Optimization of Buffer Conditions for Flaviviral Protease Assays

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Abstract

Flaviviruses such as Dengue virus and Zika virus are prevalent in tropical countries and can infect and even kill millions. They are mainly spread through arthropods such as ticks and mosquitoes and can cause a host of symptoms such as fever, body aches, headache, and joint pain [6]. The need to find an effective virus inhibiting compound has continued to increase in recent years, as the number of those affected by flaviviruses also continues to increase steadily. This study attempts to find optimized buffer conditions to assay and compare the effectiveness of various antiviral compounds to inhibit the protease of flaviviruses, which play a role in viral reproduction. The research studied the rate at which the enzyme and substrate reacted with various buffers using a spectrofluorometer to track the rate of reactions. A variety of factors were tested such as buffer concentration, buffer base, NaCl concentration, and glycerol concentration. Optimized buffer conditions for the enzyme-substrate assay were found to be 10 mM Tris, without NaCl with 30% glycerol at pH 9.5. This optimization can be used to assay a variety of protease inhibition compounds to compare their effectiveness. This work has applications in order to assay various antiviral compounds in the future to lessen the spread and virulence of flaviviruses globally.

Introduction

Flavivirus is a genus of viruses in the family Flaviviridae. This genus includes the West Nile virus, dengue virus, tick-borne encephalitis virus, yellow fever virus, Zika virus, and several other viruses. Flaviviruses are more prevalant in tropical countries, such as Southeast Asia, South America, and Africa. In conjunction with the growth of population, flaviviruses are a growing problem in these countries. Symptoms of flaviviruses include fever, body aches, headache, and joint pain, and can sometimes even prove to be fatal. Inhibiting the viral protease, which plays a role in viral replication can limit the transmission and growth of the virus (Ali, 2017). Past research has assayed compounds that can possibly inhibit the flavivirus protease with suboptimal buffer conditions (Padmanabhan, 2009). Past studies have also attempted to study the effects of specific components of buffers such as detergents (Steuer, 2009) to study their specific effect on enzyme-substrate rates. Our research project optimizes buffer conditions to assay these compounds, using spectrofluorometers to analyze the quickest enzyme-substrate reaction rate (Antoniou, 2017). The optimized buffer conditions can result in finding a wider variety of compounds to inhibit Flavivirus protease (Padmanabhan, 2007). Though this research used Dengue protease, the findings can also be applied to the protease of other flaviviruses. This research assayed multiple buffers at different conditions with enzyme and substrate to optimize viral protease activity by studying enzyme-substrate reaction rates. Studying the inhibition of protease activity can lower the virulence of Flaviviruses across the world, as we can find antiviral compounds to prevent the spread of the disease. In addition, the development of these antiviral compounds can be developed at lower costs to widely distribute the compounds to the tropical countries they frequently affect.

Materials and Methods

Kinetics

In order to determine whether the enzyme follows Michaelis-Menten kinetics or if the enzyme deviates from these kinetics, the enzyme and substrate were assayed initially. The enzyme, QRPro, and substrate: (Bz)-Nle-Lys-Arg-arg-AMC were assayed prior to assaying the chemical compounds (Figure 1). The original protease concentration was 8.8 µM and the original substrate concentration was 50 mM.

The following calculation was used to ensure that a 30.8 nM enzyme concentration will be used: $8.8\ \mu M * 7\ \mu l = X * 2 * 1000\ \mu l \rightarrow X = 0.0308\ \mu M = 30.8\ nM$

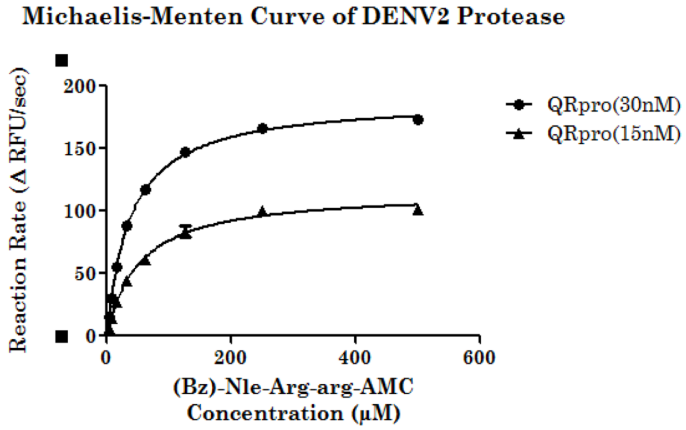

Figure 1.Enzyme& Substrate Assay to confirm enzyme followed Michaelis Menten Kinetics

Since the reaction volume is split half-and-half between the enzyme and the substrate, the concentration was 15 nM for 50 µl of substrate and 50 µl of enzyme. Since the original substrate concentration was very high, the substrate was serially diluted from 500 µM to 3.90 µM. The final reaction volume was 100 µl. Measurements were carried out with a 96 well microtiter plate spectrofluorometer: excitation 355 nm and emission 460 nm. The reactions were performed at 37°C and readings were taken every 90 seconds for 30 minutes.

30nM concentration was tested, which was confirmed as optimal by previous results using the same enzyme and substrate tested at different conditions.

Protease Assay - Compound Inhibition

In this test, 10 different chemical compounds were assayed at two different concentrations (25 μM, 10 μM) and performing tests of each compound at each concentration were performed twice.

The compounds were serially diluted with DMSO in order to reach the desired 25 μM and 10 μM concentrations and were incubated at room temperature before the addition of substrate.

The protease was diluted to a final concentration of 30 nM using 10 mM Tris PH 8.5, 20% glycerol, 1 mM chaps. 50 μl of the enzyme was added to each of the wells. 1 μl of the diluted compound was added to the wells, and 50 μl substrate was added to the wells last to ensure that the enzyme and substrate did not begin reacting preliminarily.

Two negative controls were tested at each concentration, one with just 1 μL of DMSO and another with just the enzyme and substrate. Tolcapone (tested in wells A3, A4, D3, D4) was used as the positive control and is a well-established enzyme inhibitor.

The microtiter plate was laid out as depicted in Table 1 below:

Table 1. Arrangement of compounds assayed: The first three rows (colored blue) are the compounds tested at the 25 μM concentration. The next three rows (colored orange) are the compounds tested at the 10 μM concentration. Readings were taken from a microtiter plate spectrofluorometer reader every 90 seconds for 30 minutes.

	1	2	3	4	5	6	7	8	9	10	11	12

A	A17	A17	Tolc.	Tolc.	C1	C1	C5	C5				
B	C4	C4	C3	C3	D5	D5	D2	D2				
C	D6	D6	C6	C6	1 µl (DMSO)	1 µl (DMSO)	-	-				
D	A17	A17	Tolc.	Tolc.	C1	C1	C5	C5				
E	C4	C4	C3	C3	D5	D5	D2	D2				
F	D6	D6	C6	C6	1 µl (DMSO)	1 µl (DMSO)	-	-				
G												
H												

Table 1. Arrangement of compounds assayed: The first three rows (colored blue) are the compounds tested at the 25 µM concentration. The next three rows (colored orange) are the compounds tested at the 10 µM concentration. Readings were taken from a microtiter plate spectrofluorometer reader every 90 seconds for 30 minutes.

Buffer Preparation

New buffers were made so different conditions could be tested in order to isolate the different factors. With these buffers, the effect of NaCl, different glycerol conditions, different buffer bases, various detergents, and different buffer concentrations were compared for optimum enzyme-substrate activity.

Michaelis–Menten Assay (Using different buffers)

In these assays, 3 µl of substrate was diluted into 300 µl of the specified buffer and a serial dilution was performed to dilute from a concentration of 500 µM down to 250 µM, 125 µM, 62.5

µM, 31.25 µM, 15.63 µM, 7. 81 µM, 3.91 µM, and 1.95 µM. 5 µl of the enzyme was diluted into 1.5 ml of the specified buffer. All of the assays were conducted with a final reaction volume of 100 µl. Measurements were carried out with a 96 well microtiter plate reader: excitation 355 nm and emission 460 nm. The reactions were performed at 37°C and readings were taken every 90 seconds for 30 minutes.

For the first test, two new buffers (10 mM Tris pH 9.5 and 200 mM Tris pH 8.5) were made and 6 mM of NaCl was added to a previously made solution of 200 mM Tris pH 9.5. All three of these buffers had a 30% glycerol content and 0.1% CHAPS.

Results

Enzyme activity was analyzed in Relative Fluorescence Units per minute (RFU/min) through a viral protease assay using a spectrofluorometer. Compounds were initially assayed using the same spectrofluorometer machine, but the percent inhibition was calculated using a negative control of a DMSO only well. The positive control was Tolcapone.

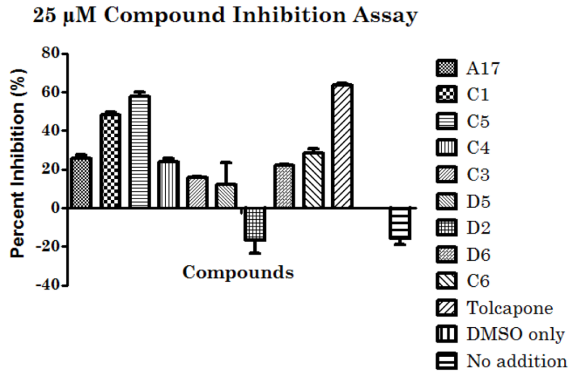

Figure 2: Class A,C, and D protease inhibiting compounds were assayed with 10 mM Tris pH 8.5, 20% glycerol, 1 mM chaps buffer to standardize a control before optimization. Compounds are assayed at 25 µM and were diluted with DMSO.

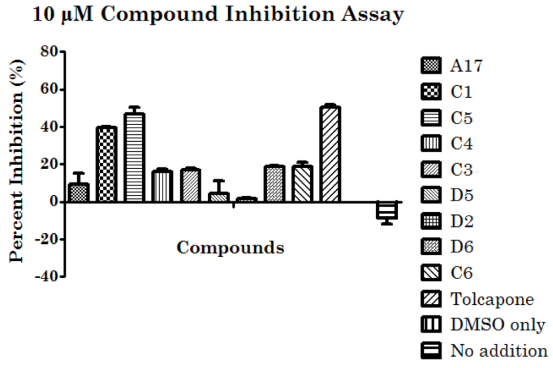

Figure 3: Class A, C, and D compounds were assayed at the same conditions as figure 2, with the exception of Compounds being assayed at a concentration of 10 µM.

Antiviral Compounds of class A, C, and D (Figure 2 and 3) were initially assayed at arbitrary conditions of 10 mM Tris pH 8.5, 20% glycerol, 1 mM chaps before optimization at 2 different concentrations. The graphs show similar patterns of activity with 25μM showing slightly higher activity. (Figure 2 and 3)

Assays were first optimized with different buffers: Tris concentrations at 10 mM and 200 mM Tris, pH 8.5, 20% glycerol, and 1mM chaps.

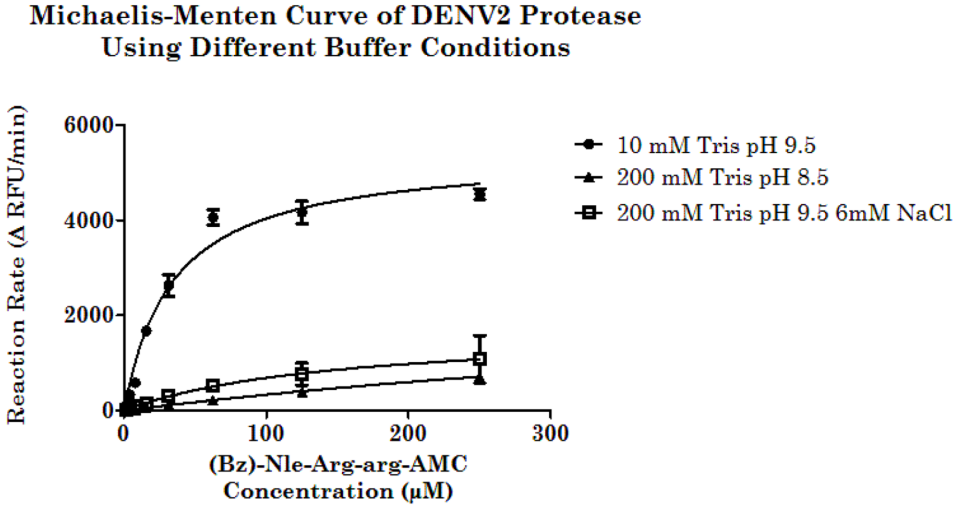

Figure 4: Buffers with different concentrations of Tris were assayed in order to determine the Tris buffer concentration with the fastest enzyme-substrate reaction rate.

At 10 mM Tris pH 9.5: Vmax: 5411 ± 293.4 Km: 34.08 ± 5.643 r^2: 0.9672
At 200 mM Tris pH 8.5: Vmax: 2904 ± 574.5 Km: 769.5 ± 191.1 r^2: 0.9943
At 200 mM Tris pH 9.5 + 6 mM NaCl: Vmax: 1716 ± 616.9 Km: 150.0 ± 105.3 r^2: 0.7769

The graph shows the quickest enzyme-substrate reaction rate to result when 10 mM Tris pH 9.5 was used. (Figure 4)

Assays were then optimized with the presence of NaCl, at 200 mM Tris and pH 9.5.

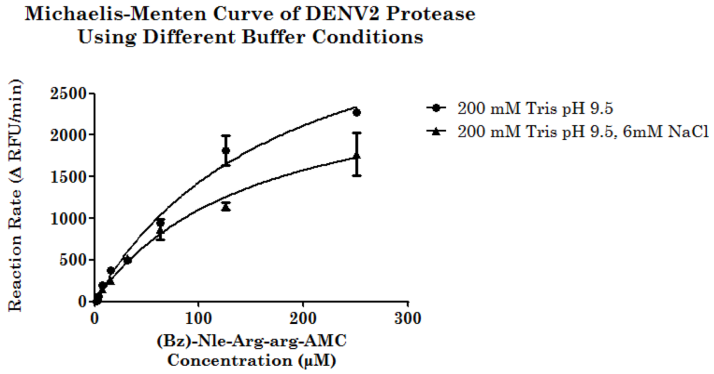

Figure 5: Two buffers were assayed with and without NaCl to determine the effect of NaCl on the enzyme-substrate reaction rate

Without NaCl: Vmax: 4016 ± 397.7 Km: 179.6 ± 32.79 r^2: 0.9829
With NaCl: Vmax: 2766 ± 354.2 Km: 150.4 ± 37.59 r^2: 0.9629

The absence of NaCl produces faster enzyme-substrate activity than the presence of NaCl. (Figure 5).

Varying Percentages of Glycerol percentages were then assayed and reaction rates were observed

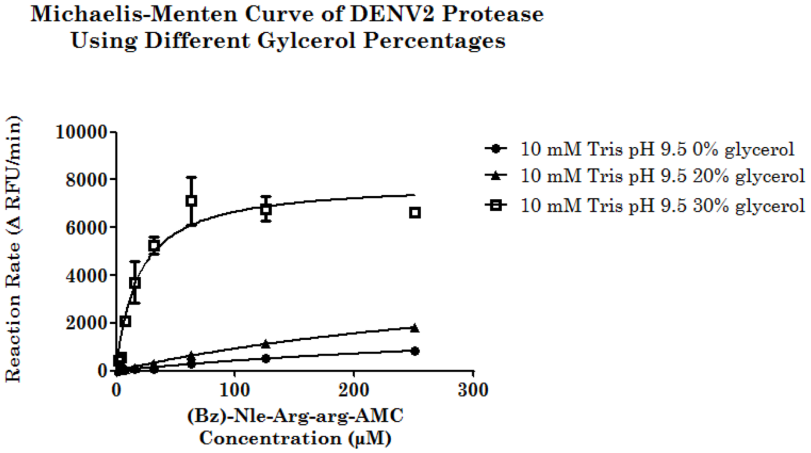

Figure 6: Buffers of 3 different glycerol concentrations were assayed to study the optimum glycerol concentration for fastest reaction rate. All buffers had 1 mM chaps.

At 0% Glycerol: Vmax: 2477 ± 647.8 Km: 490.7 ± 177.2 r^2: 0.9781
At 20% Glycerol: Vmax: 4965 ± 648.7 Km: 422.0 ± 80.66 r^2: 0.9925
At 30% Glycerol: Vmax: 1716 ± 616.9 Km: 150.0 ± 105.3 r^2: 0.9178

30% glycerol had the fastest enzyme-substrate activity by a significant margin. Meanwhile 0% and 20% glycerol had similar reaction rates with 20% glycerol being slightly quicker. (Figure 6)

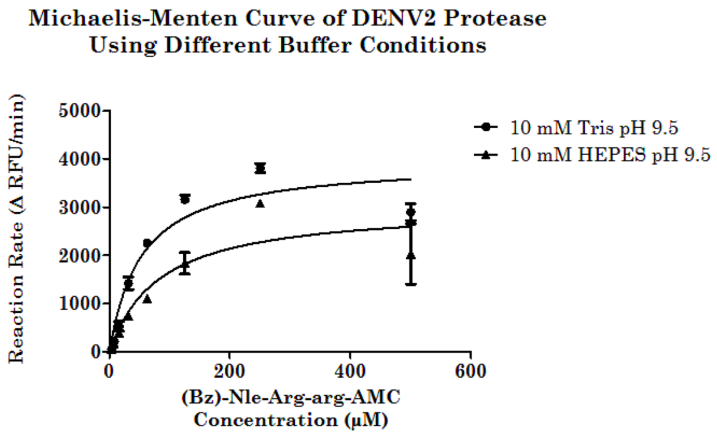

Figure 7: Two different buffers (Tris and Hepes) were assayed to compare various buffers' effects on reaction rate at 30% glycerol and 1mM chaps.

At 0% Glycerol and 8% EG: Vmax: 2477 ± 647.8	**Km: 490.7 ± 177.2**	**r^2: 0.9781**
At 20% Glycerol and 8%EG: Vmax: 4965 ± 648.7	**Km: 422.0 ± 80.66**	**r^2: 0.9925**
At 30% Glycerol and 8% EG: Vmax: 1716 ± 616.9	**Km: 150.0 ± 105.3**	**r^2: 0.9178**

An alternate buffer to Tris called Hepes at pH 9.5 was assayed in comparison to Tris. Tris produced higher enzyme activity than Hepes buffer.

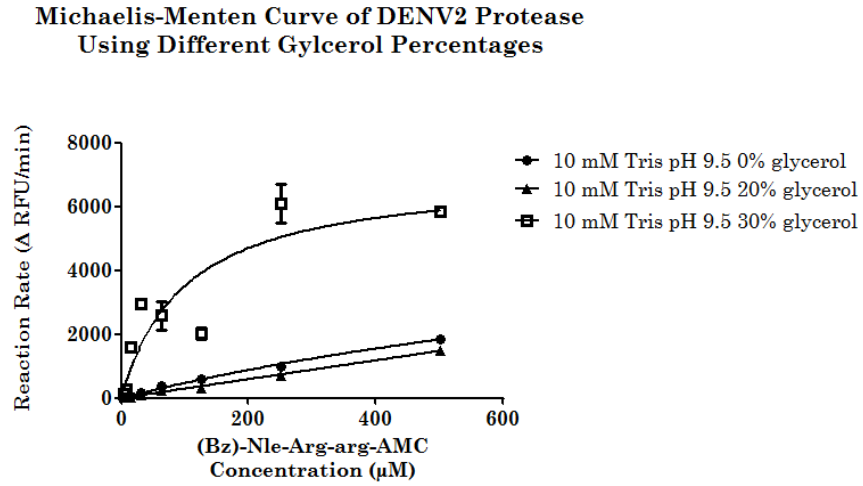

Figure 8: Tris buffers at varying glycerol concentrations were mixed with 8% ethylene glycol to determine the buffer with the fastest reaction rate using a combination of Ethylene glycol and Glycerol. 8% Ethylene Glycol was previously determined as optimum.

8% Ethylene Glycol and Varying percentages of glycerol were assayed. 10 mM Tris pH 9.5, 30% glycerol and 8% Ethylene Glycol has the highest reaction rate. (Figure 8)

Discussion

The compounds were assayed before at assay conditions of 10 mM Tris pH 8.5, 20% glycerol, 1 mM chaps in previous research (Padmanabhan, 2009) tested. In this experiment, we assayed the

compounds at these conditions for comparison, and found significantly lower reaction rate of enzyme-substrate activity which likely resulted from the deterioration of the compounds despite storage at -80 degrees Celsius.

In this experiment, we measured reaction rate at different factors. Faster reaction rates corresponded with a greater enzyme-substrate activity. Previous research from the same paper found optimized pH to be 9.5 so buffer optimization began with this pH of Tris buffer. 10 mM Tris was found to have a significantly faster reaction rate of more than 3000 RFU/ min than 200 mM Tris, signaling that buffers at lower Tris concentrations reacted faster. 10mM Tris Buffer was used in all subsequent assays. NaCl optimization followed, in which the presence of 6mM NaCl slowed enzyme-substrate reaction rate by about 600 RFU/ min showing that NaCl may likely prevent the binding of the enzyme and substrate by increasing the space in the reaction for this bond. Glycerol was then optimized at 3 different percentages with 30% glycerol significantly faster enzyme-substrate reaction rate by more than 5000 RFU/min over both 0% and 20% glycerol. However, 30% glycerol was identified as a possible problem as the high viscosity of this buffer was not logical to use for high throughput screening which could prevent the use of the assay to test effective viral protease compounds. In order to combat this problem, a combination of 8% ethylene glycol (which was found to be optimum in a previous paper) and varying percentages of glycerol were tested.

However, the results were not able to be used as the ethylene glycol mixture resulted in a highly variable pH of the buffers that were difficult to measure. The last optimization assay involved comparing Tris and Hepes, which were two buffer bases. Tris had a slightly faster reaction rate of 1000 RFU/ min. Therefore, our optimized buffer conditions are 10 mM Tris, without NaCl with 30% glycerol at pH 9.5. These results can be used to assay and compare the efficiency of

various antiviral compounds to compare their power to inhibit viral protease and consequently viral reproduction.

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