Enzymatic activity in a high-throughput fluorimetric dengue virus protease assay for dengue virus serotypes

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Masters Dissertation

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Declaration

I hereby declare that this work is original and that all non-original contributions have been properly referenced with the identification of the source.

Miguel Macedo, 16th June 2023

“The only way to make sense of change is to

plunge into it, move with it, and join the dance.”

Alan Watts

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Resumo

O vírus dengue (DENV) é uma ameaça com impacto na saúde global, com a sua protease a desempenhar um papel crucial no ciclo da sua vida viral. Esta enzima é um alvo promissor para abordagens terapêuticas contra o DENV. Estabelecer ensaios robustos e replicáveis para avaliar a atividade enzimática contribui para essa missão.

Este estudo teve como objetivo estabelecer um procedimento para o design, expressão e purificação do complexo proteico de diferentes serotipos do vírus da dengue (DENVpro). As proteases foram depois utilizadas na otimização das condições do ensaio FRET, avaliando parâmetros como buffer e vários substratos. A cinética enzimática dos quatro serotipos de DENVpro foi caracterizada e o potencial inibitório de compostos inibidores selecionados foi testado.

Design, expressão e purificação dos serotipos-alvo foram conseguidas com sucesso, com todas as proteases a apresentarem atividade enzimática com as condições padrão previamente estabelecidas no grupo de trabalho. A avaliação dos parâmetros para garantir a melhor atividade catalítica de todas as estirpes de proteases foi realizada, sendo as condições utilizadas no grupo de trabalho adequadas para os outros serotipos. No entanto, podem ser feitas adaptações para otimizar a eficiência da avaliação, como o ajuste da relação protease/substrato para promover um aumento da atividade catalítica. O DENV1pro apresentou os níveis mais baixos de clivagem de substratos, o que levou à síntese de novos substratos. Contudo, os parâmetros estabelecidos permitiram a realização de ensaios de inibição de compostos previamente testados.

A ausência de ensaios estandardizados, devido à diversidade de condições de ensaio e de construções de protéases, constitui um desafio para o estabelecimento de condições ótimas na comunidade científica. Medidas destinadas a realçar a reprodutibilidade e a robustez de um ensaio otimizado e estabelecido aumentariam a coerência e o controlo de qualidade nos estudos sobre o DENV, contribuindo para o progresso das abordagens terapêuticas.

Abstract

Dengue virus (DENV) is an impactful global health threat with its protease playing a crucial role in the viral life cycle. Such enzyme is a promising target for therapeutic approaches against DENV. The establishment of robust and reproducible assays to assess the enzyme's activitys contributes to such mission.

This protocol aimed to establish a common procedure for the design, expression, and purification of different serotypes of dengue virus protease complex (DENVpro). Such proteases were then used in the optimization of FRET-based conditions, by evaluating buffer parameters and various substrates. Enzyme kinetics of the four DENVpro serotypes were characterized, and inhibitory potential of selected inhibitor compounds was tested.

The design, expression and purification of the target serotypes were successfully achieved, with all proteases displaying enzymatic activity under standard conditions previously established in the work group. The evaluation of parameters to guarantee the best catalytic activity of all protease strains was carried out, with the conditions used in the work group being suitable for the other serotypes. However, adjustments can be made to optimize assessment efficiency, such as the adjustment protease/substrate ratio to promote an increased catalytical activity. DENV1pro showed the lowest levels of substrate cleavage, which led to the synthesis of novel substrates. Nevertheless, parameters established allowed inhibition assays of previously tested compound to be carried.

The absence of standardized assays due to diverse assay conditions and protease constructs poses a challenge in establishing optimal conditions across the research community. Measures to emphasize reproducibility and robustness of an established optimal assay would enhance coherence and quality control in DENV studies, contributing to progress in therapeutic approaches.

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Abbreviations

ADE – antibody-dependent-enhancement

AMC – 7-amino-4-methylcoumarin

ATPase – adenosine triphosphatase

CHAPS - 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate

CMC – critical micelle concentration

CV – column volume

DCM – dichloromethane

DENV – dengue virus

DENVpro – NS2B-NS3 protease of dengue virus

DIPEA – diisopropylethylamine

DMF – N-dimethylformamide

DMSO – dimethyl Sulfoxide

ER – endoplasmic reticulum

FRET – Förster Resonance Energy Transfer

G_4SG_4 linker – glycine-serine linker

HATU – hexafluorophosphate azabenzotriazole

His-tag – hexa-histidine tag

HPLC - high-performance liquid chromatography

IJUP – Investigação Jovem Universidade do Porto

IMAC – immobilized meta-ion affinity chromatography

IPMB – Institute of Pharmacy and Molecular Biotechnology

IPTG – isopropyl-d-thiogalactoside

NaCl – sodium chloride

NEB – New England Biolabs

NS – non-structural

OD – optical density

PCR - polymerase chain reaction

RFU – relative fluorescence unit

RNA – ribonucleic acid

RP-HPLC - reversed-phase high-performance liquid chromatography

RTPase – ribonucleic acid triphosphatase

SDS-PAGE – sulphate-polyacrylamide gel electrophoresis

SEC – size-exclusion chromatography

TFA – trifluoriacedic acid

TIPS – triisopropylsilane

Tris-HCl - tris(hydroxymethyl)aminomethane hydrochloride

WNV – West Nile virus

List of Symbols

° C - celcius degrees

IC_{50}- half of maximal inhibitory concentration

K_{cat} – turnover number

K_m – Michaelis constant

g- gram

L- liter

M- molarity

rpm – revolutions per minute

$V_{máx}$- maximum reaction rate

1 Introduction

1.1 Dengue Virus: History and epidemiology

Dengue virus (DENV) is a mosquito-borne pathogen and it poses one of the most significant global health threats.[1] Infected people may manifest symptomatic disease that can range from dengue-fever, with symptoms such as high fever and pain, which can be recovered from. However, disease can develop into a dengue hemorrhagic fever and dengue shock syndrome which, if not correctly treated, can lead to a lethal outcome.[2, 3]

DENV, from the genus Flavivirus in the family Flaviviridae, is an arbovirus virus, transmitted by Aedes species mosquitos. The first reported cases are believed to be described in China in the 10th century, with reports of the 17th century giving evidence of widespread incidences during the expansion of trade routes.[4] The spread of DENV accelerated after World War II with the establishment and raise of movement trades, allowing the spread of the vector. With the subsequent growth of the urbanization and globalization, the burden of DENV has increased ever since.[5, 6] Currently, DENV is found in over 125 countries, causing over 10,000 deaths and 100 million symptomatic infections per year.[7-9] Southeast Asia has the highest incidence, but South America has seen a recent increase.[7-9] Outbreaks have also been reported in Africa, while sporadic cases occur in Europe and North America due to travel and migration patterns, posing a potential risk for local transmission.[7-9]

There are four known serotypes (DENV1, 2, 3 and 4) with an emergence of a fifth (DENV5) being reported.[10] These serotypes share approximately 70% of amino acid sequence similarity, but genomic alterations within a serotype result in nucleotide variations up to 8% and a 3% divergence at the amino acid level.[11, 12] While by the 1950s all serotypes had been reported, the co-circulation of different strains within a region only became common in the late 1980's.[13] Currently, it is common in the regions with higher DENV incidence for all the serotypes to co-exist.[13] For instance, surveillance studies carried in Malaysia identified all four DENV serotypes from 1990, with shifts in serotype predominance over time.[14] Similarly, in Brazil, due to increased focus in DENV spatiotemporal dynamics and the circulation across subregions, it has been identified variations in predominant serotype distribution within smaller areas in a period of a year.[15]

The co-existence of various serotypes in one area presents a serious threat to human health since the immune response of the infection of one serotype can enhance the severity of the disease when subsequently infected with another serotype. This occurrence is known as antibody-dependent-enhancement (ADE).[2, 16] Furthermore, genomic alterations within a serotype can increase the severity of clinical phenotypes.[11] Knowing these factors, the development of a therapeutic

approach targeting all serotypes is essential and so, a deeper understanding of the virus's biology and life cycle is necessary.

1.2 Dengue virus biology

DENV is a positive single-stranded RNA virus with a genome of approximately 10 kilobases. The genome encodes a polyprotein containing three structural proteins (capsid, envelope, and membrane-protein precursor) and seven non-structural (NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5) (Figure 1). Structural proteins constitute the components of the virion and non-structural proteins contribute to RNA replication.[2, 17, 18]

Due to their compact genome, DENV depend on host cells to facilitate their replication processes. DENV infects a range of host cells, attacking primarily various immune cells such as monocytes, macrophages, dendritic cells, and others.[2, 19] Infection of DENV starts after the binding to cell surface's receptors which is then absorbed through receptor-mediated endocytosis, releasing the viral genome into the host cell cytoplasm. After entry, the positive-stranded RNA is translated as a single polyprotein, by the host cell's translation machinery and then used as a template for genome replication.[2, 17-21] Concurrently, the polyprotein is processed and cleaved by viral and host proteases. For these processes to occur, it is necessary a formation of a "replication complex". Such consists of vesicular membrane structures, which will anchor the viral protein to the endoplasmic reticulum (ER) membrane by transmembrane domains. Once attached, the polyprotein is processed by host proteases, acting in the ER lumen, and viral proteases, in the cytoplasm.[2] The role of the replication complex is essential, since it enables the action of host proteases, such as furin and signalase, and its hydrophobic nature provides the viral protease the stability and proper folding to enhance its proteolytic activity.[2] With the replication and processing of the polyprotein, once there is a considerable number of RNA chains, an assembly with the structural proteins happens, followed by transportation and secretion into the Golgi compartment.[2, 17-21] Once in the extracellular space, they target adjacent cells, spreading the virus across. During this process, it was observed that the viral protease, NS2B/NS3 (DENVpro), has a key role in the maturation and cleavage of the polyprotein and, subsequently, in the viral replication.

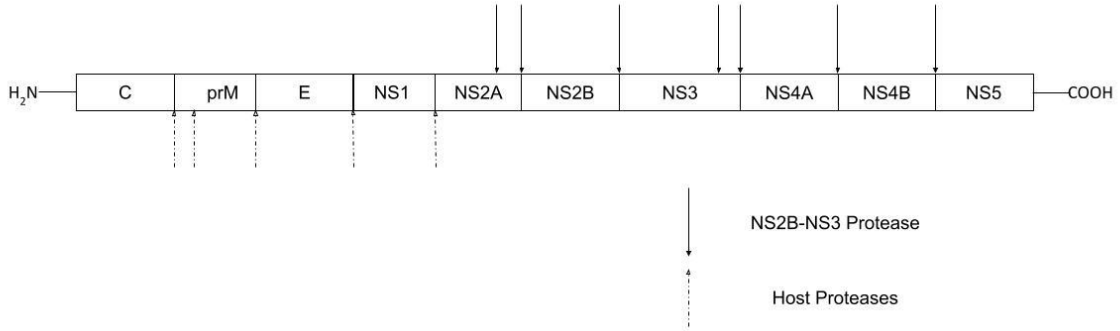

Figure 1 – Polyprotein of DENV. Cleavage sites of the viral protease, NNS3 and of the host proteases, such as furin and signalase, indicated by arrows. NS2B protein contributes to the formation of the substrate binding region of NS3 protease. (Figure adapted from Steuer *et al*, 2009

1.3 The DENVpro protease

DENV relies on the DENVpro protein complex for replication and spread. Understanding the structure and function of DENVpro is essential for identifying key targets for therapeutic interventions and exploring potential antiviral strategies. NS3 is a protein, of the chymotrypsin family, that acts as a serine protease, ATPase, RTPase and RNA helicase. While the remainder of the protein (residues 180–618) serves as the RNA helicase, the N-terminus of NS3 (residues 1-180) is known as the protease domain. The NS3 protease domain requires NS2B as a co-factor and is therefore named as the protein complex, NS2B/NS3. In this complex, NS2B, as a cofactor, guarantees the structural stability of NS3 and allows a protein folding for effective proteolytic activity, by participating in substrate recognition.[21, 22]

Being one of the most conserved proteins, with a 77% amino acid's conservation across all strains, the serotype's proteases were proven to have homologous function based on the similarity of their cleavage preferences, with a common preference at the P1 position of two basic amino acid residues, arginine and lysine.[23, 24] The conservation of the complex genome and of the recognition sequence in polypeptide substrates are a common characteristic of the Flavivirus genus, with virus such as DENV, West Nile virus, Zika virus, Japanese encephalitis virus, among others, sharing a 50% to 75% genome sequence conservation of the NS3 protease domain, across species.[25, 26]

Essentially, NS2B/NS3 belongs to a set of key factors necessary to guarantee the viability and propagation of DENV. Taking that into consideration, studies towards a potential antiviral treatment have been focusing on those key elements, leading to an increased knowledge of potential targets.

1.4 DENV treatment and drug development

Currently, there is no stablished treatment for DENV virus, with most of the treatments being supportive. The primary approach for the reduction of DENV is prevention control, targeting mostly the mosquito-vector.[27] This includes minimizing breeding places, like stagnant water sources, e.g. used tires, boxes, and water storage tanks, and implementing larvicides or insect growth regulators.[28-31] Personal protection such as the use of bed nets and windows screens are also important.[28-31] Moreover, inform and educate local communities about DENV transmission and prevention through public health campaigns is a crucial measure.[28, 32]

Nevertheless, prevention needs to be coupled with treatment. DENV has a wide spectrum of manifestations, from less to more severe outcomes. Not considering asymptomatic infections, dengue fever is the mildest manifestation of DENV, characterized by fever lasting between 2 and 7 days.[17, 30, 33] In more severe cases, dengue hemorrhagic fever can be contracted with hemorrhagic manifestations, usually during the defervescence period, period characterized by the gradual decline of fever and, in DENV infections, an indicator of either recovery or an increase of the disease severity. [30, 34] These manifestations are due to an increased capillary permeability and aberrant secretion of cytokines, resulting in plasma leakage, and severe thrombocytopenia (drastic reduction on blood's platelet, leading to prolonged bleeding or easy bruising).[17, 34] These symptoms may lead to organ failure, tissue injury and other complications that may lead to death.[17, 30, 33, 35] Currently, treatment is mostly based on symptoms relief, especially for dengue fever. In dengue hemorrhagic fever or more severe cases, fluid replacement through intravenous administration and blood and platelet transfusions are a common practise.[36, 37]

Given the global burden of DENV illness, efforts to find effective antiviral medicines are being made. Takeda, a pharmaceutical company, recently produced a vaccine and received approval in Indonesia, the European Commission and Brazil to be used, based on positive data from the initial trials.[38] Collected data in these regions already demonstrated high efficiency against hospitalized DENV cases and in individuals with DENV immunity, but a less satisfactory performance in protecting non-immune recipients, or patients with less severe clinical phenotypes.[38, 39] Despite this progress, there is still a need for more efficient therapeutic approaches, able to target all serotypes and genotypes, prevent both mild and severe outcomes and provide long-term protection with or without previous DENV immunity.

Among different therapeutic approaches, one focus on crucial viral proteins involved in viral replication as target. However, the design of antiviral medications for DENV is challenging due to genomic alterations over time and across serotypes, with emphasis for the ADE.[12] Nevertheless,

focus has aiming the viral proteins, with emphasis for NS3, NS4B and NS5 proteins. NS4B, for example, is a non-structural protein responsible to regulate the immune response from the host cell, hereby, crucial in viral replication. Its inhibition would affect viral RNA synthesis, causing the mitigation of the virus. According to Wang et al., NS4B in DENV is an interesting antiviral target, achieving inhibitors of potential preclinical development.[40] However, these same inhibitors were not effective against all serotypes. NS5 is another interesting target, mostly due to its conserved nature across the serotypes. NS5 is a protein with double function, with a N-terminal methyltransferase and a C-terminal RNA-dependent RNA polymerase, essential for the RNA synthesis, hence, vital in the replication cycle.[12, 18, 41] Due to its double function, the conceiving of antiviral agents can be designed to target either the methyltransferase or the polymerase function. Xu et al. made interesting progress targeting the latter function, by formulating a compound with inhibitory effects in all four serotypes, giving promising prospects, but still with challenges to be surpassed.[42]

Among these and various other targets and therapeutic approaches, the NS2B/NS3 protease is a well-studied subject when discussing DENV treatment. It plays a critical role in the virus replication cycle by processing the viral polyprotein. Furthermore, this is one of the most conserved non-structural proteins across serotypes with close substrate preference, making it an attractive target for a tetravalent therapeutic approach.[23] However, to accurately assess the proteolytic activity of the enzyme in vitro, assay conditions must be carefully designed to mimic the specific physiological and physicochemical requirements.

1.5 High-throughput fluorimetric assay

High-throughput fluorimetric assays have a significant role in drug discovery by providing fast screening of vast chemical libraries for potential antiviral medicines. These assays are based on fluorescence detection principles and offer several advantages over conventional screening techniques.[43]

Among such techniques used in drug development for DENV, Förster resonance energy transfer (FRET) is a reliable technique that has been employed in several studies, including within the group of Klein et al.[44] In FRET-based high-throughput fluorimetric assays, energy transfer occurs between two fluorophores: a donor and an acceptor.[43, 45-47] When exposed to a specific wavelength of light, the donor fluorophore is excited and, instead of directly emitting fluorescence, it transfers its energy to the nearby acceptor fluorophore.[43, 45-47] Consequently, there is a reduction of the donor's fluorescence and the acceptor fluorophore emits it at a different wavelength.[45] Such phenomenon is denominated quenching, with the acceptor acting as the quencher.[45] However, for the quenching

to occur in a FRET system, donor and quencher need to be in close proximity.[45] Such properties allow FRET applications to be used in understanding molecular interactions and conformational changes at small concentrations.

In the case of DENVpro, its properties as protease cleaving polypeptides can be explored by synthetizing peptide substrates containing both donor and acceptor fluorophores.[44, 48] With an intact substrate, the donor fluorophore is excited and transfer its energy to the acceptor, since the distance between these two moieties do not surpass the Förster radius.[44, 48] However, upon cleavage of the substrate by the target enzyme, both fluorophores are separated, meaning the dequenching of the donor fluorophore and a fluorescence increase.[44, 48] Such substrates are referred to as FRET substrates, and their fluorescence signal allows the screening of compound libraries to identify potential antiviral agents and serves as an indicator of the enzymatic activity.[44, 48] Alternatively to substrates that are FRET-based, substrates with fluorogenic groups next to the cleavage site can be employed to determine the enzymatic activity.[2, 44] For example, 7-amino-4-methylcoumarin (AMC) substrates are fluorogenic peptide substrates, commonly used in to determine proteolytic activity. Liberation of the fluorogenic AMC due to the enzymatic cleavage of the amine bond generates a detectable signal that allows to quantify the cleavage rate of the substrates.[49] Alternative techniques are employed among different researchers' groups. For example, HPLC-based assays for DENVpro are a precise and reliable technique used in such studies, but its time-consuming method is counterproductive when considering high-throughput analysis.[2, 50] Chromogenic substrates is other approach for the study of the protease. This substrates rely on visually detection of a coloured product upon enzymatic cleavage.[51] Even though they are employed in DENVpro studies, limitations in sensitivity and detection can affect the reliability of the assay.[2, 52] These and other measurement systems are employed in DENVpro studies, relying on different substrates and techniques, with its associated advantages and disadvantages. Aiming for a continuous improvement of such systems will benefit the evaluation of biotechnological studies.

Despite the advantages of a high-throughput fluorimetric assay, but some factors must be considered to ensure optimal conditions and accurate replication of the physicochemical environment. For example, when evaluating the substrate used for FRET assay of the DENVpro, a series of criteria should be careful analysed such as: a peptidic sequence that mimics natural cleavage sites recognized by the protease (

Table 1), chain length, donor-acceptor and their positioning, and many others.[44, 48, 53, 54] Similarly essential is the guarantee of optimal conditions of the buffer in which the assay is carried, due to their impact in the reliability of the technique. Parameters including buffer composition pH and

Table 1 – Natural cleavage sites of DENVpro for polypeptide processing (Adapted from Steuer *et. al*)

Serotype	Capsid C	NS2A/NS2B	NS2B/NS3	NS3/NS4A	NS4B/NS5
DENV-1	RRKR|SVTM	WGRK|SWPL	KKQR|SGVL	AGRR|SVSG	CGRR|GTGA
DENV-2	RRRR|XAGX	SKKR|SWPL	KKQR|AGVL	AGRR|SLTL	XTRR|GTGN
DENV-3	KRKK|TSLC	LKRR|SWPL	QTQR|SGVL	AGRR|SIAL	TGKR|GTGS
DENV-4	GRKR|STIT	ASRR|SWPL	KTQR|SGAL	AGRR|SITL	TPRR|GTGT

different additives are essential in ensuring high protease activity and minimizing interference from screening compounds.[53, 55] For example, additives such as salts affect the ionic strength of the assay, polyols contribute to protein solubility and stabilization, and detergents, prevent the formation of colloidal aggregates.[53, 55, 56]

The extended investigation and optimization of these parameters for each serotype of DENV allows to further study potential compounds with inhibitory characteristics that can aim all four serotypes. Therefore, such investigations in high-throughput fluorimetric assays for dengue virus serotypes can significantly contribute to subsequent studies in the field.

1.6 Aim of research Project

The aim of this project was to establish standard operating procedures for the design, expression, and purification of different DENVpro serotypes. It was pursued the optimization of FRET-based conditions, through the study of buffer parameters and the assessment of various substrates. Subsequently, the enzyme's activity was determined and the screening of a selection of compounds as potential inhibitors was assessed.

The main goal of this study was to contribute to the development of tetravalent therapeutic approaches against DENV by establishing an efficient and accessible biochemical assay for all serotypes.

1.7 Thesis output

The research work for this thesis was developed at IPMB – Institute of Pharmacy and Molecular Biotechnology, in department of Medicinal Chemistry at Heidelberg University, Germany. From this work resulted the following presentation:

> Macedo, M. S. C., Klein, C. D., Leuthold, M. M., Enzymatic activity in a high-throughput fluorimetric dengue virus protease assay for dengue virus serotypes. 16th Encontro de

Investigação Jovem Universidade do Porto (IJUP), 10-12th May 2023, Porto, Portugal (oral presentation).

2 Materials and Methods

2.1 Protease construct design

Expression constructs of DENV1pro and DENV3pro were designed, using pET-28a(+) (Novagen) as a vector, and a restriction-free cloning. The constructs consisted of the C-terminus of the NS2B co-factor linked by a Glycine-serine linker (G_4SG_4 linker) to the NS3 protease. NcoI and XhoI restriction sites were used for the construct insertion, for the 3' and 5' ends, respectively, ensuring a Hexa-histidine tag (His-tag) in the C-terminus.

Coding sequences of DENV 1 and DENV 3 (GenBank) were translated (Expasy translation tool) and aligned (Clustal Omega) with a DENV2pro construct (previously prepared in group), to identify regions of interest to amplify. Thereafter, using New England Biolabs builder tool software, primers were designed, assuring the incorporation of His-tag in the C-terminus, and the insertion of G_4SG_4 linker. From this design, resulted two sets of primers for each serotype (Appendix 1.1 and 1.3), purchased from Sigma Aldrich (Germany).

The New England Biolabs kit, NEBuilder® HiFi DNA Assembly, was used for the restriction-free cloning. The process started with the amplification of insertions of NS2B and NS3 via overlap-extension PCR, using the previously designed primers, followed by incorporation into the vector. Once inserted, NEB5-α E. coli (purchased from New England Biolabs) were used in the transformation of the assembled product.

Agarose gel electrophoresis and sequencing (Microsynth Seqlab GmbH) confirmed construct identity.

2.2 Protein expression and purification

DENVpro plasmids, of serotypes 1 and 3 were transformed into a E. coli BL21 DE3 cells (purchased from New England Biolabs). A pre-culture was prepared, with 40 mL of LB medium and antibiotic kanamycin (30 µg/mL), inoculated with a single colony and incubated overnight at 37 °C (160 rpm). The pre-culture was then used to inoculate 1 L of LB medium with kanamycin (30 µg/mL), forming the culture, which was incubated at 37 °C and 120 rpm until reaching an optical density (OD), at 600 nm, of 0.6. Once reached, gene expression was induced with isopropyl-d-thiogalactoside (IPTG) to a final concentration of 0.5 mM and incubation proceeded overnight at 25 °C (120 rpm). Following a period of 16-24 hours, cells were recovered by centrifugation at 3,000 g at 4 °C for 15 minutes (Sorvall RC6 Plus, Fisher Scientific, F10 - 4x1000 LEX rotor). Cell pellets were flash frozen in liquid nitrogen and stored for later use at -80 °C. Protein expression was carried for only DENV1pro and DENV3pro, since

there were already in storage, from previous works within the group, purified DENV2pro and cell pellets containing DENV4pro.

For purification, cell pellets of DENVpro serotypes 1, 3 and 4 were thawed on cold lysis buffer (25 mM Tris, 500 mM, NaCl, 5 mM imidazole, 5% [v/v] glycerol, pH 7.6). The cells were then lysed using a high-pressure cell disrupter (One Shot, Constant Systems LTD) at 1.65 kbar, and the lysate was centrifuged for 1.5 h at 50,000 g and 4 °C (Sorvall RC6 Plus, SS-34 rotor). The supernatant was subjected to immobilized meta-ion affinity chromatography (IMAC) for the purification of the proteases. The column was loaded with Chelating Sepharose Fast Flow 8GE Healthcare, charged with nickel ions beads, with a column volume (CV) of 4 mL and equilibrated with 10 CV lysis buffer. The supernatant was mixed with the column and shaken for 1 h. Afterwards, the flow-through was collected and the column was washed with 5 CV of buffers of increased imidazole concentrations, from 10 to 25 mM (20 mM Tris, 200 mM NaCl, pH 7.6). The enzyme was then eluted by multiple fractions using a CV of elution buffer, with substantially increased imidazole (20 mM Tris, 200 mM NaCl, 500 mM imidazole, pH 7.6), for each fraction. Fractions were analysed by OD and sulphate–polyacrylamide gel electrophoresis (SDS-PAGE).

Protein-containing fractions were pooled, concentrated and buffer exchanged to size-exclusion chromatography (SEC) buffer (20 mM Tris, 200 mM NaCl, pH 7.6) using Amicon centrifugation filters. Afterwards, SEC was performed using the ÄKTApurifier system (GE Healthcare). Protein solution was loaded onto the HiLoad 16/600 Superdex 200 pg column (GE Healthcare) prior to each run. The SEC buffer was used to equilibrate the column and elute the protease at a flow rate of 1 mL per minute. After the void volume (40 mL), fraction collection began, being then analysed through SDS-PAGE. Pure protein fractions were combined and concentrated. After an OD measurement at 280 nm to determine the concentration (10 μM to 150 μM, 50% glycerol), liquid nitrogen was used to freeze aliquots for storage at -80 °C.

2.3 SDS-PAGE

Samples of purified DENVPRO constructs were evaluated in SDS-PAGE analysis, with a running time of 90 min at a voltage of 120 V, in 1x SDS running buffer (25 mM TRIS, 190 mM glycine, 0.1% SDS in dH2O), incubated in a Coomassie-dye reagent (GelCode™ Blue Safe Protein Stain, Thermo Fischer Scientific) and destained with water.

2.4 Solid Phase Peptide Synthesis of FRET Substrates

All chemicals for the synthesis of precursors were obtained from Sigma-Aldrich (Germany), Alfa Aesar (Germany), Thermo Fisher Scientific (Germany/United States), Acros Organics (Belgium), TCI Europe (Belgium), and Carbolution Chemicals (Germany) and were of analytical grade. The amino acids were purchased from Carbolution Chemicals (Germany), Alfa Aesar (Germany), and TCI Europe (Belgium). Hexafluorophosphate Azabenzotriazole Tetramethyl Uronium (HATU) and Fmoc-Rink amide resin (75−150 mesh; loading capacity: 0.71 mmol/g resin) were purchased from Iris Biotech (Germany). Solvents were used as obtained from the commercial suppliers.

All sequences were assembled by stepwise solid-phase synthesis on Rink amid resin using the standard Fmoc strategy, as previously described.[44, 57, 58] Solid-phase synthesis was done manually in plastic syringes equipped with a frit. All steps were performed at room temperature under continuous shaking. Rink amide resin was preswollen in dichloromethane (DCM) for 20 min and then washed 3× with N,N-dimethylformamide (DMF). For Fmoc group removal, a piperidine solution (20% in DMF) was added 2× for 10 and 5 min. Following each deprotection or coupling step, the resin was washed 3× with DMF, 3× with DCM, and again 3× with DMF. HATU and diisopropylethylamine (DIPEA) were used for coupling steps. In detail, the coupling solution contained the Nα-Fmoc protected amino acid or the respective building block (2.0−3.0 equiv), HATU (2.0−3.0 equiv), and DIPEA (2.3−3.9 equiv) in DMF (1.0 mL per 100 mg of resin). The solution was added to the resin, and the mixture was shaken for 60−120 min. Afterward, the resin was washed as described before. Fmoc deprotection and coupling steps were iteratively repeated until the desired sequence was obtained. The resin loaded with the finished peptide was washed 5× with diethyl ether and dried under reduced pressure. The final product was cleaved 2× off the resin with trifluoriacedic acid, triisopropylsilane and H2O solution (TFA/TIPS/H2O, 95:1:4, 1−2 mL per 100 mg of resin), and the mixture was shaken for 2 h. The cleavage solution was dispensed into cold diethyl ether (35 mL per 100 mg of resin), and the resulting precipitate was centrifuged (4000g, 10 min), washed with diethyl ether/hexane, and dried under reduced pressure. If the peptides were soluble in the organic solvent, then the organic phase was washed 2 times with a small amount of water, and all solvents were removed in vacuo. The resulting residue was coevaporated with toluene several times.

2.5 Reverse-Phase High-Performance Liquid Chromatography

All synthesized compounds were purified by preparative RP-HPLC on an ÄKTA Purifier, GE Healthcare (Germany), with an RP-18 pre and main column (Rephospher, Dr. Maisch GmbH, Germany; C18-DE, 5

µm, 30 mm × 16 mm, and 120 mm × 16 mm). The following conditions were used: eluent A, water (0.1% TFA); eluent B, methanol (0.1% TFA) or eluent A, water (0.1% TFA); eluent B, acetonitrile (0.1% TFA); flow rate, 8 mL/min; and gradient, 10% B (2.5 min), 100% B (23.5 min), 100% B (26 min), 10% B (26.1 min), and 10% B (30 min). Detection was performed at 214, 254, and 280 nm. After purification, the organic solvent was evaporated, and the compounds and intermediates were freeze-dried in H2O and acetonitrile (1:1) and stored at −20 °C.

2.6 Mass Spectrometry

On a Bruker micrOTOF-Q II instrument (Bruker Daltonics), mass spectra were measured. ESI mass spectrometry was used to analyze the samples, in positive and negative mode. 100 µL of samples were prepared in a 1:1 mixture of acetonitrile and water.

2.7 Evaluation of enzymatic activity

Enzymatic activity was evaluated through biochemical assay. Protease solutions were pre-diluted with the standard buffer, (50 mM Tris-HCl, pH 9, ethylene glycol [10% v/v], 0.0016% Brij 58), which will be nominated as Brij buffer, to concentrations ranging from 0 to 5 µM. The reaction initiated by adding protease for different final concentrations (0 to 500 nm), with a final volume of 100 µL for each well. Fluorescence of released 2-Abz, from different FRET substrates, was monitored with a FLUOstar OPTIMA microplate reader (BMG Labtech), with 430 nm of emission wavelengths and with 330 nm of excitation. With a measurement time of 17 min, the protease activity was determined using the slope of the relative fluorescence units per second (RFU/s). A solution with no protease was used as a negative control and the results were expressed as a mean of the triplicates, with the respective standard deviation.

2.8 Evaluation of assay buffer composition

Different buffers parameters were tested in the biochemical assay, considering different compounds or concentrations, specifically: pH, salts, polyols and detergents. Brij buffers were considered in all assays, with variations in the parameter being tested. In pH evaluation, brij buffers were prepared with pH values ranging from 7.0 to 9.0, with a 0.5 units variation among them. Testing the influence of salts, different concentrations of sodium chloride (0, 45, 90 and 135 mM) in the brij buffer were tested to evaluate ionic strength. Regarding polyols, different concentrations of ethylene glycol (0%,

5%, 10% and 20% v/v) were tested, as well as alternatives polyols such as glycerine (5% and 20% v/v) and propylene glycol (5%, 10% and 20%). Detergents were analysed by testing various concentrations of Brij 58 (0%, 0.0008%, 0.0016% and 0.0032% v/v) in the brij buffer, followed by the Zwitteronic detergent (1 mM), and non-ionic detergents, namely, Trinton X-100 and Tween-20 (4.8 and 0.976 µM). For novel non-ionic detergents assays, 20% of their critical micelle concentration was considered, according to Hait and Moulik, to reach optimal activity.[59]

For all assays, DENV substrate was pre-diluted with Tris-HCl buffer (50 mM, pH 9) to a concentration of 1 mM and pipetted (5 µL) into wells of a black 96-well plate, resulting in a final concentration of 50 µM in the evaluated buffer. Similarly, DENVPROs from the different serotypes were pre-diluted with Tris-HCl buffer (50mM, pH 9), pipetted (5 µL) and then diluted in the assay buffer to initiate the reaction, to concentrations accordingly their enzymatic activity assessment. Fluorescence of released 2-Abz from the FRET substrates was monitored with a FLUOstar OPTIMA microplate reader (BMG Labtech), with 430 nm of emission wavelengths and with 330 nm of excitation. With a measurement time of 17 min, the protease activity was determined with RFU/s. The results were expressed as a mean of the triplicates, with the respective standard deviation.

2.9 Evaluation of different substrates

The enzymes interactions with different substrates were tested in the biochemical assay. Firstly, FRET substrates were analysed: DENV substrate and WNV substrate. Protease solutions were pre-diluted (0 to 5 µM) in brij buffer and in brij buffer with CHAPS (1 mM) as a detergent replacement (CHAPS buffer). Similarly, substrates were pre-diluted in the same buffers to a fixed concentration of 500 µM. Substrates were then pipetted (10 µL) into wells of a black 96-well plate to final concentrations of 50 µM. The reaction started by adding the prediluted protease (10 µL) at final concentrations ranging from 0 to 500 nM. Each well had a final volume of 100 µL with the specific buffer. Fluorescence of released 2-Abz from the FRET substrates was monitored with a FLUOstar OPTIMA microplate reader (BMG Labtech), with 430 nm of emission wavelengths and with 330 nm of excitation. With a measurement time of 17 min, the protease activity was determined using the slope of the relative fluorescence units per second (RFU/s). Results were expressed as a mean of the triplicates, with the respective standard deviation. The same procedure was applied to evaluate the novel substrate synthesized (DENV 2A/2B and DENV 2B/3) using only brij buffer.

Subsequently, the enzyme interaction with AMC substrates (KE3 and KE6) was tested, using two compounds, KE3 and KE6 substrate. These were prediluted in brij buffer and in CHAPS buffer with concentrations ranging from 0 to 400 µM. DENVPROs were pre-diluted to 500 nM. The substrates

were pipetted (10 µL) into wells of a black 96-well plate to concentrations ranging from 0 to 40 µM, diluted in the respective buffer. The reaction started by adding of the prediluted protease (10 µL), with a 50 nM final concentration. Each well had a final volume of 100 µL. Fluorescence of released AMC was monitored with a FLUOstar OPTIMA microplate reader, with 355 nm of emission wavelengths and with 330 nm of excitation over a 17 min period. The protease activity was determined with RFU/s. The results were expressed as a mean of the triplicates, with the respective standard deviation.

Both FRET and AMC substrates sequences are shown in Table 2.

2.10 Determination of enzyme kinetics

Enzyme kinetics were established for all proteases. Calibration curve of 2-Abz (FRET substrate) was previously established, within the group, for the conversion of RFU/s to the rate of cleaved substrate (µM/s). Thereafter, selected FRET substrates were prediluted in selected buffer conditions, and then pipetted (10 µL) into wells of a black 96-well plate to concentrations ranging from 0 to 400 µM, diluted in the respective buffer. The reaction started by adding of the prediluted protease (10 µL), with a final constant concentration established for each serotype, accordingly to their enzymatic activity assessment. Each well had a final volume of 100 µL with the specific buffer. Fluorescence of released 2-Abz from the FRET substrates was monitored with a FLUOstar OPTIMA microplate reader (BMG Labtech), with 430 nm of emission wavelengths and with 330 nm of excitation. With a measurement time of 17 min, the protease activity was determined using the slope of the relative fluorescence units per second (RFU/s). Results were expressed as a mean of the triplicates, with the respective standard deviation. Values were multiplied by the correction factor for each concentration due to the inner filter effect. K_m and $V_{máx}$ were derived from fitting the experimental data to the Michaelis-Menten equation using the GraphPad Prism 5.04 software.

Table 2 – Substrates sequences employed in the biochemical assays.

	Substrates	Sequence
FRET	DENV	2-Abz-Nle-Lys-Arg-Arg-Ser-(3-NO_2)-Tyr-NH_2
	WNV	2-Abz-Gly-Leu-Lys-Gly-Gly-(3-NO_3)-Tyr-NH_2
	2A/2B	2-Abz-Trp-Gly-Arg-Lys-Ser-(3-NO_2)-Tyr-NH_2
	2B/3	2-Abz-Lys-Lys-Gln-Arg-Ser-(3-NO_2)-Tyr-NH_2
AMC	KE3	Bz-Nle-Lys-Lys-Arg-AMC
	Ke5	Bz-Nle-Lys-Arg-Arg-AMC

2.11 Inhibition Assay

For the inhibition assay, potential inhibitor compounds were evaluated using brij buffer and DENV substrate, except for DENV1pro assay, which used DENV1 2B/3 substrate. Inhibitor compounds and substrate were prediluted (500 µM) in the buffer. Proteases were prediluted accordingly their activity assessment. Each inhibitor compound (10 µL) was incubated with protease (10 µL) and buffer (70 µL) for 15 min. Subsequently, the reaction was initiated by the addition of substrate (10 µL), for a final volume of 100 µL. The final concentration of both substrates and inhibitors was 50 µM, and protease concentration being based on its activity. Enzymatic activity was determined with RFU/s, using the FLUOstar OPTIMA microplate reader (BMG Labtech) over a total measurement time of 17 min. The percentage of inhibition was calculated relative to a positive control, with dimethyl sulfoxide (DMSO) replacing the inhibitor compound, as the mean and standard deviation of triplicates were determined. After the initial screening, compounds with significant inhibition at the established concentration were subjected to an IC_{50} (half of maximal inhibitory concentration) measurement. Similar setup and procedure as previously described were followed, but with decreasing concentrations of inhibitor. IC_{50} values were calculated with GraphPad Prism 5.4 software and plotted against the corresponding logarithmic substrate concentrations

3 Results

3.1 Construct design and cloning

Expression constructs were designed for DENV serotypes 1 and 3 (Genbank) using the pET-28a(+) vector and a restriction-free cloning method. The restrictions sites exploited were NcoI and XhoI for the 3' and 5' ends, respectively, to ensure a His-tag at the C-terminus of the construct. Moreover, the addition of a G_4SG_4 linker, to link the NS2B and NS3 protease domain was performed. The identity of the constructs was confirmed through agarose gel electrophoresis, showing the expected size, and through sequencing (Appendix 1).

3.2 Protein expression and purification

Expression of DENVpro was carried for the serotypes 1 and 3, resulting in pellet weights of 4.20 g and 3.54 g for DENV1pro and DENV3pro, respectively.

Purification of DENVpro of serotypes 1, 3 and 4 was done via IMAC with His-tag. SDS-PAGE analysis showed bands of the target molecular weight (26.7 kDa DENV1pro 1, 26.8 kDa for DENV3pro and 27.4 kDa for DENV4pro kda) in the elution fractions, but with additional bands, with different molecular weight, indicating impurities (Figure 2 and Figure 3).

Purification was improved by SEC, with new SDS-PAGE analysis indicating an increased purity, for DENV1pro and DENV3pro. DENV4pro increased its purity as well but not sufficient and so required an additional IMAC run to achieve the intended purity. Figure 2 shows corresponding SDS-PAGE analysis before and after SEC, along with the respective chromatograms from each run. Once proteases were purified, fractions were pooled for each serotype, with DENV3pro and DENV4pro forming two distinct pools (pool 1 and 2), since different fractions collected had colour intensity and purity differences. Of note, only the pools with higher concentrations were used in the following biochemical assays (Table 3). The pooled fractions were concentrated, glycerol was added, and they were flash frozen with liquid nitrogen and stored at – 80 °C. Concentration was determined by measuring the optical density (OD) at 280 nm and converting it to molarity using the known extinction coefficient (Table 4). SDS-PAGE analysis confirmed the purity of the final protease preparations, with samples with 1 and 10 µg, as shown in Figure 3. Clear bands can be observed with target molecular weight (26.7 kDa, 27.9 kDa, 26.8, kDa and 27.4 kDa for DENVpro serotypes 1, 2, 3 and 4, respectively), along with faint bands of lower molecular weight. In DENV4pro, two bands were observed, near the target molecular weight, of unknown identity. Nevertheless, it was used in the following experiments.

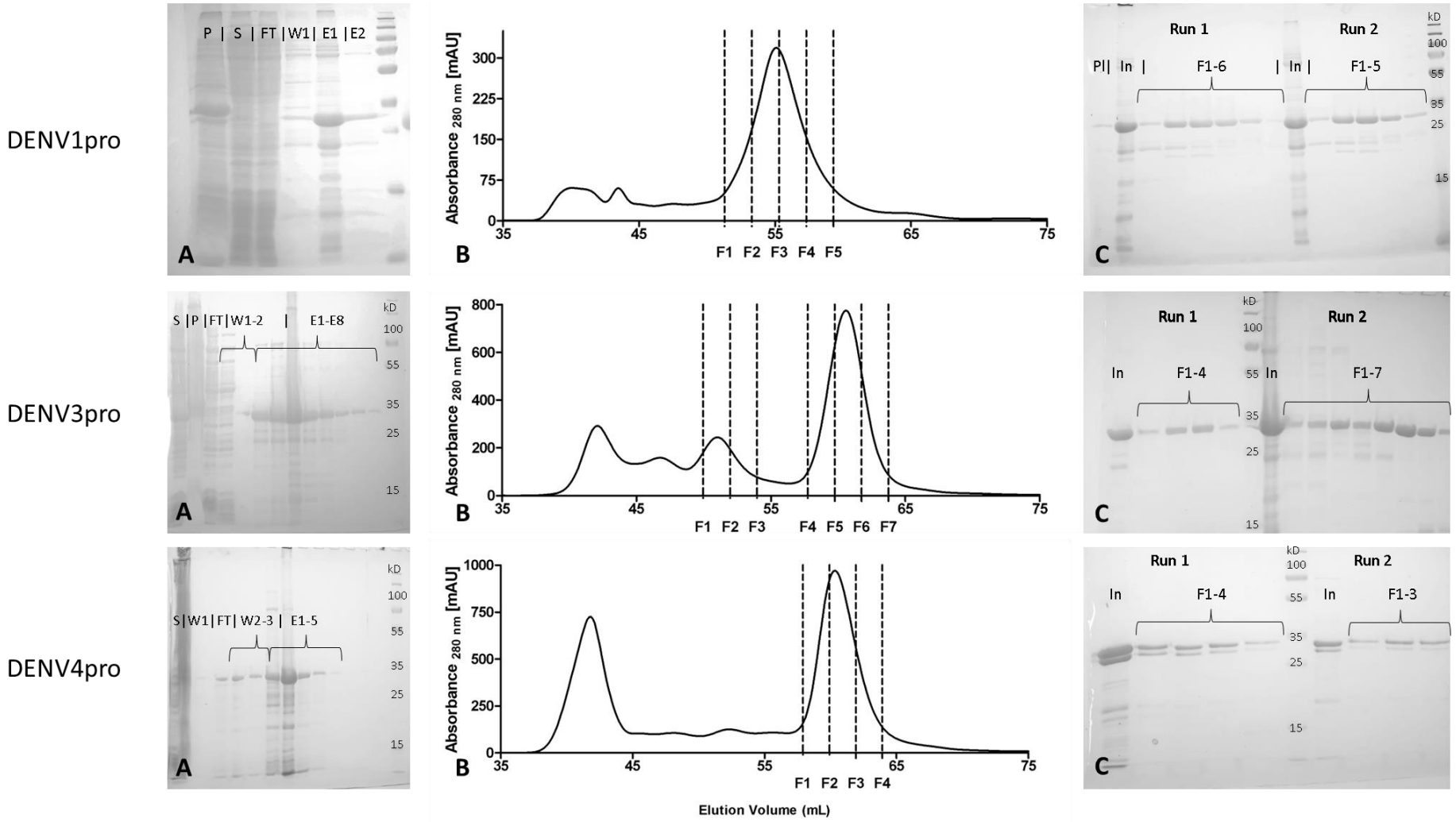

Figure 2 - DENVpro constructs analysed by SDS-PAGE before and after SEC. A - SDS-Page analysis after IMAC with samples of supernatant (S) and pellet (P) of lysate centrifuged, flow-through (FT) of IMAC, washing columns (W) andFigure 2Figure 2 various elutions (E); B- SEC chromatogram of each DENVpro; C- SDS-Page analysis after SEC with samples of pellet (PL) of elution pool centrifuged, injected protein solution (I) in SEC and fractions (F) collected

Table 3 - DENVpro constructs expression and purification yields. DENVpro serotype 1, 2 and 3 were expressed and purified. Expression and purification yields arlisted in this table.

Purified proteases	Culture volume (L)	Pellet Weights (g)	Purified protein Pool 1 / Pool 2 (mg)	Concentration of pooled protein Pool 1/ Pool 2 (mg/mL)
DENV1pro	1.0	4.20	3.794	5.6
DENV3pro		3.54	5.352/ 2.736	9.0/ 1.0
DENV4pro		3.96	2.48 / 0.700	4.6/ 1.0

Table 4 - Molecular weight and concentration of Purified DENVPRO Serotypes. Concentration was determined by measuring the optical density at 280 nm and converting it to molarity using the known extinction coefficient.

Purified proteases	Molecular weight (Da)	Extinction coefficient (M-1 cm-1)	Purified protein Pool1/ Pool 2 (mg)	Concentration of aliquots Pool 1/ Pool 2 (µM)
DENV1pro	26702	40450	7.588	69.2
DENV2pro	27875	41940	10.704	63.4
DENV3pro	26763	38960	10.704/ 5.472	115.5/ 12.8
DENV4pro	27427	40450	4.96/ 1.4	56.9/ 12.4

Figure 3 - Purified DENVpro constructs were analysed by SDS-PAGE, with 1 and 10 µg samples. Bands observed at the target molecular weight :26.7 kDa for DENV1pro, 27.9 kDa for DENV2pro, 26.8 kDa for DENV3pro and 27.4 kDa for DENV4pro.

3.3 General remarks – Biochemical assay

Enzymatic activity of DENVpros of all the serotypes was initially evaluated using DENV substrate (2-Abz-Nle-Lys-Arg-Arg-Ser-(3-NO2)-Tyr-NH2) and Brij buffer (50 mM Tris-HCl, pH 9, ethylene glycol [10% v/v], Brij-58 [0.0016%]), as previously described.[53] These conditions served as baseline for comparing effects of buffer variations and different substrates. The success of using CHAPS (1 mM) as a detergent replacement for in the Brij buffer in previous and current studies prompted further exploration, and this buffer will be referred to as CHAPS buffer.[47, 60, 61]

For all the enzymatic activity testing, a biochemical assay was performed, and the results were expressed as RFU/s.

Assays were mostly carried out with a constant substrate concentration of 50 µM, except for the evaluation of different substrates and the determination of enzyme kinetics, when applicable. Regarding proteases, these were initially assessed at various concentrations (0-500 nM) to establish the appropriate one for each serotype based on their enzymatic activity.

Inner filter effects occur, specially at higher substrate concentrations, due to overlap in the absorption spectrum of the substrate with the emission spectrum of 2-Abz.[62] To correct for the inner filter effect, a correction factor, determined by Nitsche and Klein at similar assay conditions, was considered for each substrate concentration (Appendix 2.1).[44] Such correction factor was not implemented for assays with constant substrate concentration, only for kinetic studies using different substrate concentrations.

3.4 Enzymatic activity of DENV proteases from all serotypes using the standard conditions

Using the proposed procedure and pre-diluted protease solutions and substrates, the enzymatic activity of all four DENVpros, at different protease concentrations, was evaluated. Figure 4 displays the results of the enzymatic activity of each protease, with concentrations ranging from 0 to 500 nM, with a fixed substrate concentration of 50 µM presented as the mean of triplicates with the respective standard deviation.

Results indicated that DENV4pro has the highest enzymatic activity, considering concentrations between 8 and 250 nM of the protease. However, for concentrations above 125 nM, the enzymatic activity starts to reach a plateau, with lower increases of activity, which makes concentrations similar or below 125 nM suitable for further measurements. DENV2pro show higher enzymatic activity for concentrations below 60 nM and similar activity for concentrations above, when compared to DENV3pro. DENV1pro shows the lowest enzymatic activity throughout the range of concentrations.

From the assessment of the enzymatic activity at the standard conditions, protease concentrations were established based on their activity. For the subsequent assays, the assigned protease concentrations are: DENV1pro at 400 nM, DENV2pro at 100 nM, DENV3pro at 100 nM and DENV4pro at 50 nM.

3.5 Evaluation of pH influence in assay buffer

To evaluate pH influence in the assay buffer to increase the substrate cleavage velocity, different buffers were prepared based on the Brij buffer with TRIS as buffering component and with pH values ranging from 7.0 to 9.0 (with 0.5 unit variations). Using the described parameters and fixed protease concentrations established in section 3.4 with 50 µM of DENV substrate. The results, in Figure 5, are presented as the mean value of triplicate measurements and standard deviations.

From the data collected, there is a decreased in the enzymatic activity with decreasing pH levels, similar as described previously for DENV2pro by Steuer et. al.[53] The lowest levels of activity were observed at pH 7, with decreases between 70 - 96 %, while the highest substrate cleavage occurred at the standard pH 9 conditions. Such observations apply to the four serotypes. In DENV1pro, no differences in enzymatic activity were shown.

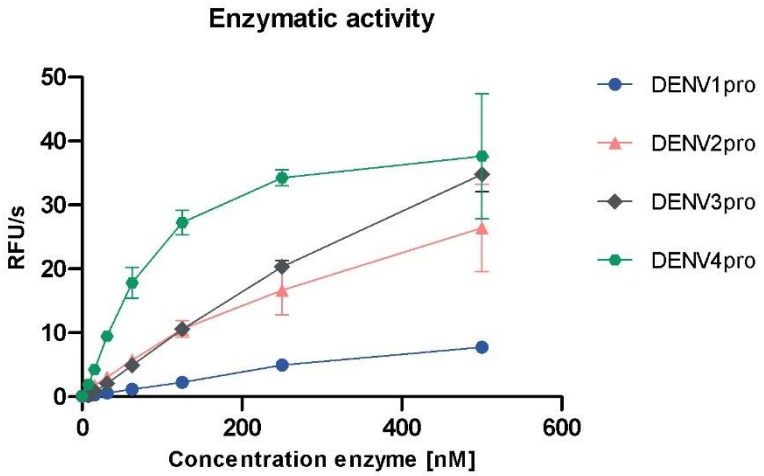

Figure 4 – Enzymatic activity of the four DENVpros. Measurements were performed with DENV substrate (50 µM) and different concentrations (0 – 500 nM) of each protease with Brij buffer

3.6 Evaluation of NaCl addition in assay

The influence of salts on the protease activity was evaluated by adding NaCl at different concentrations in the Brij buffer (0, 45, 90 and 135 mM), to assess the influence of ionic strength in the proteolytic activity. The activity was analysed as described above with 50-400nM protease concentration (depending on the serotype) and 50 µM substrate The results are presented in Figure 5 as the mean values and standard deviations.

The addition of NaCl caused a decrease of up to 65 - 95 % in the protease activity in all serotypes. Buffers with the lowest NaCl concentration of 45 mM promoted a reduction of more than 70% in protease activity, while the increasing concentrations of the salt resulted in similarly low or even lower activity results. These observations were consistent across all four serotypes.

3.7 Polyols

Polyols are frequently employed in enzymatic assays due to its action in the stabilization of the tertiary structure of the protein.[63] The influence of polyols on the substrate cleavage was tested with different concentrations of ethylene glycol in the Brij buffer, followed by alternative polyols at various concentrations, namely glycerine and propylene glycol. With ethylene glycol, concentrations of 0%, 5%,10% and 20% (v/v) were investigated. Thereafter, glycerol at 5% and 20% (v/v) and propylene glycol at 5%, 10% and 20% were analysed. The results, in Figure 5, are presented as the mean values and standard deviations.

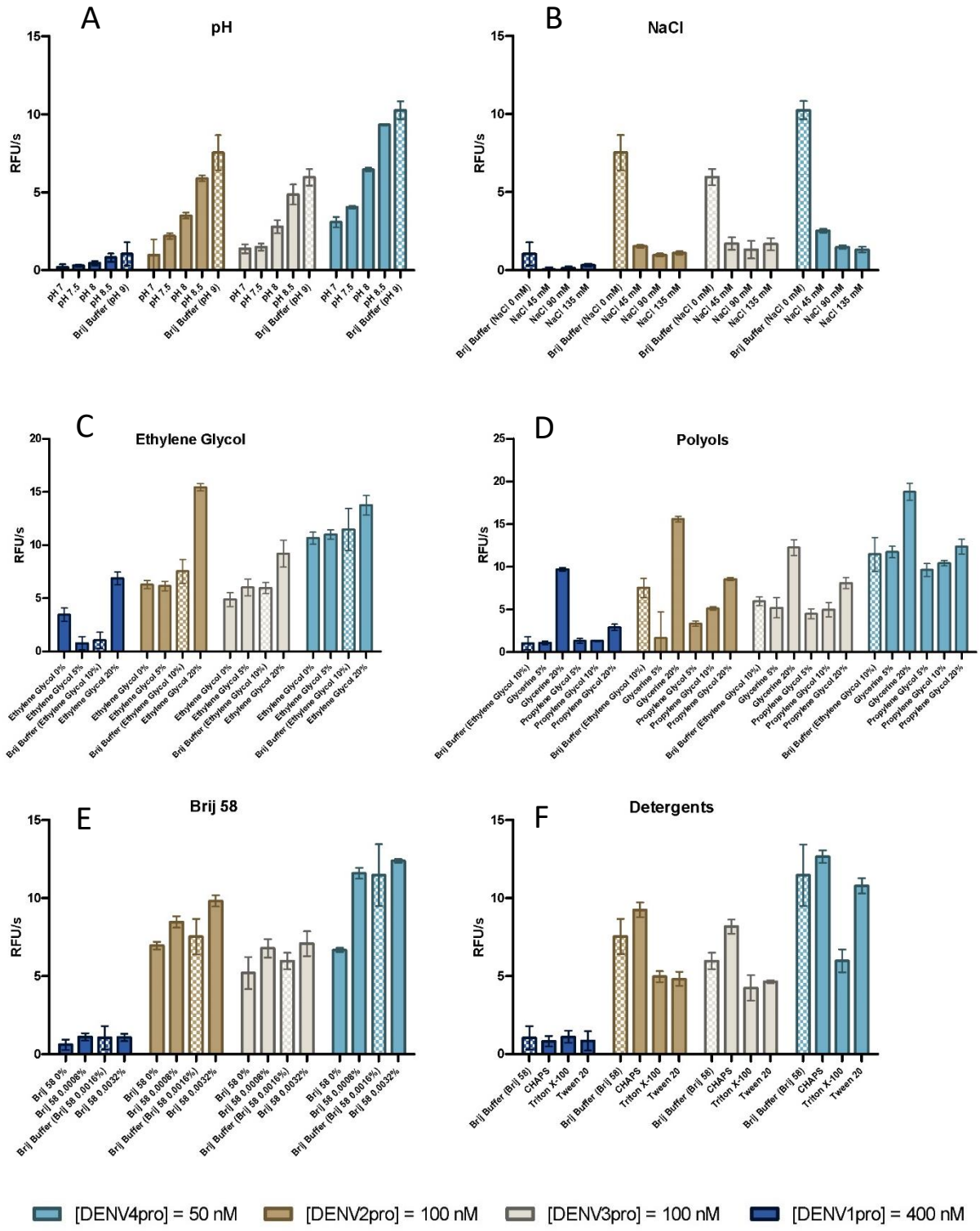

Figure 5 – Buffer optimization of the FRET assay. Measurements were performed with DENV substrate (50 µM) and 400 nM, 100 nM, 100 nM and 50 nM of DENV1-4pro, respectively. Brij buffer was used as baseline for the study of the buffer's parameters: (**A**)Comparison of substrate cleavage velocity with different pH. (**B**) Influence of NaCl. (**C**) Influence of concentration shifts in ethylene glycol. (**D**) Influence of different detergents. CHAPS at 1 mM, Triton X-100 and Tween 20 at 0.2 CMC. (**E**) Influence of concentration shifts in Brij-58. (**F**) Influence of different detergents. CHAPS at 1 mM, Triton X-100 and Tween 20 at 0.2 CMC

For DENVpro serotypes 1, 2, and 3, the highest concentration (20%) of ethylene glycol led to an increase in enzymatic activity (increase of 556 %, 105 % and 72.4 %, respectively) compared to enzyme activity in the Brij buffer. Lower concentrations of ethylene glycol did not show differences compared to the Brij buffer, except in DENV1pro where the absence of ethylene glycol led to increased activity compared to 5 and 15% but not to 20%. Considering DENV4pro, no increase or decrease in enzymatic activity were observed between the Brij buffer and different concentrations of ethylene glycol.
Moreover, buffer with 20% glycerine (v/v) showed the highest enzymatic activity in all serotypes, with activity increases, compared to Brij Buffer, of 64 % for DENV4pro, and exceeding 100 % for the remaining serotypes. In DENV1pro and DENV3pro, propylene glycol showed higher protease activity (176 and 40 % increases, respectively) compared to the Brij buffer. Nevertheless, in the remaining serotypes, the substrate cleavage was either lower or with no differences for the remaining analysed polyols when compared to the Brij buffer.

3.8 Evaluation of Detergents

Detergents are included in the assay buffer to prevent the formation of colloidal aggregates. The influence of detergents on protease activity was investigated by testing the Brij buffer with different concentrations of its detergent, Brij-58, as well as other non-ionic detergents as replacement including CHAPS, Triton X-100, and Tween-20. For Brij-58, concentrations of 0%, 0.0008%, 0.0016%, and 0.0032% (v/v) were assessed. Additionally, Triton X-100, and Tween-20 were tested at 20% of their critical micelle concentration (CMC) (Table 5). [59] The enzymatic activity was analysed as described above. The results, in Figure 5, are presented as the mean values and standard deviations.
In DENV1pro and DENV3pro, no differences in enzymatic activity were observed when comparing various concentrations of Brij-58 to the concentration in the Brij buffer. In DENV2pro, when using the buffer with 0.0032% (v/v) concentration of the detergent, the enzymatic activity was higher (increase of 30.4 %), compared to the Brij buffer concentration. Nevertheless, lower concentrations of detergent showed no differences in enzymatic activity. In the case of DENV4pro, no differences in enzymatic activity were observed at different Brij-58 concentrations, except for the absence of the detergent with a 42 % lower protease activity.

Table 5 - Critical Micelle Concentration (CMC) of Nonionic Detergents, adapted from Hait et al. [5]

Detergents	CMC [µM]
Trinton X-100	24
Tween 20	4.88

When comparing the different detergent types, DENV1pro showed no differences among the different buffers tested. In DENV2pro, CHAPS buffers manifested the highest enzymatic activity (22.6 % increase), followed by Brij buffer, while buffers with Triton X and Tween 20 exhibited lower enzymatic activity (34.1 and 36.0 % decrease, respectively). Similarly, in DENV3pro, CHAPS buffer had the highest substrate cleavage velocity with a 53.1 % increase in enzymatic activity, followed by the Brij buffer, followed by Triton X and Tween 20 buffers (26.7 and 19.6 % decrease). Finally, in DENV4pro, CHAPS and Tween 20 buffers had no difference in enzymatic activity when compared to the Brij buffer, displaying the highest enzymatic activity, in contrast with Triton X, with the least protease activity (47.9 % decrease).

3.9 Cleavage efficiency of different FRET and AMC substrates in standard conditions

Using the proposed procedure, the enzyme-substrate interactions were evaluated. The FRET substrates employed are internally quenched substrates with peptide sequences that, once cleaved, increases fluorescence. On the other hand, AMC substrates rely on the liberation of the fluorogenic group AMC, due to enzymatic cleavage, to determine the proteolytic activity.

Firstly, FRET substrates available in the group were tested, DENV substrate and WNV substrate (2-Abz Nle-Lys-Arg-Arg-Ser-[3-NO2]-Tyr-NH2 and 2-Abz-Gly-Lys-Lys-Arg-Gly-(3-NO3)-Tyr-Ala-Lys-NH2, respectively), with two buffers, Brij buffer and CHAPS buffer. Such substrates are employed in the group for studies with DENV and WNV virus.[64] In their synthesis, a peptide sequence that mimics the natural cleavage sites of their respective virus protease was implemented, to increase the cleavage rate. Following the biochemical assay, a microplate reader was used to quantify the fluorescence, and RFU/s was used to calculate the protease activity at different protease concentrations (0 to 500 nM) and with a fixed substrate concentration (50 µM). The test included a negative control with no protease. Figure 6 displays the results of the enzymatic activity of each protease for the respective substrate, presented as the mean of triplicates with the corresponding standard deviations. Moreover, to provide a clearer understanding of the protease activity of each serotype interacting the different substrates, results are presented in Table 6, with selected protease concentrations close to the values

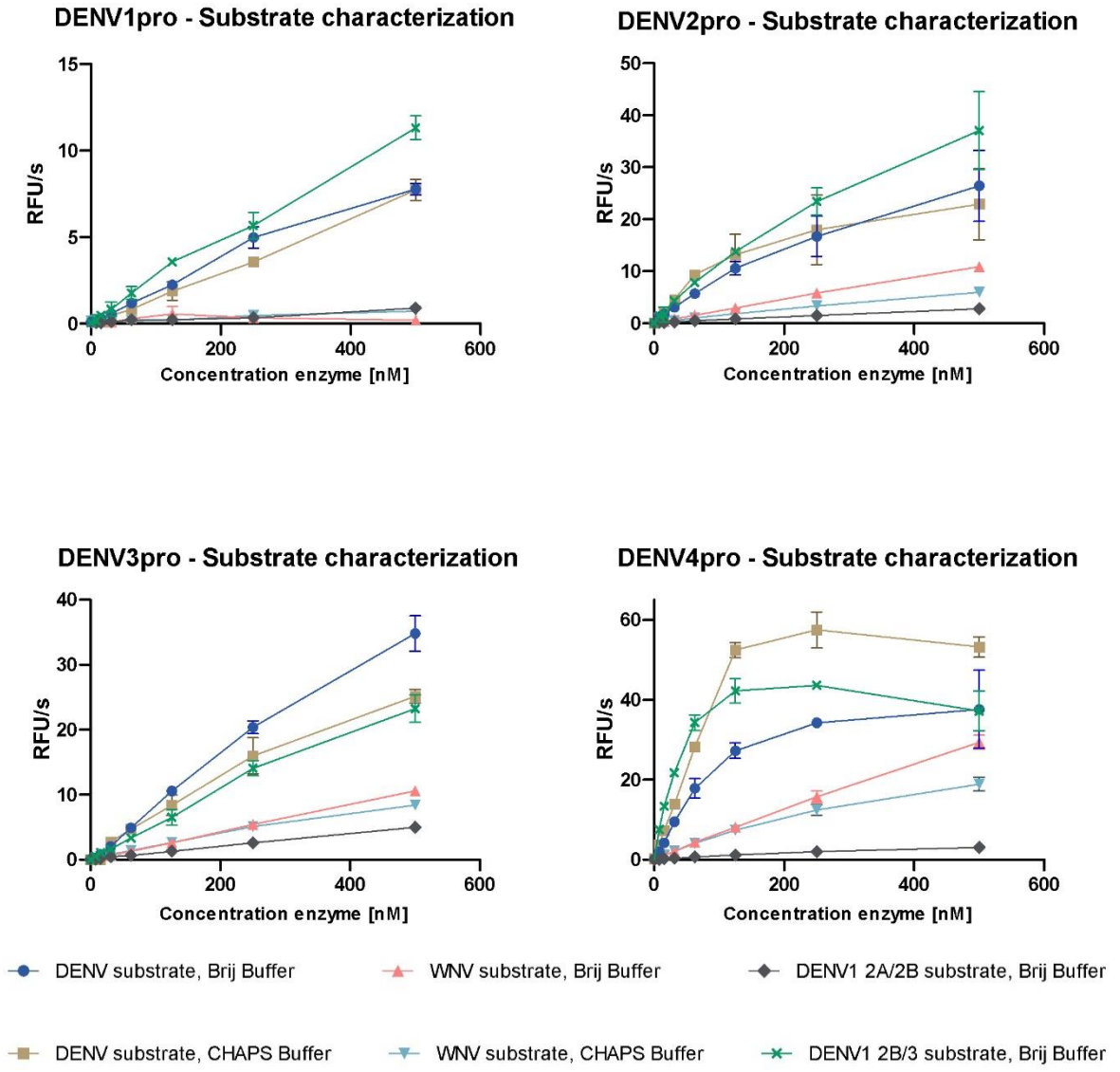

Figure 6 - Enzymatic activity of DENVpros with different FRET substrates. Measurements were performed with DENV, WNV, 2A/2B and 2B/3 substrates (50 µM) at different concentrations of each protease (0 – 500 nM). Brij buffer was used in all substrates assessments, CHAPS buffer was used in DENV and WNV substrate.

assigned in section 3.4 (500 nM and 62.5 nM for DENV1pro and DENV4pro, respectively, and 125 nM for DENV2pro and DENV3pro), and with 50 µM of different substrates.

From the results, a decrease in enzymatic activity is observed when cleaving WNV substrate (36 %, 60 %, 70 % and 75 % decreases for DENV1-4pro, respectively) compared to DENV substrate, leading to its exclusion from further assays. Looking at DENV substrate, similar cleavage rates were observed with both buffer conditions for DENV1pro and DENV2pro. DENV3pro showed increased activity with Brij buffer at protein concentrations above 125 nM in comparison to CHAPS buffer. In contrast, DENV4pro had a higher enzymatic activity with DENV substrate when using CHAPS buffer compared to Brij buffer.

Table 6 – Enzymatic activity of DENVpros with different buffer conditions (Brij and CHAPS buffer) and FRET substrates (DENV, WNV, 2A/2B). The table displays the relative fluorescence units per second (RFU/s) for a fixed concentration of substrate (50 µM) and protease concentrations that were experimentally tested and closely aligned with the pre-established conditions mentioned in section 3.4. The enzymatic activity of DENV1-4pro proteases is assessed under these varied buffer conditions and FRET substrates.

		Enzymatic activity [RFU/s]			
FRET substrates [50 µM]	**Buffers**	**DENV1pro [500 nM]**	**DENV2pro [125 nM]**	**DENV3pro [125 nM]**	**DENV4pro [62.5 nM]**
DENV	**Brij**	7.775 ± 0.328	10.62 ± 1.29	10.58 ± 0.36	17.85 ± 2.41
	CHAPS	7.730 ± 0.619	13.17 ± 3.93	8.399 ± 1.595	28.18 ± 0.29
WNV	**Brij**	N/a	2.930 ± 0.043	2.62 ± 0.080	4.370 ± 0.212
	CHAPS	0.741 ± 0.122	1.841 ± 0.145	2.643 ± 0.062	4.159 ± 0.207
2A/2B	**Brij**	0.696 ± 0.072	0.840 ± 0.049	1.319 ± 0.199	0.682 ± 0.176
2B/3	**Brij**	11.37 ± 1.78	13.77 ± 0.38	6.530 ± 1.228	34.29 ± 1.90

The evaluation of the new substrates, 2A/2B and 2B/3 was followed. Such substrates have a peptide sequence based on DENV1pro natural cleavage sites, originating substrates 2A/2B and 2B/3 (2-Abz-Trp-Gly-Arg-Lys-Ser-(3-NO2)-Tyr-NH2 and 2-Abz-Lys-Lys-Gln-Arg-Ser-(3-NO2)-Tyr-NH2, respectively). These were only tested in the Brij buffer. The two substrates performed very contrary. In the assay with 2A/2B, the substrate was not well cleaved by any of the serotypes, with RFU/s ranging from 0.682 - 1.319 RFU/s. This substrate was not used for further assays. In contrast, 2B/3 substrate, showed the better cleavage rate in most cases. Looking at Table 6**Error! Reference source not found.**, DENV1pro, D ENV2pro and DENV4pro had the highest enzymatic activity, when cleaving 2B/3 substrate, compared

to DENV substrate. However, in DENV3pro, the assay with 2B/3 substrate showed lower enzymatic activity when compared to DENV substrate in Brij buffer. Important to add that, in the case of DENV4pro, higher concentrations of protease reached stagnation points in the enzymatic activity, when in the following assay conditions: 2B/3 substrate with Brij buffer and DENV substrate with both buffers.

In addition, the enzymatic activity of DENVpro with AMC substrates was tested. KE3 and KE6 (Bz-Nle-Lys-Lys-Arg-AMC and Bz-Nle-Lys-Arg-Arg-AMC, respectively), previously synthesized within the group, were applied in such assessments.[65] Enzyme activity with AMC substrates (KE3 and KE6) was also evaluated, using Brij buffer and CHAPS. The enzymatic activity at different concentrations of substrate (0 to 40 μM) and a fixed protease concentration (50 nM) was determined. The test included a negative control lacking protease.

In case of DENV1pro, due to the lack of activity with the used concentration, an additional test was conducted to assess enzymatic activity, with increasing protease concentration (0 to 200 nM) at a fixed substrate concentration (50 µM) of KE3 (Appendix 2.2). Based on the results of this assessment, a fixed protease concentration of 200 nM was chosen for subsequent evaluations involving DENV1pro. Figure 7 displays the results of the enzymatic activity of each protease presented as the mean of triplicates with the respective standard deviation.

Considering the results, generally assays with KE6 had a higher substrate cleavage compared to KE3 assays. Only with DENV1pro such was not observed, where, for example, at 50 µM of KE3 substrate, DENV1pro had an increase of 69 % and 48 %, compared to KE6 in Brij and CHAPS buffer, respectively. Analysing the buffer conditions, assays with CHAPS buffer showed higher proteolytic activity across all serotypes, while assays with Brij buffer showed mostly a lower activity. However, two punctual cases

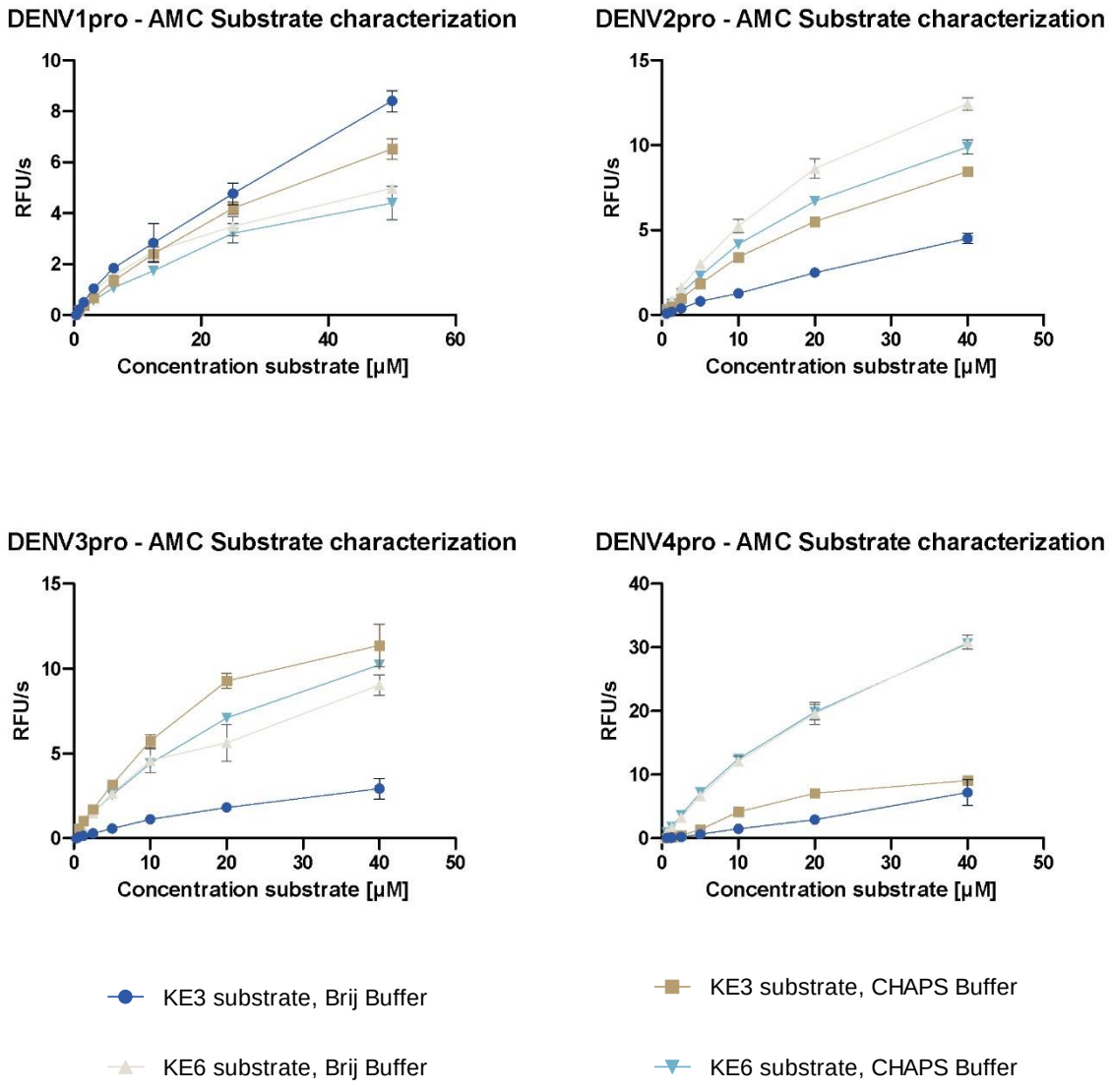

Figure 7 - Enzymatic activity of DENVpros with AMC substrates. Measurements were performed with different concentrations of KE3 and KE6 substrates (0 – 40 µM) and fixed concentrations of each protease (DENV1pro at 200 nM, DENV2-4pro at 50 nM). Brij and CHAPS buffer were used in all substrates assessments.

were identified, DENV1pro assay with KE3 and DENV2pro with KE6, where, assays in Brij buffer resulted in higher enzymatic activity. Notably, DENV3pro showed a distinct pattern, with assays using KE3 substrate showing both the highest and lowest activity, using CHAPS and Brij buffer, respectively.

3.10 Kinetics

Michelis-Menten kinetics were determined for DENV1-4pro using DENV substrate. Moreover, DENV1pro was further explored with 2B/3 substrate. Key parameters in enzyme kinetics include the maximum reaction rate ($V_{máx}$) and the substrate concentration at which the reaction rate is half of the $V_{máx}$ (K_m). Such parameters provide important information about effectiveness and affinity of a substrate for its substrate.

The following procedure was used to calculate the enzyme kinetics. DENV substrate and Brij buffer were the assays conditions implemented. Moreover, 2B/3 substrate in Brij buffer was also considered in the analysis of DENV1pro. Substrate solutions were added to wells of a black 96-well plate at concentrations ranging from 0 to 400 µM. The protease reactions were initiated by adding the prediluted protease at the predetermined constant concentration based on their enzymatic activity evaluation in section 3.4. To account for the inner filter effect, the measured values (RFU/s) for each substrate concentration were adjusted by multiplying with the concentration-dependent correction factor (Appendix 2.1).[44] The rate of cleaved substrate (µM/s) was calculated using a calibration curve of 2-Abz, previously generated within the group, to convert RFU/s to µM/s (Appendix 2.2).[65] K_m and $V_{máx}$ values were obtained by fitting the experimental data to the Michaelis-Menten equation using GraphPad Prism 5.04 software. The results, in Figure 8, are presented as the mean values and standard deviations, with Michaelis-Menten curves. Once obtained K_m and $V_{máx}$, such allowed to determine the turnover number, K_{cat}, and the catalytical efficiency of enzymes, K_{cat} / K_m. Table 7 summarizes the kinetic parameters for all DENVpro with the FRET substrates analysed.

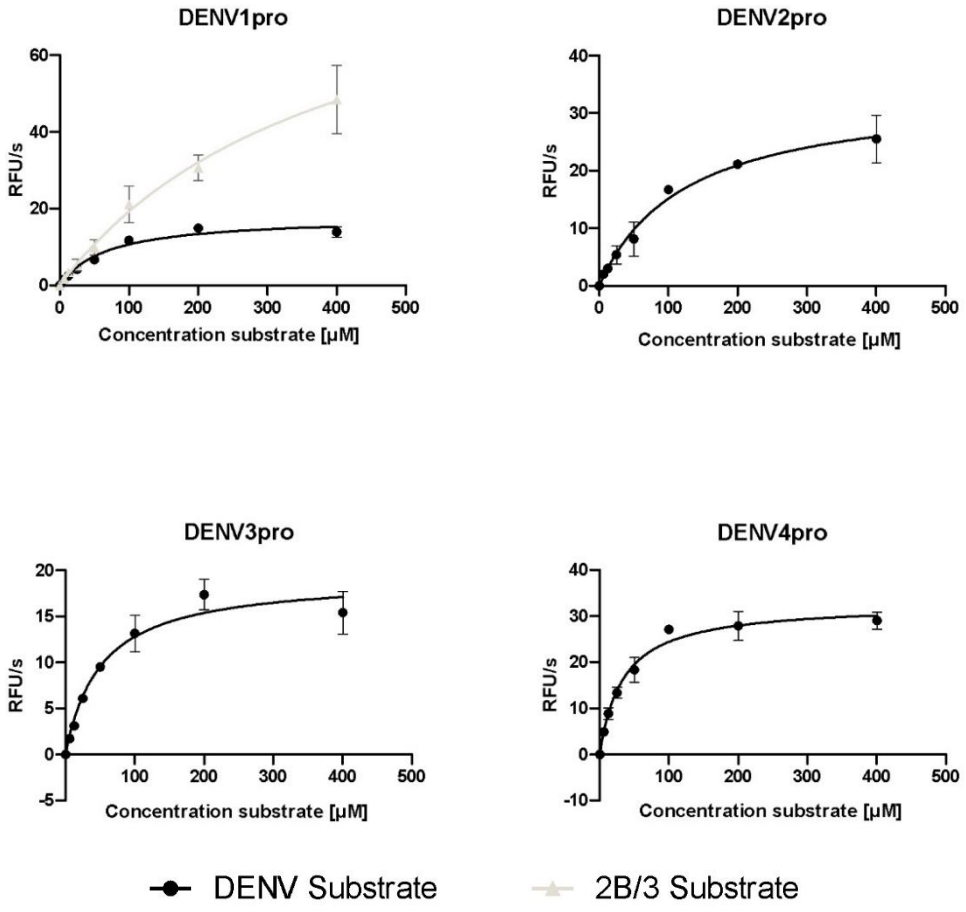

Figure 8 – Michaelis-Menten Kinetics for DENVpro and DENV and 2B/3 substrate. Activity of DENV1-4pro measured at fixed concentrations (400 nM, 100 nM, 100 nM and 50 nM, respectively) using DENV substrate at concentrations ranging from 0 µM to 400 µM in Brij buffer. Activity of DENV1pro was measured for same conditions for 2B/3 substrate.

Considering only DENV1pro, the assay with 2B/3 showed both a higher K_m and $V_{máx}$. However, such assay presented the highest standard deviation for both parameters, specifically 26 % and 16 % for K_m and $V_{máx}$, respectively. Looking at assays with DENV substrate employed, DENV2pro and DENV4pro had the highest $V_{máx}$, and DENV1pro and DENV3pro the lowest. However, it should be taken into account the different concentrations employed for each serotype. Regarding K_m, the highest value was observed in DENV2pro, followed by DENV1pro and DENV3pro, and finally DENV4pro with the lowest K_m value among the serotypes.

Table 7 - Enzymatic Kinetic Parameters of DENVpros with Different Substrates. Km, Vmáx, Kcat, and Kcat/Km values for DENV1-4pro, at fixed concentrations, interacting with different substrates.

Protease	Substrate	K_m [μM]	$V_{máx}$ (x10-3) [μM/s]	K_{cat} [s-1]	K_{cat}/K_m [M-1s.1]
DENV1pro [400 nM]	2B/3	399.3 ± 104.9	64 ± 10	0.16 ± 0.02	400
DENV2pro [100 nM]	DENV	68.59 ± 9.6	11.9 ± 0.6	0.030 ± 0.001	437
DENV3pro [100 nM]		124.0 ± 20.7	22.6 ± 1.6	0.23 ± 0.02	1855
DENV4pro [50 nM]		50.8 ± 8.4	12.8 ± 0.7	0.128 ± 0.007	2520
		33.9 ± 3.9	21.8 ± 0.7	0.44 ± 0.01	12981

3.11 Inhibition Assay

An inhibition assay was carried out, where potential inhibitory compounds were incubated with protease and substrate and the enzymatic activity was evaluated. The percentage of inhibition was calculated relative to a positive control with DMSO in the place of inhibitor. Brij buffer and DENV substrate, were the conditions employed in this assay. In the case of DENV1pro, the 2B/3 substrate was preferred.

A series of available compounds with inhibitory potential were implemented in such assay. MB-8 and MB-53, previously reported for DENV2pro, are commonly used in the group as reference compounds.[66] Also to validate the screening assay, aprotinin, a potent commercially available inhibitor, was also employed.[67] The remaining inhibitor tested were synthesized and are mostly published in inhibition research of dengue virus.[68-70]

In an initial screening several inhibitory compounds were tested at a concentration of 50 μM. The mean percentage of inhibition and standard deviation for each compound are shown in Table 8. The results showed varying degrees of inhibition for the tested compounds. The following compounds,

with relative inhibition above 90% were exploited for IC_{50} determination: aprotinin, MB-53, SB-63, MJ-04-32. MB-8 is a reference inhibitor, along with MB-53, and so was also studied in the assay.

For the IC_{50} assay, inhibitors were serially diluted starting from of 1 µM, 50 µM or 100 µM concentration, until at least a relative inhibition above 50 % was achieved. The mean and standard deviation of triplicates was plotted against the corresponding logarithmic concentrations to determine IC_{50} of the compounds, using nonlinear dose–response curves with variable slopes. From the experimental data, IC_{50} of each analysed compound was calculated, for each DENVpro, and are represented in Table 8.

In a further look to the results obtained, aprotinin was the most potent inhibitor tested. Such was expected , since it is a known and commercially available protease inhibitor.[71] MB-8 and MB-53 are reference compounds previously applied in DENV2pro assessments, hence its evaluation.[64] Considering the two tested successful inhibitor compounds, the IC_{50} of both MJ-04-32 and SB-63 were inferior to 10 µM in all assessments, except the interaction of MJ-04-32 with DENV1pro. In fact, in the presence of DENV1pro, SB-63 also showed its highest IC_{50} in this study. Nevertheless, despite being its highest, SB-63 has 10-fold lower IC_{50} in such serotype, compared to MJ-04-32. In the case of MJ-04-32, lowest IC_{50} is noticed in the DENV2pro, while in SB-63 is observed in DENV3pro, but with DENV2pro showing similar results. Such compounds are available in the group but are yet to be published (Appendix 2.4).

Table 8 – Relative inhibition of DENVpros activity at 50 **µM inhibitor concentration.**

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	Relative Inhibition at 50µM (%)			
Compounds	**DENV1pro (200 nM)**	**DENV2pro (100 nM)**	**DENV3pro (100 nM)**	**DENV4pro (50 nM)**
Control	0.0 ± 8.4	0.0 ± 2.5	0.0 ± 7.3	0.0 ± 4.6
MB-8	50.2 ± 5.6	70.4 ± 1.5	60.5 ± 6.7	59.8 ± 1.5
MB-53	94.5 ± 1.3	99.2 ± 0.6	105.5 ± 8.3	98.6 ± 0.8
Aprotinin	100.8 ± 1.6	98.6 ± 1.8	98.9 ± 4.9	98.3 ± 1.6
GG-92-1	14.2 ± 5.4	58.1 ± 4.0	55.3 ± 10.6	17.6 ± 3.4
MJ-04-32	97.5 ± 1-1	100.8 ± 2.1	102.5 ± 3.2	99.7 ± 2.0
NK-157	2.1 ± 5.5	17.6 ± 3.8	22.8 ± 10.8	-4.4 ± 6.9
NK-193	33.4 ± 4.6	14.0 ± 2.2	6.4 ± 12.2	2.2 ± 1.6
SB-63	94.2 ± 1.5	100.3 ± 3.2	98.0 ± 5.9	100.1 ± 1.3

Table 9 – IC_{50} valuesdetermined for compounds and DENVpro. MB-8, MB-53 and aprotinin are used as reference compounds

	IC $_{50}$ [µM]			
Compounds	**DENV1pro (200 nM)**	**DENV2pro (50 nM)**	**DENV3pro (50 nM)**	**DENV4pro (50 nM)**
MB-8 (0 - 100 µM)	43.8	43.2	48.9	34.3
MB-53 (0 – 50 µM)	1.16	0.895	1.07	0.207
Aprotinin (0 – 1 µM)	63.7×10^{-3}	99.3×10^{-3}	41.0×10^{-3}	23.7×10^{-3}
MJ-04-32 (0 – 50 µM)	13.2	0.593	0.934	4.95
SB-63 (0 – 50 µM)	1.17	0.235	0.963	0.174

4 Discussion

4.1 Design, expression and purification of DENVpro constructs

The enzymatically active DENV protease consists of the NS3 protease domain and needs NS2B as co-factor for proteolytic activity. However, parts of the NS2B and NS3 protein are membrane anchored, meaning hydrophobic which makes it a difficult task for soluble protein expression in E. coli.[72]
Concerning DENVpro construct design, a truncated NS2B with a 47-residue soluble core domain linked via a glycine-serine linker (G_4SG_4) to the protease domain of NS3, located in the N-terminally (approximately 185 amino acids) has been established.[24, 52, 73] Other researchers designed constructs with the full length of both proteins, including the hydrophobic sequence of NS2B and the helicase domain of NS3, with no linker and it resulted in the absence of proteolytic activity.[74] In this work, construct DENV1pro and DENV3pro were cloned using a pET-28a(+) vector, incorporated via restriction-free cloning. Such method proofed suitable, confirmed via agarose gel electrophoresis and sequencing, and was used for enzymatic testing. Various protocols employed similar strategies with successful outcomes. Indeed, Leung et al. developed and expressed such construct from DENV 2, with relevant levels of enzymatic activity and less prone to auto catalytical cleavage and precipitation, when expressed in E. coli. The difference to the approach of our study was opting for a pTM1 vector.[52] On the same study, a similar expression construct but without a glycine linker was obtained, but with significantly less catalytical activity.[52] Nevertheless, the DENVpro construct design protocol followed by the work group led to success in the steps to come.
Subsequently, a successful expression and purification of DENV1pro, DENV3pro and DENV4pro were achieved. Steps used are standardized in biochemical studies, including in the work group, however aiming for the optimization of DENVpro's case will benefit subsequent studies. In this project, impurities of lower molecular weight were detected after a two step purification of IMAC and SEC in all constructs via the SDS-PAGE analysis. Additionally, a second IMAC run in DENV4pro was necessary, due to purification inefficiency from the previous one. In order to improve purification, increasing volumes of washing buffer can be considered. In these wash buffers, small concentrations of imidazole were present. Imidazole competes with histidine residues for binding to metal ions, leading to the displacement of the target protein from the column.[75] In small concentrations, the imidazole can remove non-specific proteins with weak binding to the column, hence its use in such buffers. In this work, 10 mM of imidazole were used in wash buffers to remove untargeted proteins. However, increased volumes of such buffer would benefit te purification. An increase of imidazole concentration in wash buffers could aid in the non-specific proteins' removal. For example, in the purification of

DENV2pro,such approach was consider by Götz et al., where they applied 20 mM Imidazole in buffers previous to the elution of the target protein, followed by SEC.[47] Nevertheless, such measure might aim to target-proteins, affecting the yield of final product. Indeed, considering fig. x, SDS-PAGE analysis of IMAC samples show bands with the target molecular weight when analysing the washing flowthroughs. Alternatively, lowering the pH of wash buffer is another option.[75] Lowering the pH promotes the protonation of the imidazole nitrogen atom present in the histidine residue., disrupting the bond between the histidine residue and the transition metal. Considering endogenous proteins containing neighbouring histidine residues, these may be washed by lowering the protein. However, to do so, optimal pH for the washing step are protein dependent and need to be determined experimentally to avoid elution of the target protein.[75] Moreover, lower pH may damage the target protein.[75] Additional SEC runs would increase protein purification, but the loss of yield and the time consumption makes it a not preferred method. In the case of DENV4pro, there were two enhanced bands with the target molecular weight observed in the SDS-PAGE analysis, possibly indicating protein autocatalytical cleavage, despite these being more associated with non-truncated versions of the expression construct, which is not the case in this study.[72] To a deeper understanding, Western blot and in-gel proteolysis analysis could be performed to identify the nature of these bands. Lastly, two pools were formed for DENV3pro and DENV4pro, due to fractions collected from either different SEC runs, or different peaks observed in the SEC chromatogram, which showed slight differences in purity in the SDS-PAGE analysis. Therefore, two pools were formed to not jeopardize the purity of the product. Nevertheless, the employed protocol resulted in purified enzymes that successfully carried the purpose of the subsequent tests. Even though such procedures are well-established, changes in the protein expression and purification are used when studying the DENVpro complex. Bodenreider et al., achieved their intended purification levels with only an IMAC procedure, with a linear gradient of imidazole concentration from 20 to 300 mM being used in the elution steps.[76] Nevertheless, due to the standardization and proven record of the protocol followed, such should be considered, with the optimization modifications already mentioned in this discussion.

4.2 Biochemical assay conditions

Establishing parameters for the catalytical activity of DENVpro in vitro is crucial for achieving reliable results with physiological and physicochemical characteristics and can be considered in vitro/ in cellulo/ in vivo correlations. Various buffer parameters and substrates are employed among different studies. Such variations require a deeper understanding of the protease's functional properties, and its potential as therapeutic target can be achieved.

Initially, the enzymatic activity of the purified DENVpro constructs was assessed under standard assay conditions applied in the group.[44] From such results and in order to optimize the enzyme-to-sustrate ratio to achieve a considerable enzymatic activity, the following studies were carried out at the following concentrations: DENV1pro at 400 nM, DENV2pro and DENV3pro at 100 nM, DENV4pro at 50 nM. Iempridee *et. al* reported similar enzymatic activities, with DEN4pro has the highest, and DENV1pro has the lowest. [77] However, the enzymatic activity is dependent on assay conditions, including substrate recognition. In studies comparing the serotypes, despite DENV4pro showing consistently higher enzymatic activity, the difference between both serotypes shifts with the substrate changing. [24, 77] With such observations, one might consider that the synthesis of a specific substrate may lead to the shift of behaviour, making DENV1pro the most active protease.

The influence of different buffers parameters was investigated, similar to Steuer et al., where it was shown that it is worth optimizing the buffer conditions to get increased enzymatic activity and at the same time reduce pipetting mistakes due to viscosity or false detection of promiscuous inhibitor.[53] Due to time-consumption issues and to mitigate operator's error, substrate and protease utilized in the study of buffer's parameters assays were diluted in Tris-HCl buffer (50 mM, pH 9). First, the influence of pH was tested. Considering that Tris-HCl buffer has a pH range between 7 and 9, 0.5 variations among these values were considered. A decrease in substrate cleavage happens with the decrease of pH basicity for all serotypes. In the case of DENV1pro, such observations are less notable, but still observable with a slight shift from a pH 9 to 8.5 resulting in a decrease of 63 %. However, due to general low activity performances and high deviation, the reliability of the results should be questioned. Such observations for DENVpro 1 are applicable to most of the buffer parameters assays. The results obtained from the assessment of the pH influence are supported by previous studies that consider pH variations, where optimal activity was obtained with pH 9 and noticeable decrease for less basic buffers.[53, 78]

The addition of NaCl to test the influence of ionic strength was evaluated. The presence of the salt resulted in a big decrease of protease activity, with the raising of salt concentration resulting in similarly low or marginally lower concentrations. However, in DENV1pro, highest concentrations of NaCl had no significant difference when compared to standard conditions, probably due to the same observations previously stated. Kim *et al.* also tested the inclusion of NaCl in the protease complex of flavivirus, specifically DENV2 and WNV, with a decrease of substrate cleavage when compared to its absence.[79] In the same study, potassium chloride was also analysed, with similar decrease of enzymatic activity. Other groups also performed such tests, with similar outcomes as the ones from this study, leading to the conclusion that DENVpro has a low ionic strength tolerance.[53, 72]

Moreover, the testing of different polyols and concentrations was performed. Polyols are frequently employed in enzymatic assays due to their action in the stabilization of the tertiary structure of the protein. Moreover, they convey hydrogen bonds and lipophilic interactions among proteins and the different molecules.[53, 63] In general, higher concentrations of polyols meant higher enzymatic activity. However, such high concentrations can enhance the formation of aggregates, which will result in activity detection of promiscuous inhibitors, and false positives in inhibitor screenings. Therefore, an optimal concentration should be the target.[53] Furthermore, buffers containing 20% of glycerine resulted in considerable increases of activity in all serotypes. Glycerine is indeed commonly used in such enzymatic assays, with concentrations varying from 5 % to 30%, due to a positive contribution in the substrate cleavage.[80-83] However, besides the high concentrations related issues, glycerine has a high viscosity which, in turn, negatively affects the mixing and pipetting process. Steuer *et al.* analysed less viscous polyols replacements to overcome such detrimental effects, including propylene glycol and ethylene glycol, with both showing suitable results to carry enzymatic assays.[53] An inclusion of 20% propylene glycol showed either similar or higher enzymatic activity, when compared to the assays using standard conditions, 10% ethylene glycol. However, higher concentration constraints previously mentioned lead to consider 10 % ethylene glycol as the optimal balance between enzymatic activity and assay reliability.

Detergents are included in the assay buffer in order to increase solubility and maintain protein stability, preventing the formation of colloidal aggregates, by providing a membrane-like microenvironment, by mimicking the properties of natural lipid bilayers.[2] When testing different concentrations of Brij 58, the general results indicated lower activity in the absence of the detergent, but no relevant influence in the concentration shifting. Thereafter, different detergents influence in the assay's buffer were tested. CHAPS buffer was prepared with 1 mM of the detergent, concentration commonly used in this and several other groups.[76, 84, 85] The remaining detergents were prepared with 0.2 times the respective CMC, to avoid the formation the micelles that would affect the assay reliability.[59] When assessing different detergents, CHAPS presence in the assay buffer had the highest substrate cleavage throughout all serotypes, with no relevant differences compared to the standard detergent for DENV1pro and DENV4pro, even though the previous reasons stated for DENV1pro assays and a high deviation of the standard buffer assay in DENV4pro was observed. Indeed, CHAPS detergent at 1 mM is used in several DENV assays among research groups, due to its increasing effect on enzymatic activity.[50, 60, 73, 85] However, when considering inhibition assays, CHAPS presence in buffer may affect the reliability of the tests. Being a zwitterionic detergent, there is an increased risk of CHAPS interacting with charged inhibitor moieties, leading to inactivation and false-negative results.[2] Since the goal of this study is to contribute to the elaboration of a buffer for

reliable and efficient inhibitor screening campaign, other options should be taken into account, and so non-ionic detergents shall be considered. A study compared the influence of different detergents in the activity of DENV2pro, including CHAPS and Trinton X-100, a non-ionic detergent. In such study, although there was a decrease in enzymatic activity in the Trinton X-100 assay compared to CHAPS, Leung et al. observed no relevant difference among the different detergents, leading to consideration non-ionic detergents. [71] Aware of the constraints in using CHAPS, Steuer et al. tested different non-ionic detergents showing promising results for Brij 58 and Triton X-100.[53] Ultimately, considering the results and the literature, CHAPS is a viable option for testing enzymatic activity, but due its limitation in reliability for inhibitor screening, Brij 58 should be preferred. Considering results and observations, further investigations consider both Brij buffer (50 mM Tris-HCl, pH 9, 10 % ethylene glycol and 0.0016 % Brij 58) and CHAPS buffer (50 mM Tris-HCl, pH 9, 10 % ethylene glycol and 1 mM CHAPS).

Once established buffer and protease conditions, the research proceeded with the investigation of different substrates. For such, interactions with FRET and AMC substrates were assessed, with increasing and fixed concentrations of protease and substrate, respectively. Other substrates are employed in different studies, such as chromogenic substrates with thiobenzyl ester moiety in p1', but AMC and FRET substrates are the more commonly employed in such assays. Starting with FRET substrates, the assessment of the interaction between the protease and two FRET substrates was made: DENV (2-Abz-Nle-Lys-Arg-Arg-Ser-(3-NO2)-Tyr-NH2) substrate and WNV (2-Abz-Gly-Lys-Lys-Arg-Gly-(3-NO3)-Tyr-Ala-Lys-NH2) substrate. These were tested for the four different serotypes, using Brij and Chaps buffer conditions. The DENV substrate was designed considering the natural cleavage sites of DENV3pro, and it is commonly employed in biochemical assays of the protease within the group.[44, 53] The WNV substrate was initially designed for the evaluation of WNV virus biochemical assays. However, due to the structural and biochemical similarities shared among flaviviral proteases, it was decided to explore its activity.[53] Considering the obtained results, a relevant decrease in enzymatic activity, in the assay containing WNV substrate, was observed in all serotypes when compared to the assays with DENV substrate. It can be considered that WNV substrate's peptide sequence, 2-Abz-Gly-Leu-Lys-Arg-Gly-Gly-Tyr(3-NO3)-NH2, fails to closely mimic DENVpro natural cleavage sites in the viral polypeptide, hence the decrease of the activity. Interactions of the DENV substrate with different DENV serotype proteases, as previously observed, showed relevant protease activity, except for DENV1pro. Moreover, comparing buffer conditions, substrates in CHAPS buffer were generally cleaved at higher rates. This is supported by researches made in the analysis of detergent influence.[52] Therefore, two novel substrates were synthesized by solid-phase peptide synthesis using the Fmoc strategy. In the conception of such substrates, similar strategies were

considered as the DENV substrate, with equal peptide sequence length, and same fluorophore and quencher in similar locations, P4 and P1', respectively. However, the peptide sequence replicated the ones of DENV1pro natural cleavage sites between NS2A and 2B or NS2B and 3. From these considerations, and looking at table 1, resulted 2A/2B substrate, 2-Abz-Trp-Gly-Arg-Lys-Ser-(3-NO2)-Tyr-NH2, and 2B/3, 2-Abz-Lys-Lys-Gln-Arg-Ser-(3-NO2)-Tyr-NH2. Such substrates were evaluated in their interactions with DENVpro from all serotypes, but only using DENV buffer. Similar to WNV substrate, 2A/2B application in the biochemical assay resulted in low enzymatic activity in all DENVpros, including DENV1pro, and will not be considered in further investigations. The implementation of 2B/3 in the assay resulted in considerable substrate cleavage, generally showing higher activity compared to DENV substrate in the same buffer. Such was true except for DENV3pro, where DENV substrate exhibited higher enzymatic activity. This observation can be attributed to the fact that the peptide sequence of the DENV substrate corresponds to a natural cleavage site of DENV3pro, suggesting a higher affinity between the enzyme and substrate, as supported by the experimental data.

AMC substrates are also heavily employed in the assay of DENVpro.[24, 73, 84](who does that as well?) Therefore, we assessed this type of substrates in this biochemical assay. Two substrates, previously synthesized for Zika virus related assays, namely KE3 (Bz-Nle-Lys-Arg-Arg-AMC) and KE6 (Bz-Nle-Lys-Lys-Arg-AMC) were used in this study. Under fixed protease concentrations, the enzyme activity was observed using different substrates concentrations. KE6 demonstrated higher enzymatic activity compared to K6. Notably, DENV1pro exhibited a distinct behaviour, showing preference for KE3 over KE6, with increases up to 69 % of the enzymatic activity. These substrates differ in the P2 amino acid, with KE3 having an arginine ad KE6 a lysine. Both, along with the similar P1, dibasic sequences which usually means strong affinity with DENVpro. [2] Considering the natural cleavage sites, both are replicated in many cases. It is worth to mention that these results were achieved using half of the enzyme concentration and with a 10-fold lower substrate concentration compared to FRET-based substrate assays. Exploring AMC substrates in the enzymatic assays, with wider ranges of protease and substrate concentration could potentially optimize the assay, with less enzymatic material being necessary. However, due to limitations of substrate availability, and time constraints to perform new synthesis, further considerations and kinetics studies were not possible in this study.

4.3 Enzyme kinetics

The meticulous study of assay conditions, with selection of appropriate buffer and substrate, are aimed to ensure the reliability of in vitro experimental setup. Such achievement allows a further

experimental study of the enzyme kinetics. These studies offer valuable insights into the enzyme behaviour and functionality, such as substrate affinity, reaction rates and catalytic properties.

In order to characterize enzymes and their interactions with substrates, several enzymatic parameters were determined, including K_m, $V_{máx}$, K_{cat}, and K_{cat} / K_m. However, comparing results across different studies can be challenging due to variations in assay conditions, protease constructs, and substrate options. To ensure consistency, the standard conditions in this work, including buffer parameters and substrate, were the same employed within the group. Comparing the enzyme kinetics parameters of DENV2pro from this study to those obtained within the group, it was found that values such as K_m were very similar (124 ± 20.7 µM in comparison to 105 µM in previously published work).[68, 86, 87] Such results support the successful implementation of the protocol and enhance the reliability of the obtained results. With similar conditions all the serotypes in this work were tested. Observing the K_{cat} / K_m of the different strains, the most catalytic efficient protease in this study was DENV4pro, with 12 861 $M^{-1}s^{-1}$, followed by DENV3pro, DENV2pro and, lastly, DENV1pro with 435.5 $M^{-1}s^{-1}$. Iempridee et al. also performed biochemical analysis to the four dengue virus serotypes and observed similar catalytical efficiency, with the construct from DENV 4 as the most efficient and, oppositely, DENV 1 as the less efficient.[77] In such study, similar expression constructs were used, assay buffer with slight changes and a FRET-based substrate, but with a different peptide sequence.

Additionally, it was decided to evaluate, in the case of DENV1pro, the novel 2B/3 substrate (2-Abz-Lys-Lys-Gln-Arg-Ser-(3-NO2)-Tyr-NH2), to improve the cleavage efficiency. Even though the turnover number, K_{cat}, had a superior value, when compared to the previous tested substrate, the Michaellis constant K_m also was far superior. Therefore, the interaction of DENV1pro with the two different substrates resulted in a similar catalytic efficiency. Based on these results, the adoption of the novel substrate in future studies of this research group can be considered. Although there was no increase in catalytic efficiency, the higher RFU/s detected in FRET assays could make it more suitable for inhibitors screening campaigns. However, the lack of data with this substrate should be noted, making it an obstacle when correlations with previous works needs to be taken into account.

4.4 Inhibition assay

The in-depth study of enzyme expression and purification and optimization of assay conditions had as main goal to establish a high-throughput fluorometric assay for evaluating potential inhibitors of the dengue protease. The successful implementation of this assay would further enhance the credibility of the previous stages.

A selected set of inhibitor compounds, available and tested in the group for DENV2pro, were evaluated against all four proteases.[66, 68-70] A screening of compounds resulted in a follow-up IC_{50} assay for the more potent inhibitors, enabling the characterization of each compound.[69] Aprotinin was the most potent inhibitor, with IC_{50} values reaching the nanomolar range. Aprotinin is a polypeptide with reported high affinity to proteases, including DENVpro.[88] Due to the successful record and commercial availability, this compound is usually employed as a reference compound. Similarly, MB-53 and MB-8, usually employed in DENV2pro biochemical assays, are also used as reference compounds in such assays.[64] Even though results showed expected behaviour, from previous assessments, a more in-depth understanding of the inhibition properties of MB-53 and MB-8 with DENVpro benefits the efficiency and reliability of future screening tests.

SB-63 and MJ-04-32 showed the highest affinity for the four serotypes, excluding the reference compounds, indicating significant relevance in these systems and their potential for future studies and publications.

Although no inhibition was assessed, Kühl *et al.* reported inhibition properties in NK-157 and NK-193 in DENV2pro.[69] However, the choice of the assay conditions, such as the preference for a CHAPS detergent, may influence thestudy outcomes. Therefore, further assessment of these compounds, with the different DENVpro serotypes under more favourable assay conditions is recommended to fully understand the potential of NK-157 and NK-193.

5 Conclusion

The aim of this project was to, initially, establish a common protocol for the design, expression, and purification of different DENVpro serotypes. Subsequently, the optimization of FRET-base conditions was studied, through the evaluation of buffer parameters and the assessment of various substrates. Additionally, the characterization of enzyme kinetics of the four DENVpro serotypes was performed, along with the testing of inhibiting potential from a selection of established DENV2pro inhibitor compounds.

The design, expression and purification of the target serotypes were successfully achieved, although with potential for further improvements, since all proteases displayed enzymatic activity under standard conditions previously established in the work group.

The evaluation of parameters to guarantee optimal catalytic activity of all protease strains was conducted. The conditions employed by the group in the assessment of DENV2pro, including buffer pH, additives and substrate preference, appeared to be suitable for the remaining serotypes. Adjustments can still be performed to optimize the assessment efficiency. Considering variations in catalytical activity among serotypes and adapting the protease/substrate ratio, accordingly, is one measure consider. Notably, DENV1pro manifested the lowest levels of substrate cleavage, and despite efforts in the design and synthesis of novel substrates based on its natural cleavage sites, unsatisfactory results were achieved. Therefore, further study into the substrate specificity of DENV1pro is recommended for the deeper understanding of this particular serotype.

Inhibition assays that were successfully applied with DENV2pro, in previous works from the group, also demonstrated inhibitory properties against other serotypes. However, this study failed to replicate the assay buffer conditions, particularly the usage of CHAPS detergent, which were employed in such research. Consequently, different outcomes were observed, specifically the absence of inhibition. New screening of these compounds using assay conditions that have been validated would benefit the deeper understanding of these compounds and their interaction with the different proteases.

Despite the increase and progress of recent studies, the multitude of assay conditions and protease constructs promotes the absence of standardized assay, impeding to establish optimal conditions across the research community. The lack of uniformity can be beneficial, in the sense that several different approaches can arise, but also be a challenge in the advance of therapeutic approaches for dengue virus. Therefore, focus on the reproducibility and robustness, across laboratories, of an established optimal assay can be crucial. Such measures would increase coherence and quality control in DENV studies, contributing for the progress of therapeutic measures.

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Appendix

1 Cloning

1.1 DENV1pro expression construct

Primer's sequences DENV1pro

Table A 1 – Primer's sequences of DENV1pro expression construct

Name	Primer 5' (ovelap/ANNEAL) 3'
NS2B_fwd	actttaagaaggagatataccatgGCCGATTTATCACTGGAG
NS2B_rev	gccaccaccaccgctaccaccaccaccATCTCTCTCTTCATCTTTTATC
N3S_fwd	ggtggtggtggtagcggtggtggtggcTCAGGAGTGCTATGGGAC
N3S_rev	agtggtggtggtggtggtgctcgagTTTCCTAAACACCTCGTCC

Nucleotide sequence of DENV1pro expression construct

CTCTAGAATAATTTTGTTTAACTTTAAGAAGGAGATATACCATGGCCGATTTATCACTGGAGAAAGCGGCTGA
GGTCTCCTGGGAAGAAGAAGCAGAACACTCTGGTGCCTCACACAACATATTAGTGGAGGTCCAAGATGATGG
AACCATGAAGATAAAAGATGAAGAGAGAGATGGTGGTGGTGGTAGCGGTGGTGGTGGCTCAGGAGTGCTAT
GGGACACACCTAGCCCTCCAGAAGTGGAAAGAGCAGTTCTTGACGATGGTATCTATAGAATCATGCAGAGAG
GACTGTTGGGCAGGTCCCAAGTAGGGGTAGGAGTTTTTCAAGAAAACGTGTTCCACACAATGTGGCATGTCA
CCAGGGGAGCTGTACTCATGTATCAAGGGAAGAGACTGGAACCGAGCTGGGCCAGTGTCAAAAAAGACCTG
ATCTCATATGGAGGAGGTTGGAGGCTTCAAGGATCCTGGAACACGGGAGAAGAAGTGCAGGTGATTGCTGTT
GAACCAGGGAAAAACCCCAAAAATGTACAAACAGCGCCAGGCACCTTTAAGACTCCTGAAGGCGAAGTTGGA
GCCATTGCCCTAGATTTTAAACCCGGCACATCTGGATCTCCCATCGTGAACAGAGAAGGGAAAATAGTAGGTC
TTTATGGAAATGGAGTAGTGACAACAAGTGGAACCTATGTCAGTGCCATAGCTCAAGCCAAAGCATCACAAG
AAGGGCCCCTACCAGAGATTGAAGACGAGGTGTTTAGGAAACTCGAGCACCACCACCACCACCACTGAGATC
CGGCTGCTAACAAAGCCCGAAAGGAAGCTGAGTTGGCTGCTGCCACCGCTGAGCAATAACTAGCATAACCCC
TTGGGGCCTCTAAACGGGTCTTGAGGGGTTTTTTGCTGAAAGGAGGAACTATATCCGGATTGGCGAATGGGA
CGCGCCCTGTAGCGGCGCATTAAGCGCGGCGGGTGTGGTGGTTACGCGCAGCGTGACCGCTACACTTGCCAG
CGCCCTAGCGCCCGCTCCTTTCGCTTCTTCCCTTCCTTTCTCGCCACGTTCGCCGGCTTTCCCCGTCAAGCTCTAA
ATCGG

Amino acid sequence of DENV1pro expression construct

MADLSLEKAAEVSWEEEAEHSGASHNILVEVQDDGTMKIKDEERDGGGGSGGGGSGVLWDTPSPPEVERAVLD
DGIYRIMQRGLLGRSQVGVGVFQENVFHTMWHVTRGAVLMYQGKRLEPSWASVKKDLISYGGGWRLQGSWN
TGEEVQVIAVEPGKNPKNVQTAPGTFKTPEGEVGAIALDFKPGTSGSPIVNREGKIVGLYGNGVVTTSGTYVSAIAQ
AKASQEGPLPEIEDEVFRKLEHHHHHH-

Molecular weight: 26701.75 D

Ext. coefficient 40450

Abs 0.1% (=1 g/l) 1.515

1.2 DENV2pro expression construct

Amino acid sequence of DENV2pro expression construct

MADLELERAADVKWEDQAEISGSSPILSITISEDGSMSIKNEEEEGGGGSGGGGAGVLWDVPSPPPVGKAELEDG
AYRIKQKGILGYSQIGAGVYKEGTFHTMWHVTRGAVLMHKGKRIEPSWADVKKDLISYGGGWKLEGEWKEGEEV
QVLALEPGKNPRAVQTKPGLFKTNAGTIGAVSLDFSPGTAGSPIIDKKGKVVGLYGNGVVTRSGAYVSAIAQTEKS
DNPEIEDDIFRKLVPRGSTSSVDKLAAALEHHHHHH-

Molecular weight: 27875.21 D

Ext. coefficient 41940

Abs 0.1% (=1 g/l) 1.505

1.3 DENV3pro expression construct

Primer's sequences DENV3pro

Table A 2 - Primer's sequences of DENV3pro expression construct

Name	Primer 5' (ovelap/ANNEAL) 3'
NS2B_fwd	actttaagaaggagatataccatgGCAGACCTCACTGTAGAAAAAG
NS2B_rev	gccaccaccaccgctaccaccaccaccCTCAGTCTCGTCATCTTTTATTC
N3S_fwd	ggtggtggtggtagcggtggtggtggcTCCGGCGTCCTATGGGAC
N3S_rev	agtggtggtggtggtggtgctcgagCTTTTTGAACATCTCTTCTTCCAACTCTG

Nucleotide sequence of DENV3pro expression construct

GAATAATTTTGTTTAACTTTAAGAAGGAGATATACCATGGCAGACCTCACTGTAGAAAAAGCAGCAGATGTAA
CATGGGAGGAAGAGGCCGAGCAAACAGGAGTGTCCCACAATTTAATGATCACAGTTGATGATGATGGAACAA
TGAGAATAAAAGATGACGAGACTGAGGGTGGTGGTGGTAGCGGTGGTGGTGGCTCCGGCGTCCTATGGGAC
GTACCCAGCCCCCCAGAGACACAGAAAGCGGAACTGGAAGAAGGGGTCTATAGGATCAAACAGCAAGGAAT
TTTTGGGAAAACCCAAGTGGGGGTTGGAGTACAGAAAGAAGGAGTTTTCCACACCATGTGGCACGTCACAAG
AGGGGCAGTGTTGACACACAATGGGAAAAGACTGGAACCAAACTGGGCTAGCGTGAAAAAAGATCTGATTTC
ATACGGAGGAGGATGGAGATTGAGTGCACAATGGCAAAAGGGGGAGGAGGTGCAGGTTATTGCCGTAGAG
CCTGGGAAGAACCCAAAGAACTTTCAAACCATGCCAGGCATTTTTCAGACAACAACAGGGGAAATAGGAGCA
ATTGCACTGGATTTCAAGCCTGGAACTTCAGGATCTCCCATCATAAACAGAGAGGGAAAGGTAGTGGGACTG
TATGGCAATGGAGTGGTTACAAAGAATGGAGGCTATGTCAGTGGAATAGCGCAAACAAATGCAGAACCAGAT
GGACCGACACCAGAGTTGGAAGAAGAGATGTTCAAAAAGCTCGAGCACCACCACCACCACCACTGAGATCCG
GCTGCTAACAAAGCCCGAAAGGAAGCTGAGTTGGCTGCTGCCACCGCTGAGCAATAACTAGCATAACCCCTT
GGGGCCTCTAAACGGGTCTTGAGGGGTTTTTTGCTGAAAGGAGGAACTATATCCGGATTGGCGAATGGGACG
CGCCCTGTAGCGGCGCATTAAGCGCGGCGGGTGTGGTGGTTACGCGCAGCGTGACCGCTACACTTGCCAGCG
CCCTAGCGCCCGCTCCTTTCGCTTTCTTCCCTTCCTTTCTCGCCACGTTCGCCGGCT

Amino acid sequence of DENV3pro expression construct

MADLTVEKAADVTWEEEAEQTGVSHNLMITVDDDGTMRIKDDETEGGGGSGGGGSGVLWDVPSPPETQKAEL
EEGVYRIKQQGIFGKTQVGVGVQKEGVFHTMWHVTRGAVLTHNGKRLEPNWASVKKDLISYGGGWRLSAQWQ
KGEEVQVIAVEPGKNPKNFQTMPGIFQTTTGEIGAIALDFKPGTSGSPIINREGKVVGLYGNGVVTKNGGYVSGIAQ
TNAEPDGPTPELEEEMFKKLEHHHHHH

Molecular weight: 26763.83 D

Ext. coefficient 38960

Abs 0.1% (=1 g/l) 1.456

1.4 DENV4pro expression construct

Nucleotide sequence of DENV4pro expression construct

AAGGAGATATACCATGGCAGACCTGTCACTAGAGAAGGCCGCCAATGWGCAGTGGGATGAGATGGCAGAC
ATAACAGGCTCAAGCCCAATCATAGAAGTGAAGCAGGATGAAGATGGCTCTTTCTCCATACGGGACATCGAG
GAAACCGGTGGCGGWGGCAGCGGTGGCGGTGGCTCAGGAGCCCTGTGGGACGTCCCCTCACCTGCTGCCGC
TCAGAAAGCCACACTGACTGAAGGAGTGTACAGGATCATGCAAAGAGGGTTGTTTGGGAAAACTCAGGKTGG
AGKAGGGATACACRTGGAAGGKGTATTTCMCACAATGTGGCATGTAACAAGAGGATCAGTGATCTGCCATGA
GACTGGGAGACTGGAGCCATCTTGGGCTGACGKCAGGAACGACATGATATCATACGGTGGGGGATGGAGGC
TCGGAGACARATGGGACAAAGAAGAAGATGTTCAGGTTCTAGCTATAGAACCAGGAAAAAATCCGAAACATG
TCCAAACGAAGCCCGGCCTTTTCAAAACCCTAACTGGAGAAATTGGAGCAGTGACATTGGACTTCAAACCCGG
AACATCTGGKTCTCCCATCATTAACAGGAAAGGAAAAGTTATCGGACTCTACGGAAATGGAGTAGTCACTAAA
TCAGGTGATTACGTCAGTGCTATAACGCRAGCCGAAAGAACTGGTGAGCCAGATTATGAAGTGGATGAGGAC
ATTTTTCGAAAGAAAAGACTGGTGCCGCGCGGCAGCCACCACCACCACCACCACTGAGATCCGGCTGCTAACA
AAGCCCGAAAGGAAGCTGAGTTGGCTGCTGCCACCGCTGAGCAATAACTAGCATAACCCCTTGGGCCTCTAA
ACGGTCTTGAGGGGTTTTTTGCTGAAAGGAGGAACTATATCCGGATT

Amino acid sequence of DENV3pro expression construct

MADLSLEKAANXQWDEMADITGSSPIIEVKQDEDGSFSIRDIEETGGXGSGGGGSGALWDVPSPAAAQKATLTEG
VYRIMQRGLFGKTQXGXGIHXEXVFXTMWHVTRGSVICHETGRLEPSWADXRNDMISYGGGWRLGDXWDKEE
DVQVLAIEPGKNPKHVQTKPGLFKTLTGEIGAVTLDFKPGTSXSPIINRKGKVIGLYGNGVVTKSGDYVSAITXAERT
GEPDYEVDEDIFRKKRLVPRGSHHHHHH-

Molecular weight: 27559.04

Ext. coefficient 40450

Abs 0.1% (=1 g/l) 1.468

2 Biochemical assay

2.1 Correction factors for the inner filter effect at higher concentrations

Table A 3 Correction factors for the inner fi lter effect of the substrate at eight

Substrate concentration (µM)	Correction factor
25	1.0492
50	1.0495
75	1.1784
100	1.3743
150	1.5357
200	1.6451
300	1.9582
400	2.3748

2.2 Substrate characterization

Biochemical assay for determination of the enzymatic activity with AMC substrates

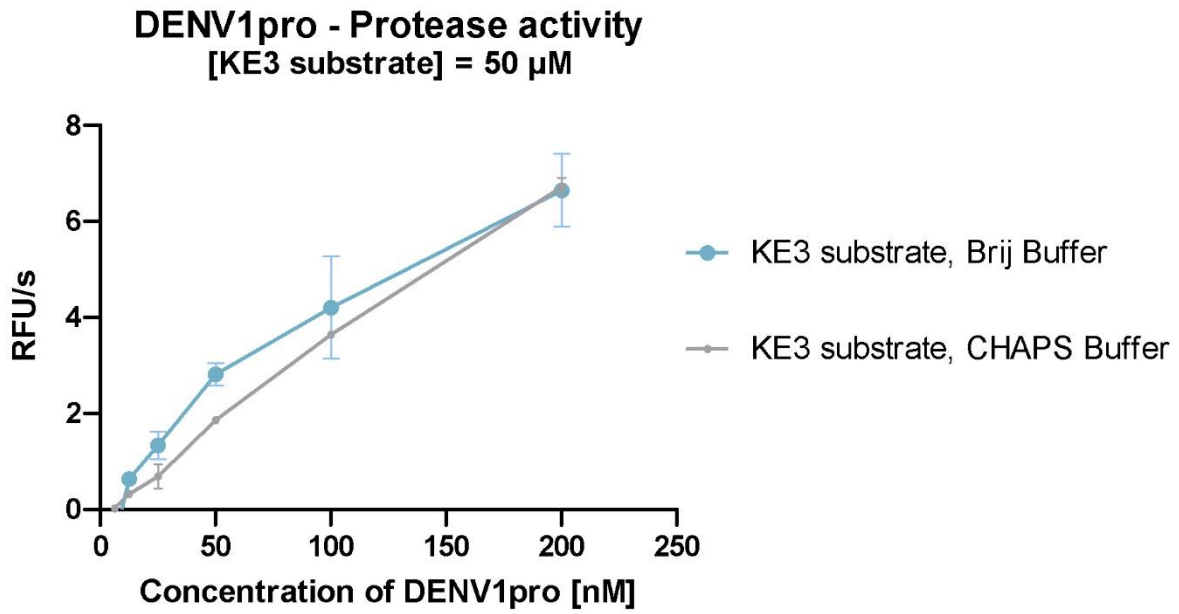


Figure A9 - - Enzymatic activity of DENV1pro with different AMC substrates. Measurements were performed with KE3 and KE6 (50 µM) at different concentrations of each protease (0 – 200 nM), with both Brij and CHAPS buffer was used in all substrates assessments, CHAPS buffer was used in DENV and WNV substrate.

2-Abz calibration curve

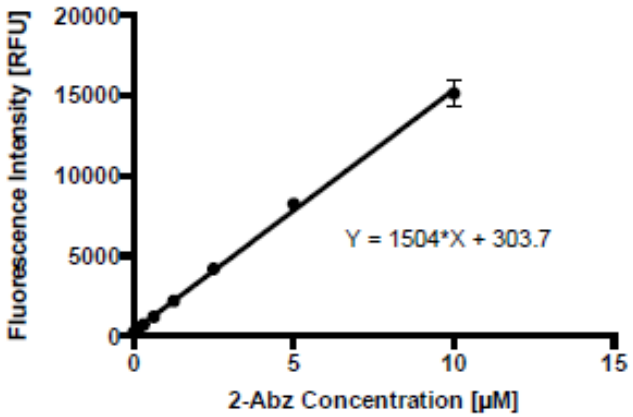


Figure A10 - 2-Abz calibration curve. Fluorescence of free 2-Abz detected at eight different concentrations ranging from 0 to 10 µM 2-Abz. Measured fluorescence values were plotted against the 2-Abz concentrations and linear regression was performed. The slope of the curve was used for conversion of RFU/s to µM/s.

2.3 Inhibition Assay

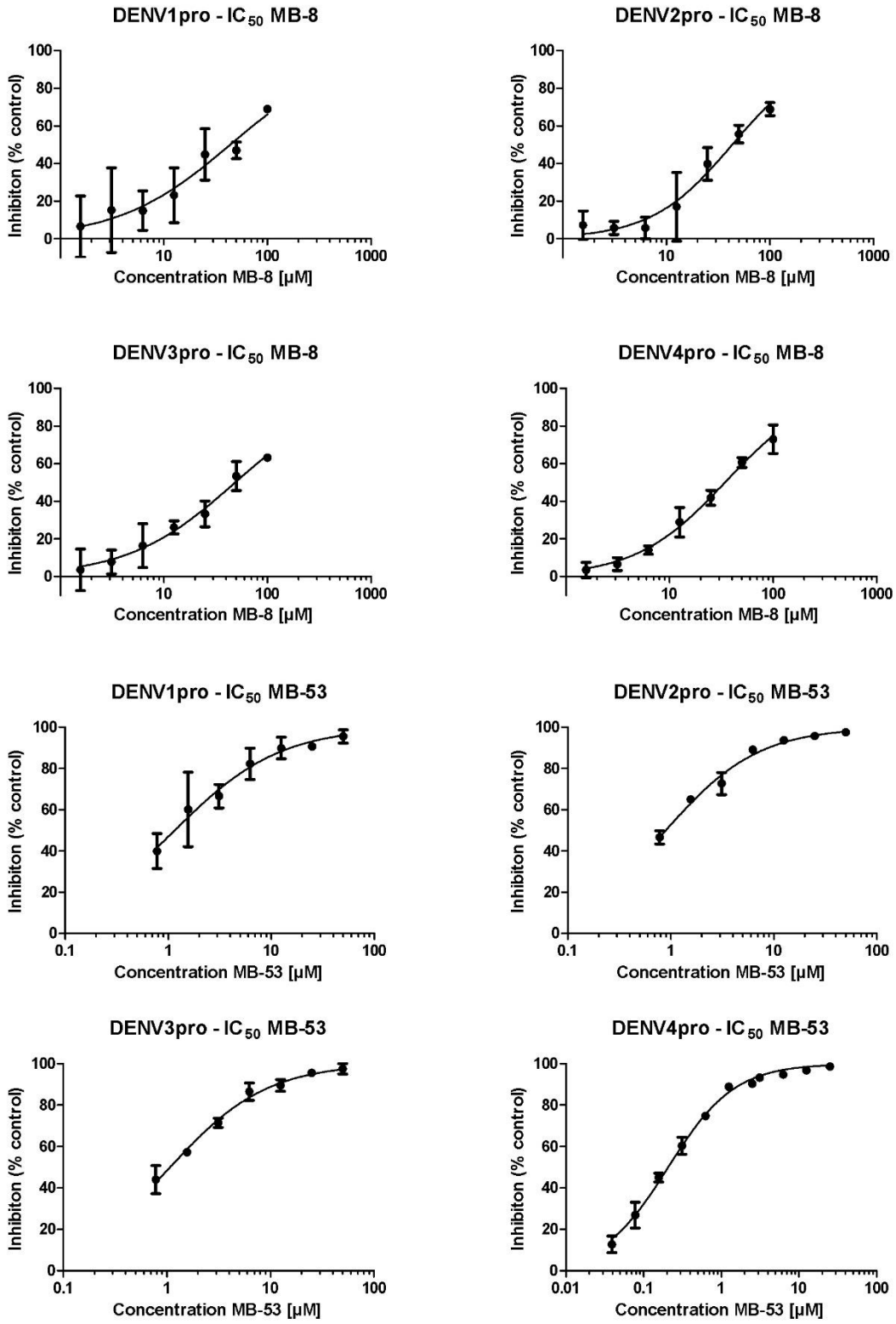


Figure A11 - IC_{50} for MB-8 and MB-53 and DENV1-4pro. A. IC_{50} curve for MB-8 and MB-53 generated with GraphPad Prism (log(inhibitor) vs. normalized response – variable slope).

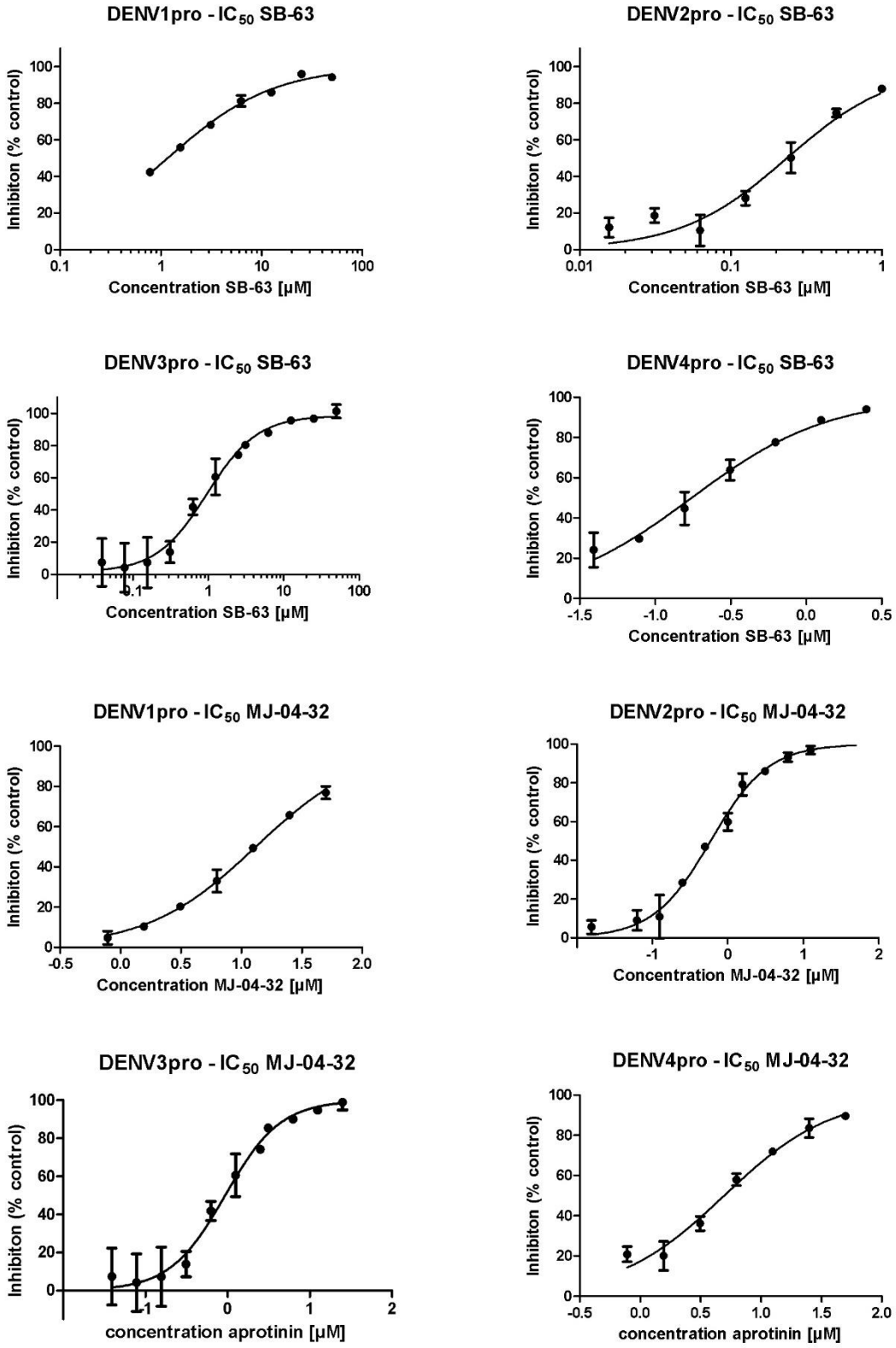


Figure A12 - IC_{50} for SB-63 and MJ-04-32 and DENV1-4pro. A. IC_{50} curve for SB-63 and M-04-32 generated with GraphPad Prism (log(inhibitor) vs. normalized response – variable slope).

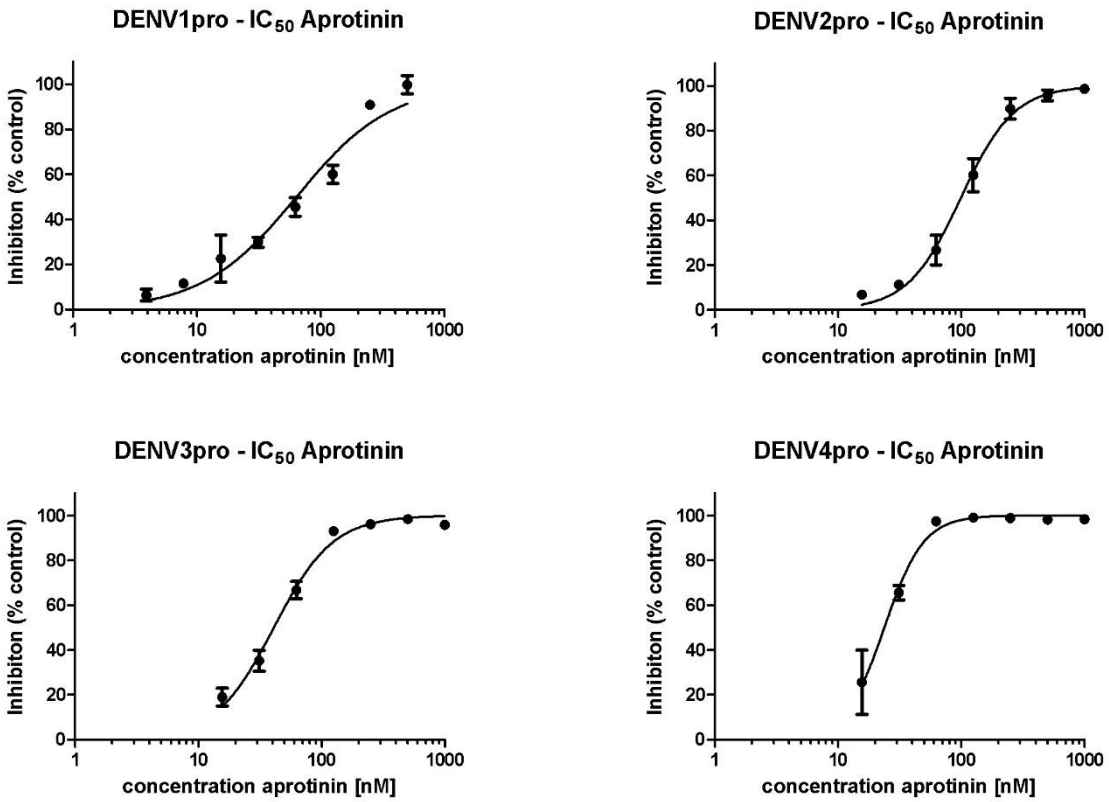
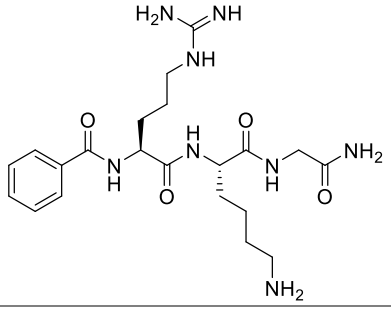
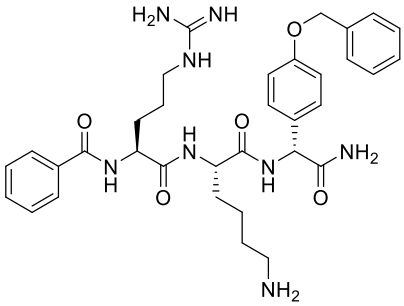
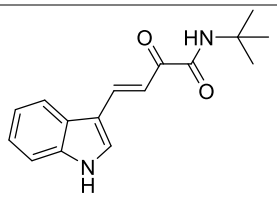
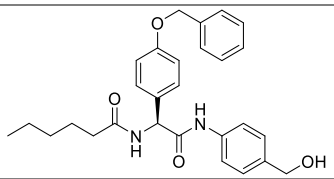
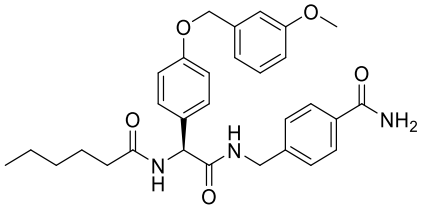


Figure A13 - IC_{50} for aprotinin and DENV1-4pro. A. IC_{50} curve for aprotinin generated with GraphPad Prism (log(inhibitor) vs. normalized response – variable slope).

2.4 Inhibtors Compounds

Table A2 – Inhibitors compounds and respective structural formula and source

Name	Structural formula	Source
MB-8		*J. Med. Chem*. **2015**, 58, 9354−9370. doi:10.1021/acs.jmedchem.5b01441
MB-53		
GG-92		*Bioorg. Med. Chem*. **2011**, 19, 4067–4074. doi:10.1016/j.bmc.2011.05.015
MJ-04	Unpublished	?
NK-157		https://doi.org/10.1021/acs.jmedchem.0c02042 *J. Med. Chem*. **2021**, 64, 4567−4587
NK-193		
SB-63	Unpublished	?