3CL SARS-CoV-2 3CL Protease Analyzing

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Chapter 1

Protein Purification

1.1 *E.coli* Lysis: Homogenizer

1.1.1 Materials

- Buffer Lysis
  - PBS (Phosphate Buffered Saline: HPO_4^{2-} / $H_2PO_4^-$): 20 mM (pH=7.3)
  - NaCl: 500 mM
  - Imidazole: 10 mM
  - DTT (Dithiothreitol: Reducing Agent): 1 mM
  - PMSF (Phenylmethanesulfonyl Fluoride: Serine Protease Inhibitor): 1 mM

1.1.2 Experimental Procedure

- 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 Materials

- Buffer A
  - PBS (Phosphate Buffered Saline: HPO_4^{2-} / $H_2PO_4^-$): 20 mM (pH=7.3)
  - NaCl: 500 mM
  - Imidazole: **10 mM**
- Buffer B
  - PBS (Phosphate Buffered Saline: HPO_4^{2-} / $H_2PO_4^-$): 20 mM (pH=7.3)
  - NaCl: 500 mM
  - Imidazole: **500 mM**
- Buffer C
  - PBS (Phosphate Buffered Saline: HPO_4^{2-} / $H_2PO_4^-$): 40 mM (pH=7.3)
  - NaCl: 100 mM
  - EDTA: 1 mM

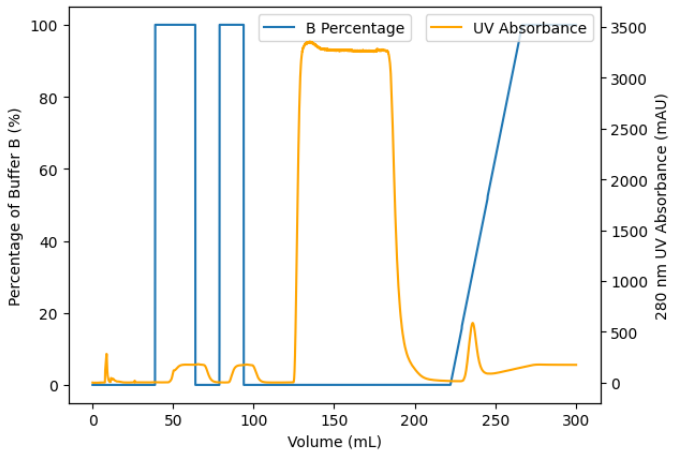

Figure 1.1: Affinity Chromatography: UV Absorbance with Percentage of Buffer B

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

Experimental Procedure

We purified 3CL protease fist by affinity chromatography. Immobilized Ni^{2+} ion had been attached to the column while His-tag (six histidine in the terminal of protein) had been fused with 3CL protease.

The Ni^{2+} column has the capacity of 5 mL. We washed the column before loading using buffer A and buffer B in turn (A(25 mL)-B(25 mL)-A(15 mL)-B(15 mL)-A(15 mL)) as shown in the blue curve in Figure 1.1 and Figure 1.2. We set pressure difference ($\Delta P = 0.3$ MPa) to be the control threshold with the pre-column pressure ($P_{pre} = 0.5$ MPa). The flow rate was set to be 1 mL/min for above operation.

Then we loaded 55 mL sample after lysis into the column with the flow velocity to be 2 mL/min. After that, buffer A was constantly pumped into to column to elute impurities until the signal of UV absorbance went back near 0 mAU. Once the eluting was complete, we started to raise the proportion of buffer B linearly to form a linear gradient. The threshold to collect product was set to be 20 mAU. The fraction collecting was performed automatically with the amount of 1.8 mL/tube. Finally, we collected tubes from number 3 to number 8, totally $6 \times 1.8 = 10.8$ mL.

Condensation with centrifuging filtering and solvent changing was used condense 10.8 mL solution to nearly 1.5 mL. The centrifuging was in 4°C with the rotating speed of 3,500 rpm for 10 minutes. In each turn, we decanted 5 mL solvent for the Gel-filtration Column to replace the buffer for Ni^{2+} column containing lots of imidazole.

Results

The tracking of UV absorbance changing and conductance changing with varied buffer B proportion can be seen in Figure 1.1 and Figure 1.2. The major peak in the middle of the Figure 1.1 corresponds to the impurities (protein that has UV absorbance companied by nucleic acid). Meanwhile the conductance drops down (Figure 1.2) due the poor conductivity of protein compared with inorganic salts.

From the Fourth Column in Figure 1.5 we can clearly see the effective purification produced by affinity chromatography compared with the lysis mixture in second and third column.

1.2.3 Gel-filtration Chromatography

Experimental Procedure

The Gel-filtration Column has the capacity of 24 mL. We set pressure difference ($\Delta P = 1.8$ MPa) to be the control threshold with the pre-column pressure ($P_{pre} = 5.0$ MPa). Careful washing of the column and sample ring was performed. As for the sample ring, 4 mL ultra-pure water with 4 mL buffer assay were used to expel the air. The two direction of washing(up to down and vice versa) with careful injector operation was included to ensure any bubble would be excluded from the column.

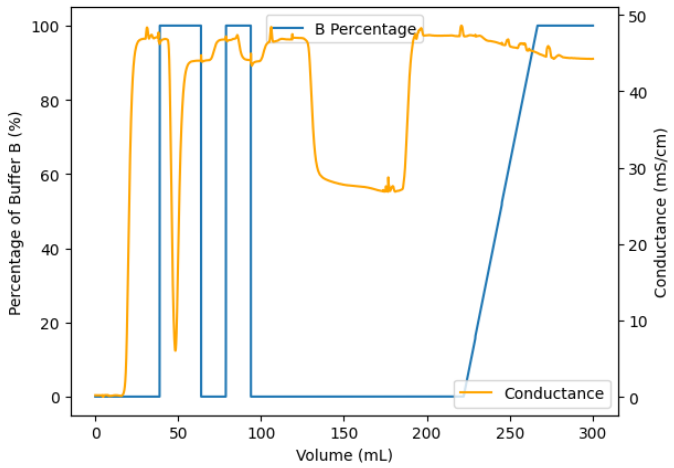

Figure 1.2: Affinity Chromatography: Conductance with Percentage of Buffer B

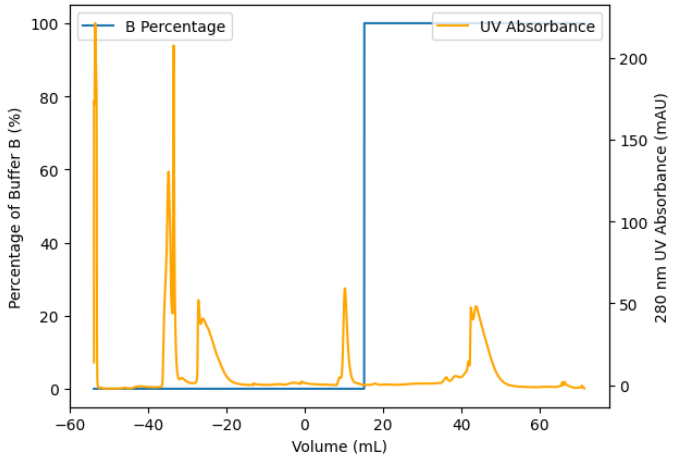

Figure 1.3: Gel-filtration Chromatography: UV absorbance with Percentage of Buffer B

The loading amount should be 500 μL with the concentration of 1 mg/mL. To ensure the sample concentration to be around 1 mg/mL, we detected the concentration of the sample by nano drop. The result was 6.737 Abs equivalent to $6.737 \times 1.07 = 7.21$ mg/mL. That means around 70 μL concentrated protease was needed with 430 μL buffer assay. However we enlarged the system by two fold which means diluting 140 μL concentrated protease with 860 μL buffer assay in case of failure.

We collected according to the UV absorbance as shown in Figure 1.4 and Figure 1.3. Then condensed the sample as previous process in affinity chromatography.

$$A = \epsilon c l \tag{1.1}$$

$$A = 6.737\ Abs \tag{1.2}$$

$$\epsilon = 0.937\ \mathrm{L/g \cdot cm} \tag{1.3}$$

$$l = 1\ \mathrm{cm} \tag{1.4}$$

$$c \approx 7.21\ \mathrm{mg/mL} = \frac{7.21\ \mathrm{g/L}}{35 \times 10^3 \mathrm{g/mol}} = 205.4\ \mu\mathrm{M} \tag{1.5}$$

Results

The effect can be seen in Figure 1.5. Compared with the fourth column, the last column after gel-filtration chromatography does not show additional purification. Probably, the solution had been purified to an extreme extend after affinity chromatography, which leads to the unobvious improvement.

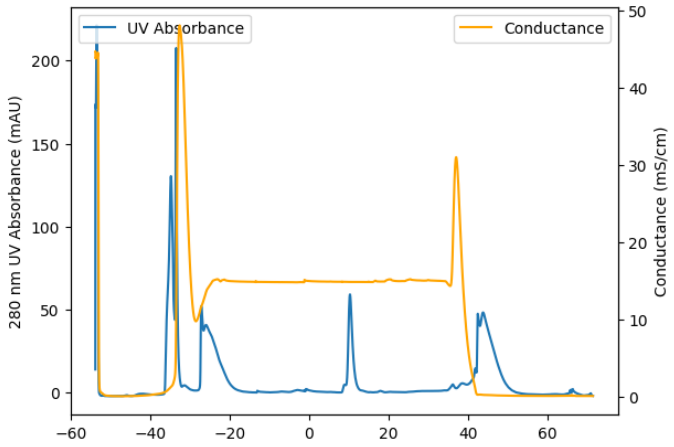

Figure 1.4: Gel-filtration Chromatography: UV absorbance with Conductance

1.3 Analyzing: SDS-PAGE

1.3.1 Experimental Procedure

In this experiment, we used precast polyacrylamide gel. However, we still need to prepare the buffer(500 mL) for electrophoresis by hands.

Four type of samples were prepared for SDS-PAGE to check the effect of protein purification.

- No.1 *E.coli* lysate
- No.2 Supernate of lysate after centrifuging
- No.3 Sample after affinity chromatography
- No.4 Sample after gel-filtration chromatography

In each well, we put 20 μL sample with 5 μL loading buffer except for No.3 which only left 8 μL. To control the concentration in consistency, only 2 μL loading buffer was added to No.3.

We mixed the solution by **Vortex Genie 2** and centrifuge for instant. Then heated at 94 °C for 3 minutes. Finally, loaded marker (2 μL) as well as the four types of sample (10 μL) into the polyacrylamide gel in sequence.

The electrophoresis apparatus was set at 150 V working for 38 minutes.

After electrophoresis, the gel was stained using Coomassie Brilliant Blue with heating in microwave oven for three times. In each turn, the paint was heated until boiling. H_2O plus acetic acid (3%) was used to destain with the similar procedure in microwave oven.

1.3.2 Results

As shown in Figure 1.5, the effect of purification is obvious. The most effective method should be affinity chromatography while other steps are also playing an important role in the whole process. Besides, we can also figure out the molecular weight of 3CL protease which is near 35 kDa.

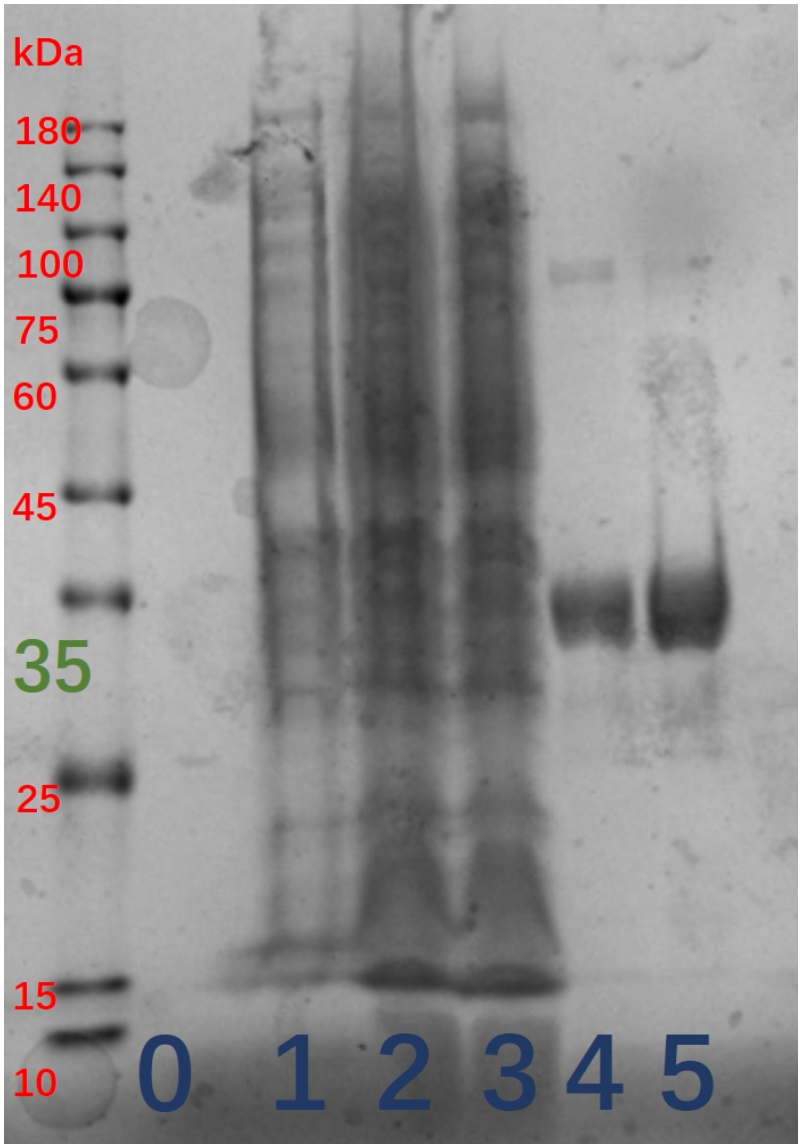

Figure 1.5: Results of SDS-PAGE: The Second Column is the solution after lysis; The Third Column is from supernate; The Fourth Column is after affinity chromatography; The Last One is after gel-filtration chromatography. The First Column has the same composition as the second one just with additional loading buffer. The leftmost one is marker.

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 Introduction

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 Experimental Procedure

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

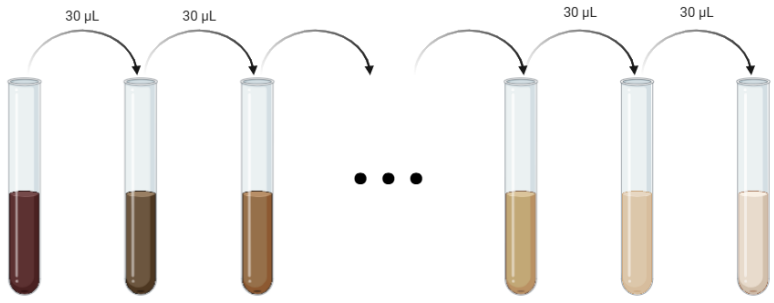

Figure 2.1: Serial Dilution of Inhibitor

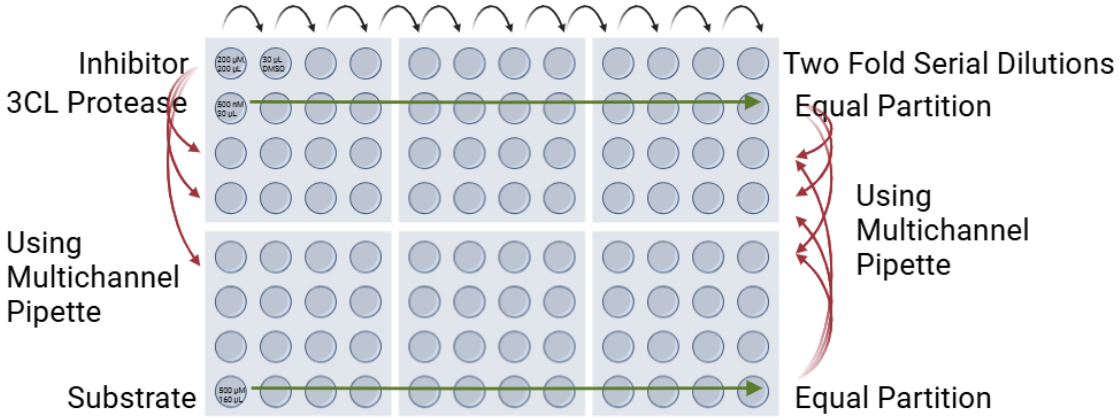

Figure 2.2: Solution Configuration in Microplate

Table 2.1: Substrate 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

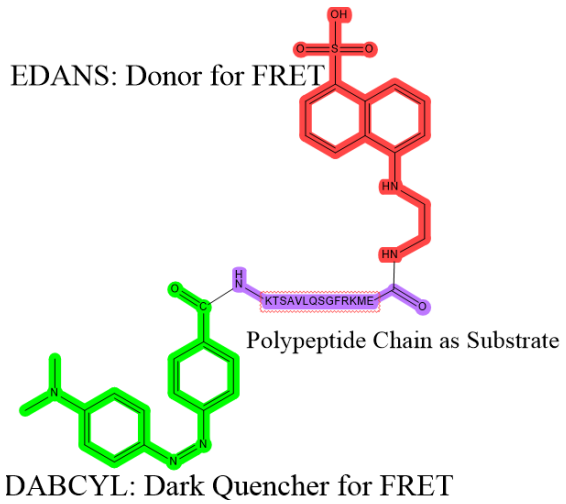

Figure 2.3: Structure of Fluorescent Substrate

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

The Composition of the System (100 μL):

- Buffer Assay (80 μL, 80%)
  - Na_2HPO_4 / NaH_2PO_4 (40 mM)
  - NaCl (100mM)
  - EDTA (1mM)
  - Triton X-100 (0.1%)
- SARS-CoV2-3CL Protease (10 μL, 10%) (before mixing: 500 nM, after mixing: 50 nM)
- Fluorescent Substrate (10 μL, 10%) (before mixing the different concentrations can be seen in Table 2.1).
- Temperature: 25°C

3CL protease was preserved in fridge at -80°C, which was then melted in ice box. The initial concentration of 3CL protease was 206 μM, and then we took 2 μL diluting it with 2 mL Buffer Assay to get 500 nM protease solution. The buffer assay was prepared by adding 50 μL Triton X-100 into 50 mL buffer solution at the very beginning.

Both buffer assay and 3CL protease were partitioned equally into eight different wells in the same microplate, and were mixed using multichannel pipette in other three rows. Fluorescent substrate was stored in another microplate while was prepared according to Table 2.1. Also, multichannel pipette was used to transfer substrate into the system.

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.

$$V_{max} = 20 \text{ RFU/second}$$

$$K_m = 28.99 \ \mu\text{M}$$

2.2 Detecting Half-maximal Inhibitory Concentration

2.2.1 Introduction

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.

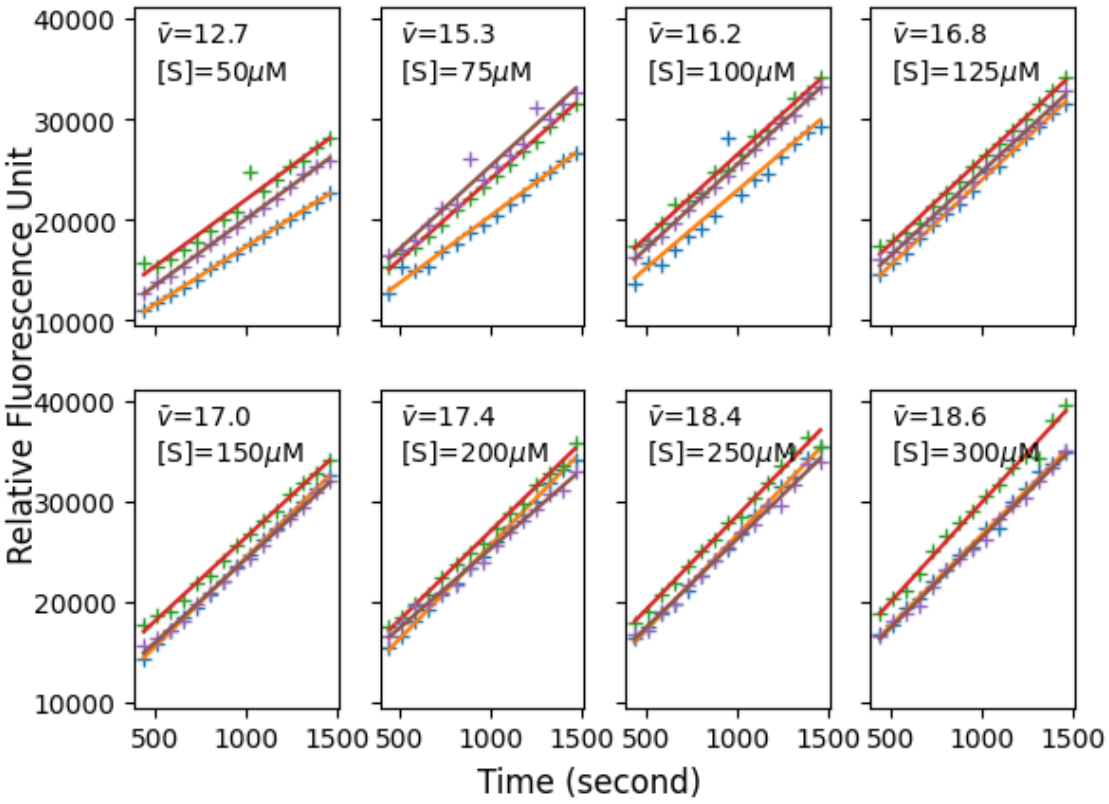

Figure 2.4: Reaction Rate versus Substrate Concentration: 8 groups of experiments, each has 3 parallel groups

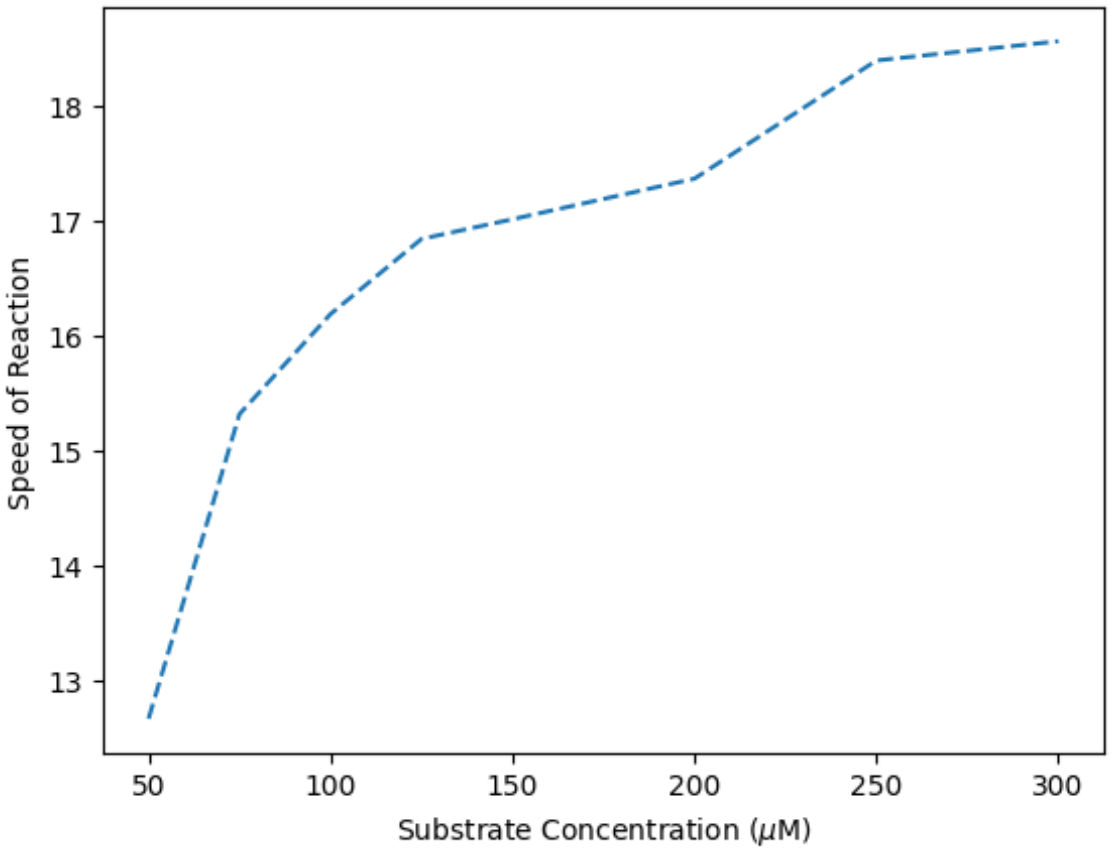

Figure 2.5: Reaction Rate versus Substrate Concentration: in normal axes

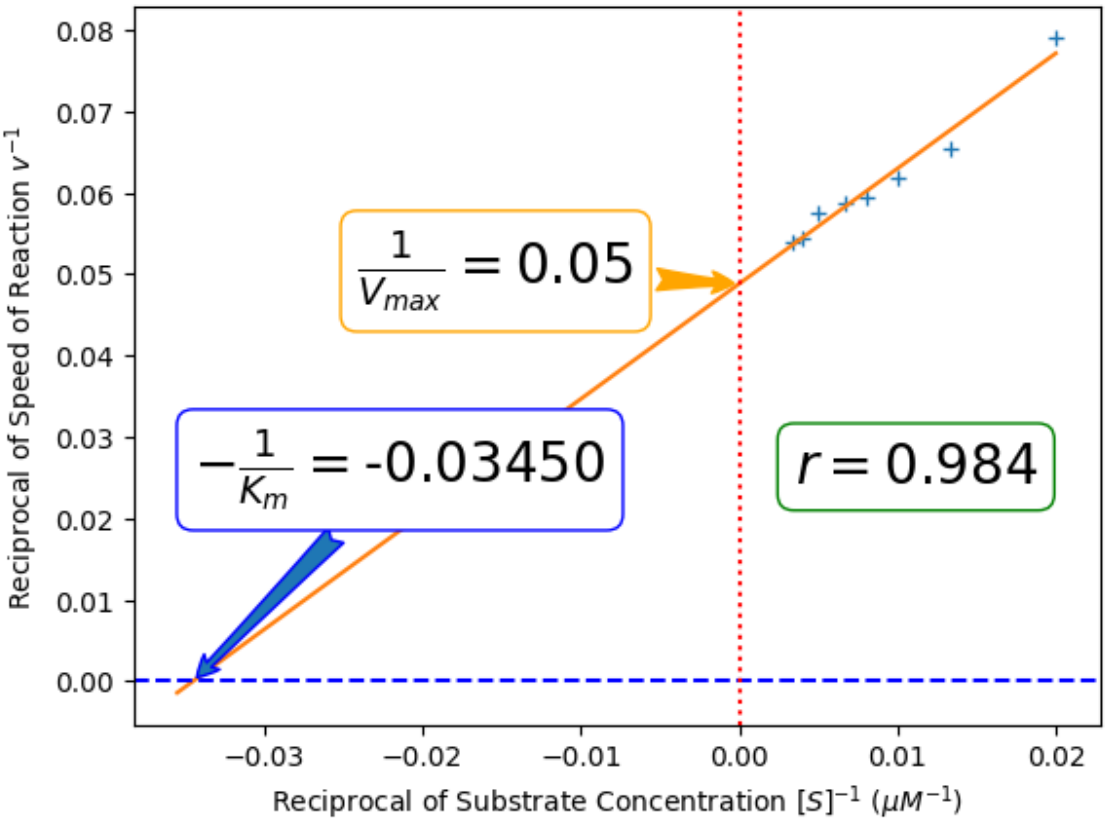

Figure 2.6: Reaction Rate versus Substrate Concentration: in reciprocal axes

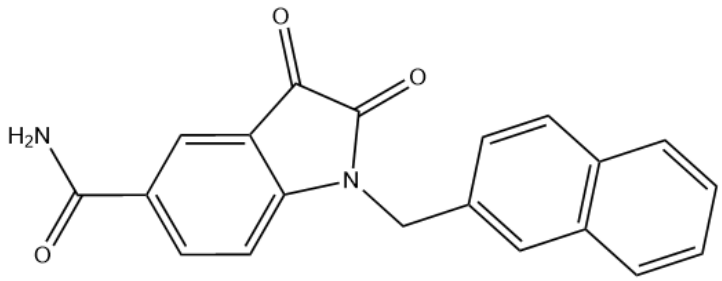

Figure 2.7: Structure of Inhibitor

2.2.2 Experimental Procedure

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.

The Composition of the System (100 μL):

- Buffer Assay (80 μL, 80%)
  - Na_2HPO_4 / NaH_2PO_4 (40 mM)
  - NaCl (100mM)
  - EDTA (1mM)
  - Triton X-100 (0.1%)
- SARS-CoV2-3CL Protease (10 μL, 10%) (before mixing: 500 nM, after mixing: 50 nM)
- Fluorescent Substrate (5 μL, 5%) (before mixing: 500 μM, after mixing: 25 μM)
- Inhibitor (5 μL, 5%) (after mixing the serial concentration can be seen in Figure 2.8).
- Temperature: 25°C

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 $IC_{50} = 0.095\ \mu M$. Somehow, the value is higher than the reported value $IC_{50} = 0.095\ \mu M$.

$$IC_{50} = 0.095\ \mu\text{M}$$

Besides, we can also figure out the inhibitory rate with different inhibitory concentration [I]. The intuitive recognition can be acquired in Figure 2.9 while the precise values are shown in Table 2.2.

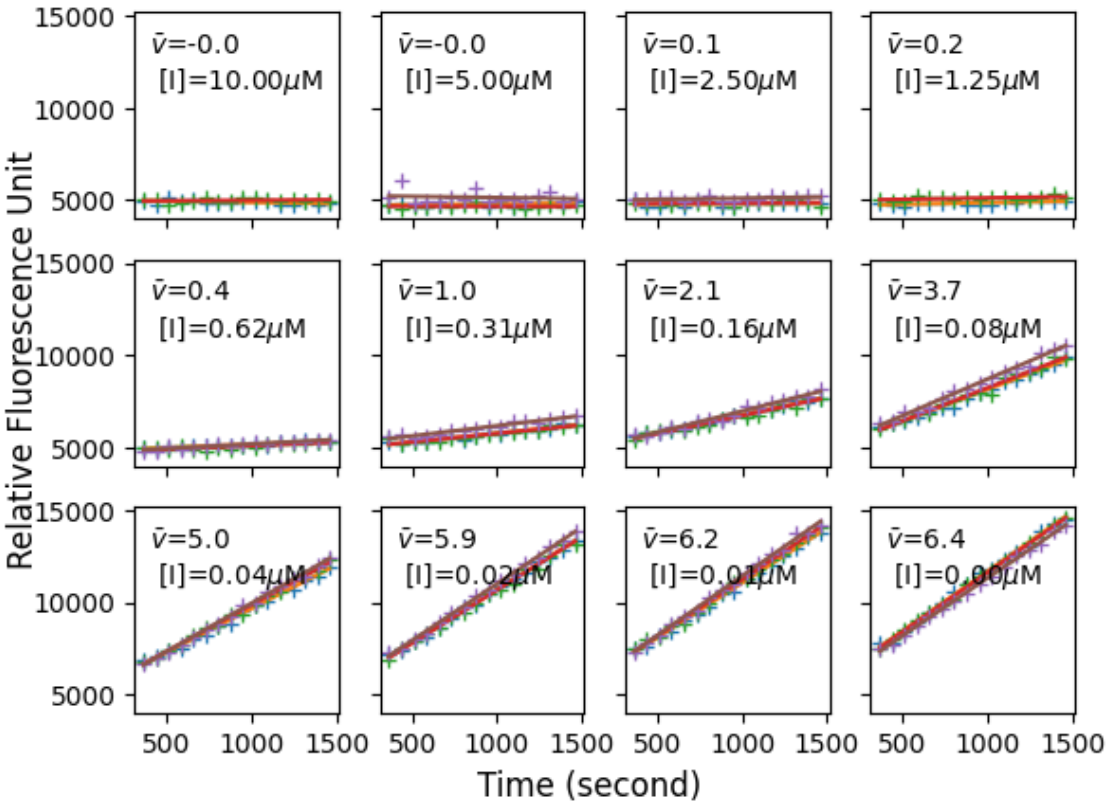

Figure 2.8: Reaction Rate versus Inhibitor Concentration: 12 groups of experiments, each has 3 parallel groups

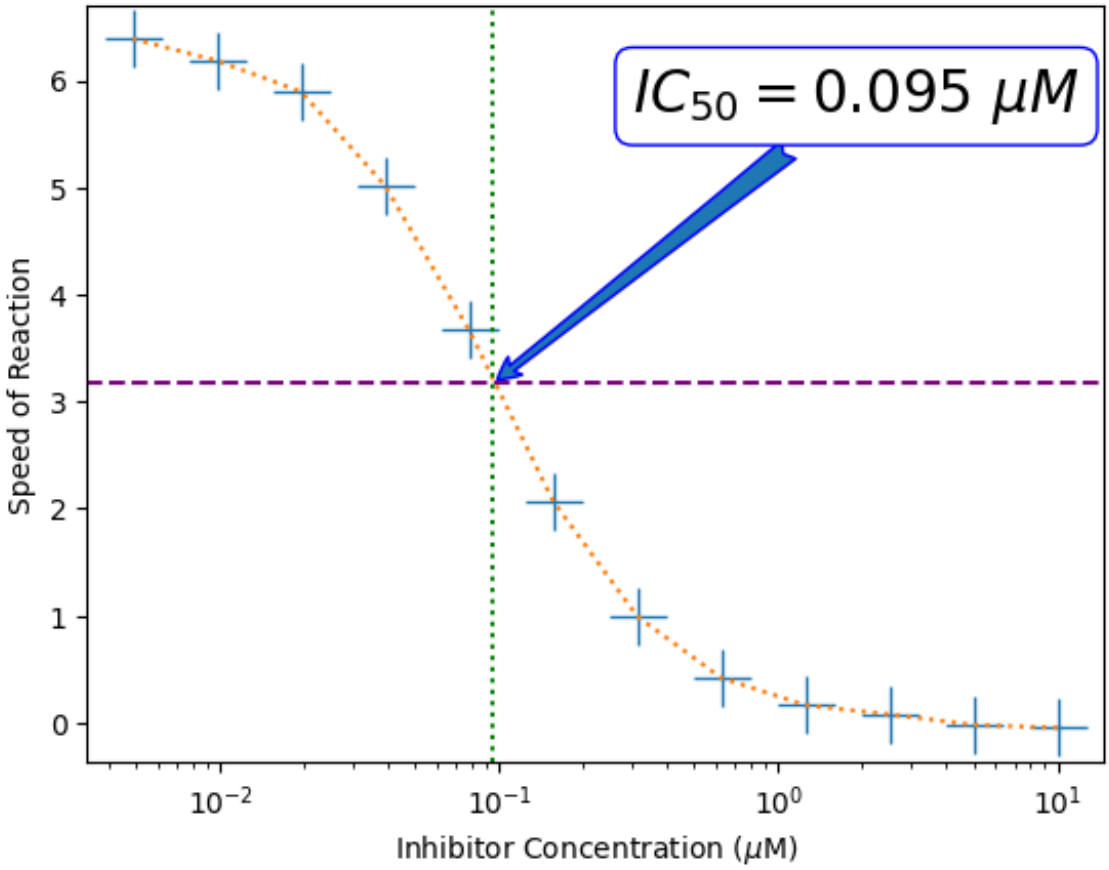

Figure 2.9: Reaction Rate versus Inhibitor Concentration: determining IC50

Table 2.2: Inhibitory Rate with Different Inhibitor Concentration

	Inhibitor Concentration (μM)	Inhibitory Rate
0	10.00	100.00 %
1	5.00	99.59 %
2	2.50	98.07 %
3	1.25	96.72 %
4	0.62	92.77 %
5	0.31	83.84 %
6	0.16	67.12 %
7	0.08	42.29 %
8	0.04	21.31 %
9	0.02	7.65 %
10	0.01	3.15 %
11	0.00	0.00 %

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