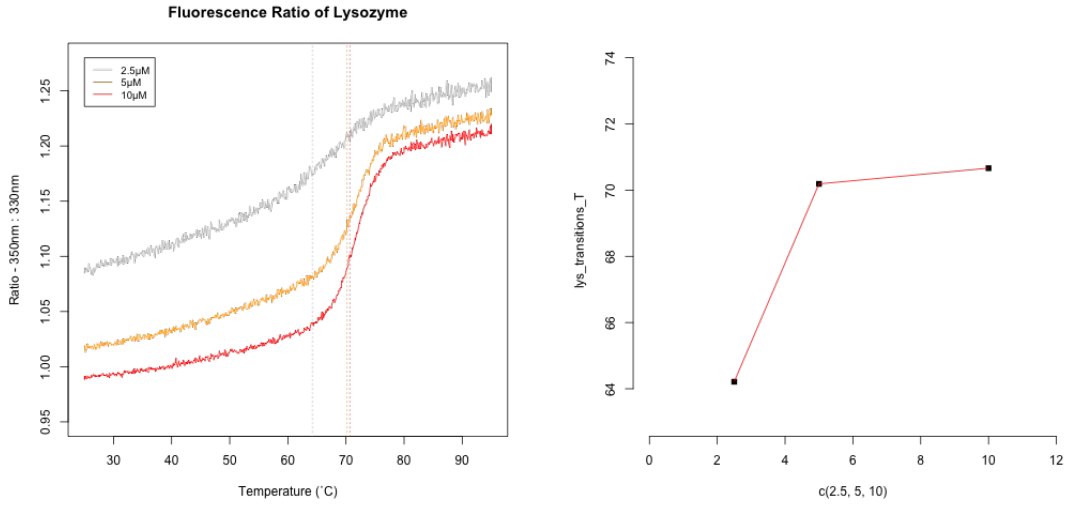
(a) (b)

Figure 2: BSA Fluorescence at wavelengths (a) 350nm and (b) 330nm

Analysis

The Fluorescence Ratio graph (Figure 3 and 4) was used to approximate the transition midpoint, while the protein's heat capacity was derived from the plot's derivative. These graphs allow the observation of the transition of proteins in both their folded and unfolded states. Stability in the folded state arises from various interactions within the polypeptide. However, upon surpassing a threshold temperature, the polypeptide transitions to an unstable state. Consequently, as temperature increases, the population of unfolded molecules also rises. This process behaves differently for the two proteins.

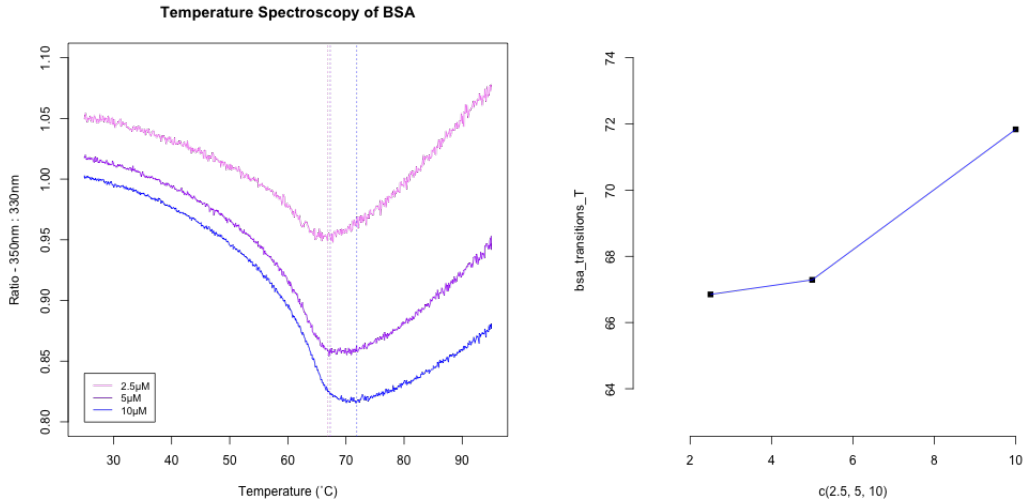
For Lysozyme, a low ratio can be observed at the beginning, which indicates that the folded state predominates. With increasing temperature, this confirmation changes up to a threshold temperature and the transition of the molecules into the unfolded state, and accordingly the ratio also increases.



(a) (b)

Figure 3: Ratio of Fluorescence at 350nm to 330nm for (a) Lysozyme and (b) BSA

The opposite can be observed for BSA. Here, the ratio is already high at the start of the process and falls down to a certain threshold temperature. From this point, the ratio increases for all concentrations and thus also the amount of proteins in the unfolded state.



(a) (b)

Figure 4: Ratio of Fluorescence at 350nm to 330nm for BSA

2 Lysozyme Aggregation

In this experiment, aggregation of Lysozyme was studied to understand protein aggregation, where unfolded polypeptide chains of the proteins Lysozyme and Hsp26 associate irreversibly, leading to the formation of large particles that scatter light. Throughout the experiment, Hsp26 was used to analyze its effect on the aggregation of Lysozyme with presence of TCEP.

Measurements

Poor absorbance spectrometer used to do a poor-man's version of scattering. The scattering is not actually detected. What we do is choose a wavelength where we know nothing in the mix absorbs anything and we use that fact to say all of the intensity reduction is because of the scattered light.

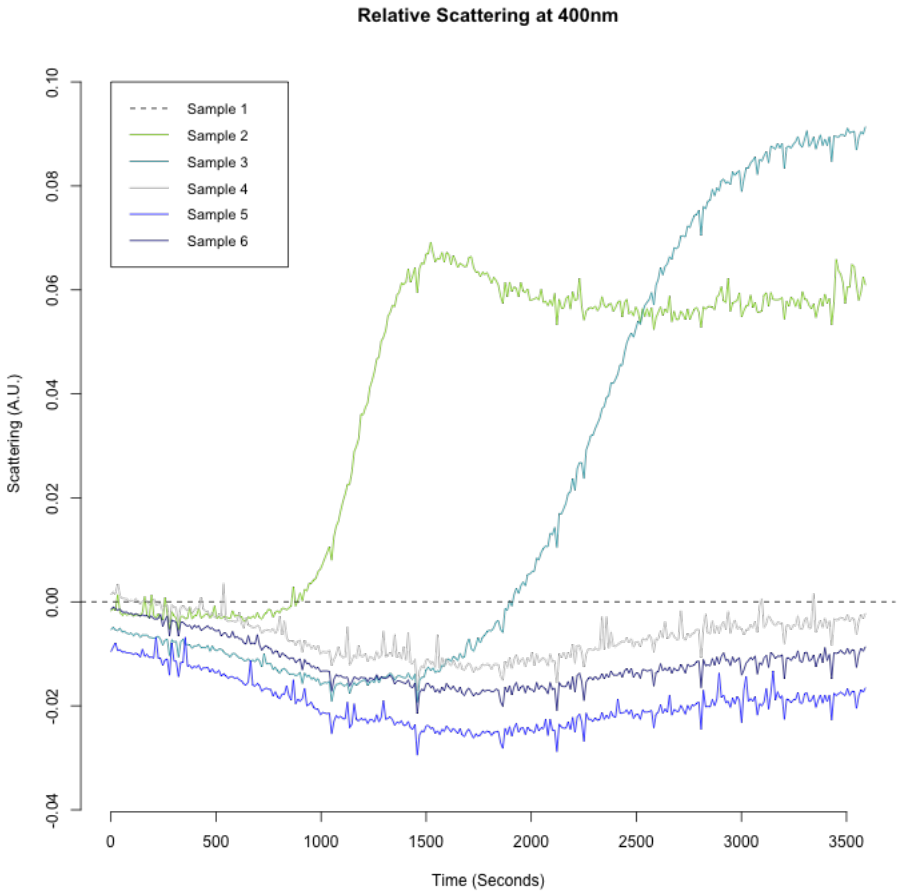


Figure 5: Scattering recorded as a function of time.

Analysis

As protein aggregation increases, more light is absorbed by the sample. Introducing Hsp26 into the sample solutions can regulate aggregation and, consequently, absorption. At low Hsp26 concentrations (Samples 1 to 3 in Figure 5), Lysozyme aggregation isn't effectively inhibited. Thus, the presence of Hsp26 in the solution increases overall absorption. But it can also be seen that a higher concentration of 5 µM delays the aggregation.

However, when the concentration of Hsp26 matches that of Lysozyme, aggregation is prevented, resulting in lower absorbance (Sample 4). The control samples lacking Lysozyme and denaturant exhibit no absorbance change at 400nm (Samples 5 and 6). Only Samples 2 and 3 allow for the determination of an apparent halftime, as their curves display a change of confirmation.

3 Michaelis-Menten Kinetics

Lactatdehydrogenase (LDH) catalyzes the reaction of the substrate pyruvate into the product, lactate. In order to measure its activity, the conversion of NADH, the cofactor of LDH, NAD^+ was observed in this experiment through spectroscopy of NADH.

Measurements

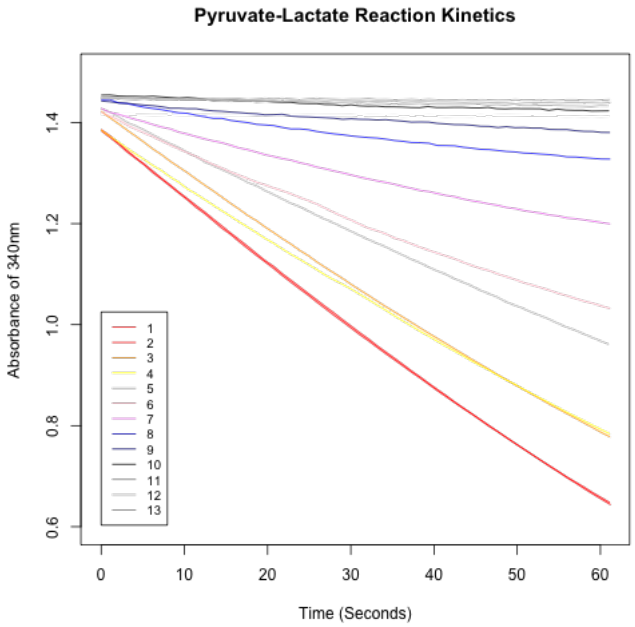
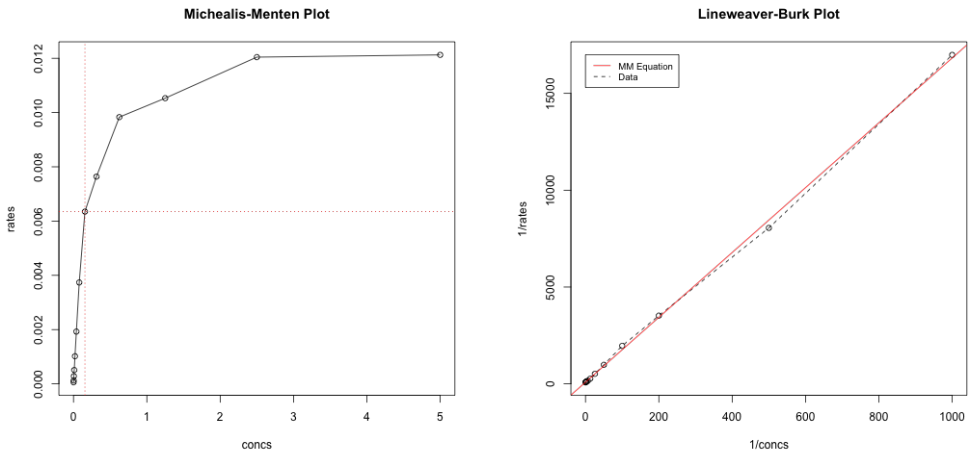


Figure 6: Rates

Analysis

The Michaelis-Menten plot (see figure x left) could be plotted from the reaction rates and the according concentration of substrate (NADH). It shows that for low substrate concentrations, the rate of the reaction increases linearly with increasing substrate concentration. As the substrate concentration increases, the rate of the reaction or velocity eventually reaches a maximum value (V_{max}). In this region, the active sites of the enzyme are largely occupied by the substrate, and the rate becomes independent of further increases in substrate concentration.



(a) (b)

Figure 7: Rates

Derived from the Michaelis-Menten plot, the Lineweaver-Burk plot provides a linear transformation. It is used for determining the kinetic constants, the maximum reaction rate V_{max} and the Michaelis constant K_m. The result for $V_{max} = 0.0116$ µM/s and for $K_m = 0.1942$ µM, respectively.

4 UV/VIS Spectroscopy of NADH

Analysis