3CL SARS-CoV-2 3CL Protease Analyzing

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Abstract

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Keywords:

Chapter 1

Protein Purification

1.1 *E.coli* Lysis: Homogenizer

- Stir the buffer lysis containing *E.coli* until without obvious lumps and precipitates in an ice bath.
- Filter the mixture (50 mL) by gauze and then pour into the **homogenizer** [2] with 10 mL H_2O following the below settings:
  - Raise the pressure to 1,000 bar for 2 minutes.
  - Pause 1 minute.
  - Repeat the cycle 3 times.
- Centrifugate the liquid at 14,000 rpm for 30 minutes.
- Strain the product through a filter (0.45 μm) into a graduated cylinder.

1.2 Purification: Column Chromatography

1.2.1 Affinity Chromatography: Immobilized Ni^{2+} Metal Ion + His-tag

1.2.2 Gel-filtration Chromatography

1.3 Analyzing: SDS-PAGE

Chapter 2

Enzyme Kinetics

The following experiments involve tedious configuration of solutions with different concentrations, including substrate, 3CL Protease (Enzyme), and inhibitor. To summarize and visualize the complicated process of dilution, I have drawn the Figure 2.2. Generally speaking, we used **multi-channel pipette** to transfer a whole row of solution to other three different rows because of the three independent parallel groups. Before that, however, we must use **single-channel pipette** to adjust the concentration in one row. The **two fold serial diluting**, as shown in Figure 2.1, was used for inhibitor while substrate was diluted one by one differently as shown in Table 2.1.

2.1 Verifying Michaelis Menton Equation

$$E + S \rightleftharpoons ES \rightarrow E + P$$

$$V_0 = \frac{k_{cat} E_0 [S]}{K_m + [S]} = \frac{V_{max}[S]}{K_m + [S]}$$

2.1.1 Principles

The fluorescent substrate relates to **FRET**, which means the substrate will be fluorescent only when catalyzed by protease. Specifically speaking, DABCYL plays the role of dark quencher [3] while the EDANS [1] as the donor of fluorescence. The substrate (polypeptide) is stuck in the middle as shown in Figure 2.3.

The overall consequence is that, when the substrate is consumed, then, it will show fluorescence. Further process of reaction will lead to more intensive fluorescence which can be detected by **ELISA** in the unit of RFU.

2.1.2 Methods

The independent variable in Michaelis Menton Equation is the concentration of substrate $[S]$. Thus to verify the equation we must configure the substrate solution of different concentrations. We started by dissolving 2 mg solid substrate into 192 μL DMSO solvent, which has the concentration

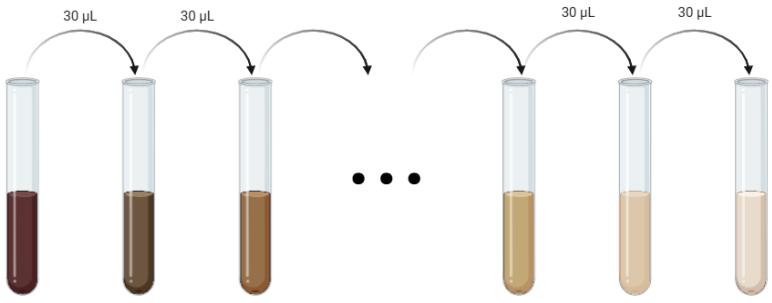

Figure 2.1: Serial Dilution of Inhibitor

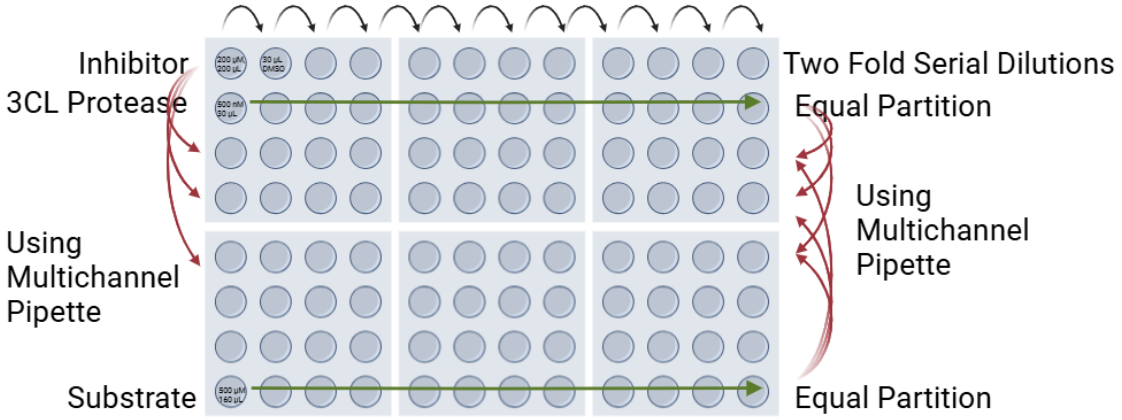

Figure 2.2: Solution Configuration in Microplate

Number of Sample	1	2	3	4	5	6	7	8
Substrate Concentration (μM)	500	750	1000	1250	1500	2000	2500	3000
DMSO Solvent (μL)	9	8.5	8	7.5	7	6	5	4
5000μM Concentrated Substrate (μL)	1	1.5	2	2.5	3	4	5	6

Table 2.1: Substrate Solution Configuration in Microplate

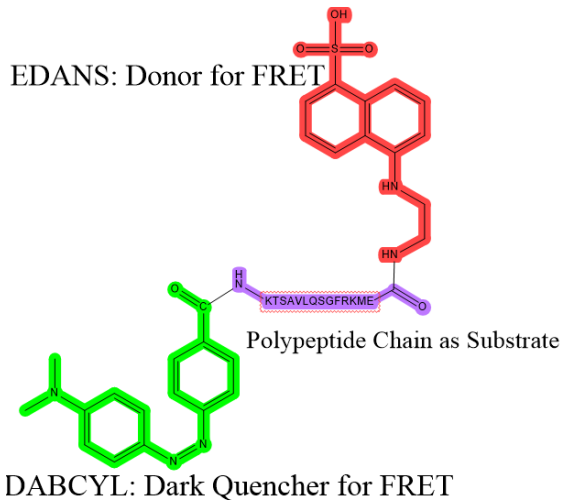

Figure 2.3: Structure of Fluorescent Substrate

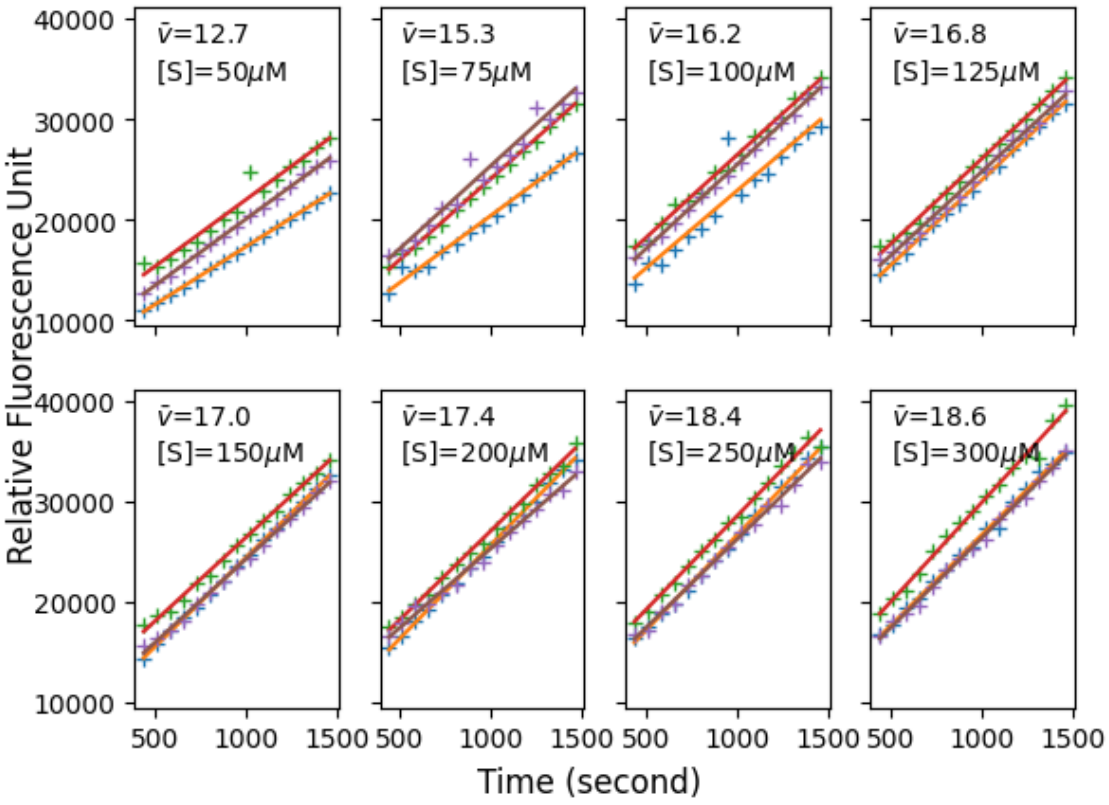

Figure 2.4: Reaction Rate versus Substrate Concentration: 8 groups of experiments, each has 3 parallel groups

of 5000 μL. Then, we adjusted the ratio of DMSO and concentrated substrate to achieve 8 different concentrations of substrate solution, as shown in Table 2.1.

We detected the speed of reaction by the concentration variation of fluorescent substrate which was identified by **enzyme-linked immunosorbent assay (ELISA)**. The original data is shown in Figure 2.4.

2.1.3 Data Analysis

The original data gotten from **ELISA** is visualized in Figure 2.4. In each subfigure, I have drawn the three parallelled groups of same substrate concentration. It should be noted that, the several initial points were omitted for keeping linear parts only in accordance with the assumptions of Michaelis Menton Equation (the [ES] has reached steady state, not changing with time).

I performed linear regression to each of them, then averaged the slope which represents the speed of reaction.

The eight slopes (speed of reaction) were plotted with respect to substrate concentration [S] then. As shown in Figure 2.5, the consequence is a curve which was similar to the prediction of Michaelis Menton Equation.

To further analyze the results, however, I converted the axes to the reciprocal of speed V_0^{-1} and substrate concentration $[S]^{-1}$. The benefit of reciprocal plotting is that we can operate linear regression again.

$$\frac{1}{V_0} = \frac{K_m}{V_{max}} \cdot \frac{1}{[S]} + \frac{1}{V_{max}}$$

$$y = kx + b$$

The vertical intercept b is $\frac{1}{V_{max}}$ and the horizontal intercept corresponds to $-\frac{1}{K_m}$, which are also annotated in Figure 2.6.

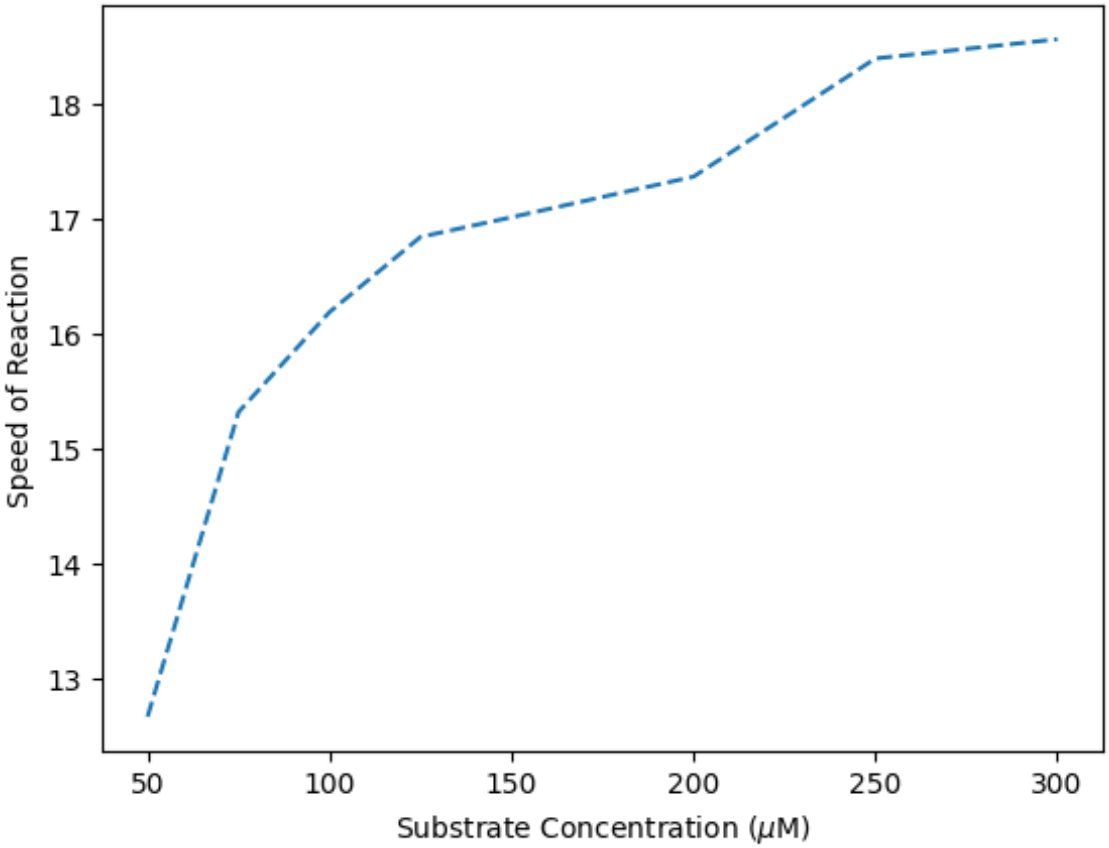

Figure 2.5: Reaction Rate versus Substrate Concentration: in normal axes

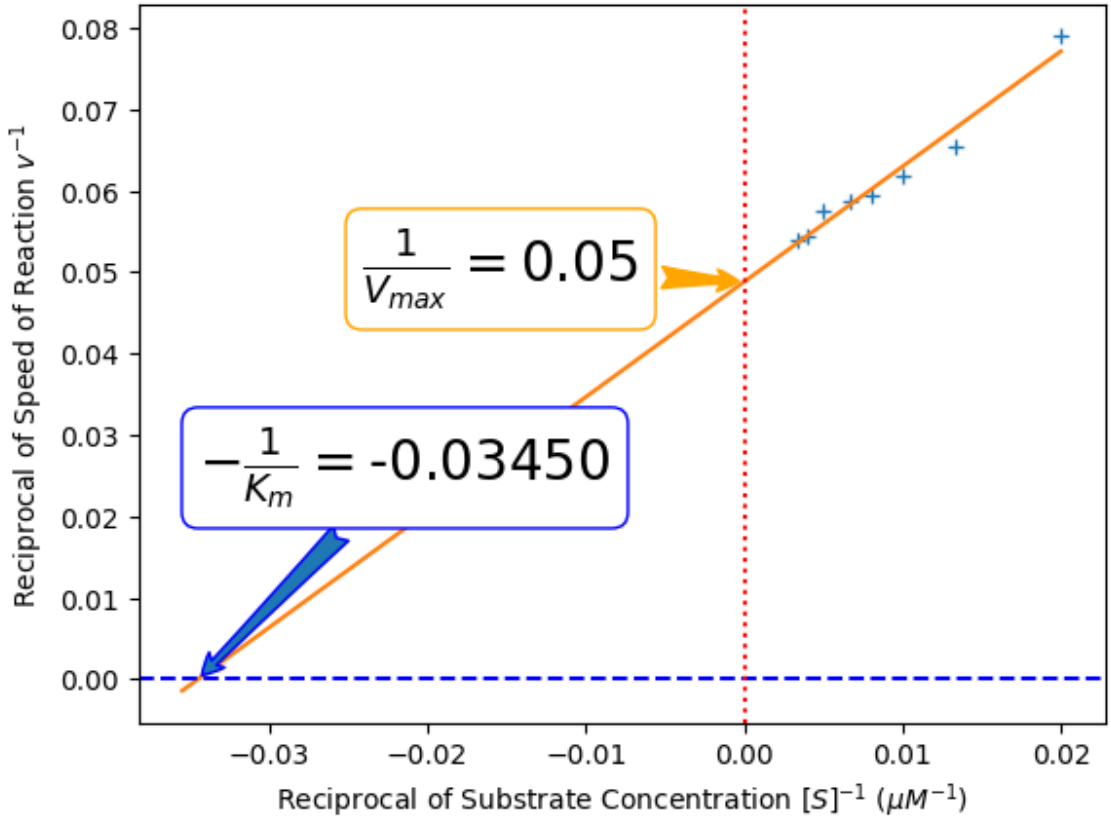

Figure 2.6: Reaction Rate versus Substrate Concentration: in reciprocal axes

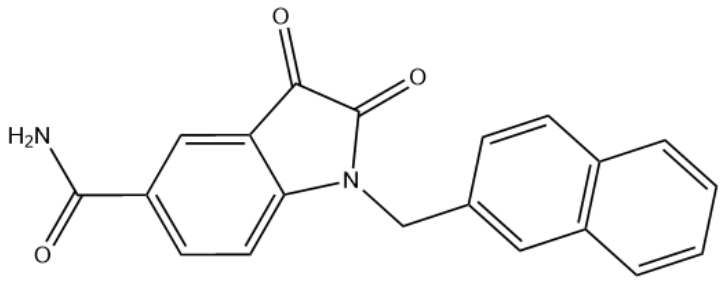

Figure 2.7: Structure of Inhibitor

2.2 Detecting Half-maximal Inhibitory Concentration

2.2.1 Principles

The structure of inhibitor is shown in Figure 2.7. It will repress the reaction catalyzed by 3CL protease probably by competitive binding to the enzyme. And we will determine the **Half-maximal Inhibitory Concentration,** IC_{50} through speed of reaction.

2.2.2 Methods

The inhibitor is consist of competitive inhibitor, uncompetitive inhibitor and mixed inhibitor. Instead of changing substrate concentration simultaneously, we varied inhibitor concentration [I] only and keep [S] under control. Also, we focused on **Half-maximal Inhibitory Concentration** (IC_{50}) only, without concerns about modified factors to V_{max} or K_m.

The following procedure was used to prepare to inhibitor solution with different concentration:

- sample 20 μL concentrated inhibitor(20 mM) solution with 180 μL DMSO as additional solvent. Then mix in 1^{st} well of microplate, which has the concentration of 200 μM.
- prepare 30 μL DMSO in each well of 1^{st} raw.
- take 30 μL solution from 1^{st} to 2^{nd} and mix in 2^{nd} well.
- repeat the step for the following wells to perform two fold serial dilution as shown in Figure 2.1.

To get the solution of substrate, we decanted 1.92 mL DMSO into 2 mg fluorescent substrate then separated it into twelve different wells in the same row. As shown in Figure 2.2, the substrate and inhibitor were in different rows. To be noted that, in reality, they were also stored in separated microplates to avoid unwanted illumination of fluorescent substrate.

2.2.3 Data Analysis

The reaction speed was also detected by **ELISA**. Twelve subfigures are shown in Figure 2.8 and each of them corresponds to a different inhibitor concentration [I]. There are two intriguing phenomena in the figure. Firstly, one group in [I]=10 μM and one group in [I]=1.25 μL exist extreme fluctuations which may caused by bubbles in solution. Secondly, the reaction speed has negative value in high concentration of inhibitor. The major cause of that absurd phenomenon is probably the unsteady state with fluctuation at early initial period. To eliminate the negative value and disturbance of it, I also decided to omit the early four points, which should reasonable and appropriate.

I performed linear regression again to each group and average the parallel groups to get the mean speed of reaction. Then the plot of mean speed $\bar{V_0}$ versus inhibitor concentration was drawn. From Figure 2.9, we can identify the **Half-maximal Inhibitory Concentration** IC_{50}, which is $IC_{50} = 0.095\ \mu M$.

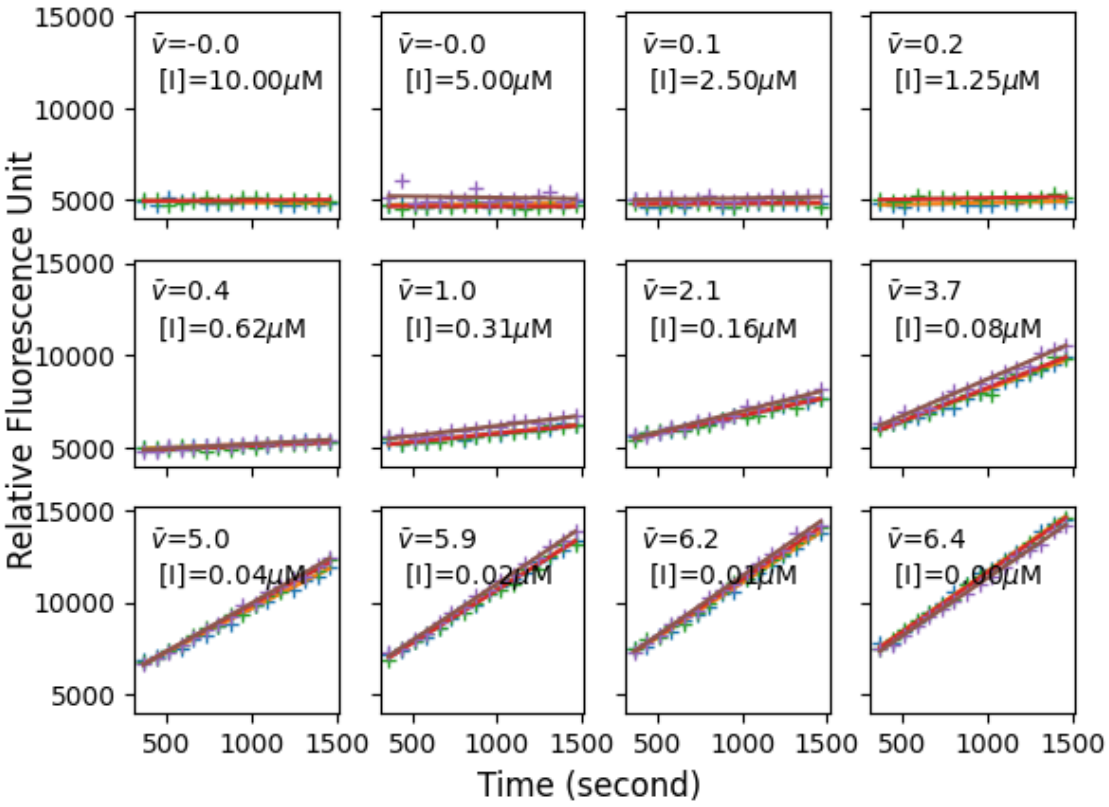

Figure 2.8: Reaction Rate versus Inhibitor Concentration: 12 groups of experiments, each has 3 parallel groups

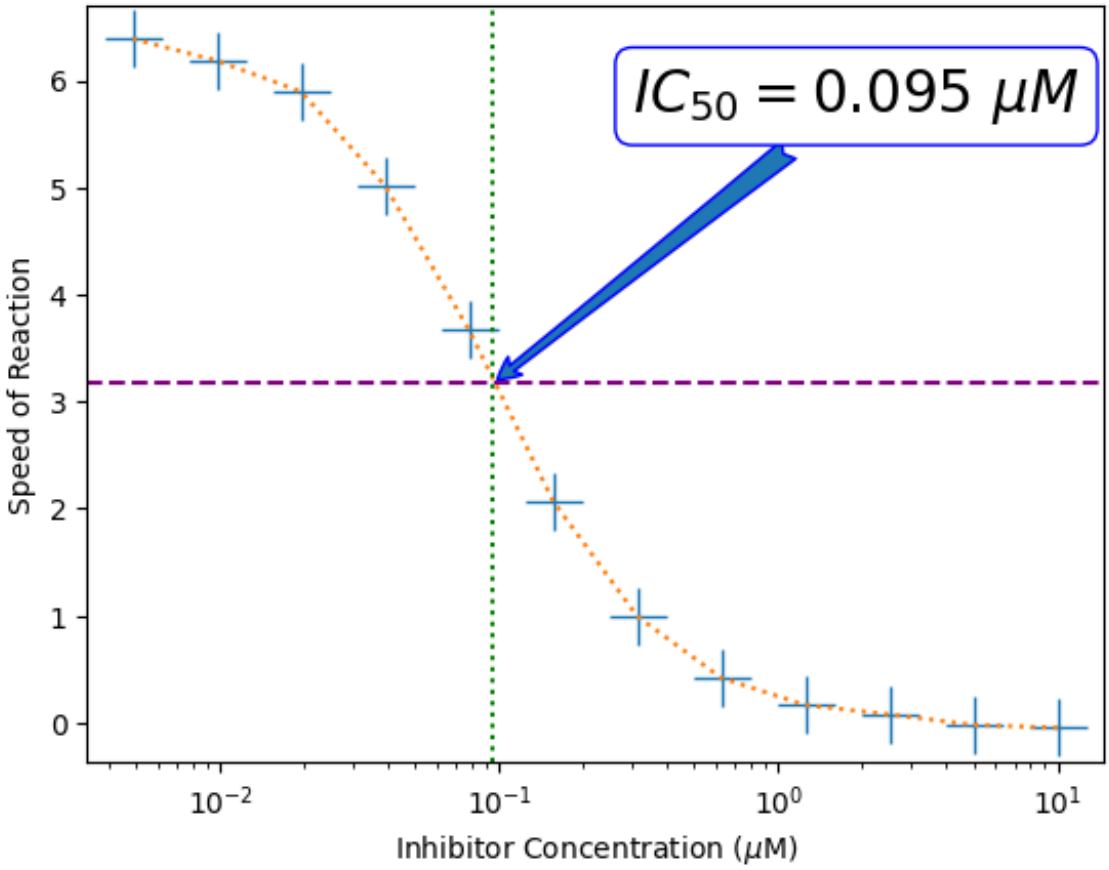

Figure 2.9: Reaction Rate versus Inhibitor Concentration: determining IC50

Bibliography

[1] Edans. https://en.wikipedia.org/wiki/EDANS.

[2] Homogenization. https://en.wikipedia.org/wiki/Homogenization_(chemistry)#cite_note-1.

[3] Quencher. https://en.wikipedia.org/wiki/Dark_quencher.