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Original Article

Design, Synthesis, and in-*vitro* Protease Inhibition Assay of Linear Pentapeptides as Potential Dengue Virus NS2B/NS3 Protease Inhibitors

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Abstract

Nowadays dengue virus infection (DENV) is one of the major health complications in the world. Although DENV is an old and common disease, unfortunately, until now, there are no specific relevant treatments available for it. This study, therefore, aimed to design, as well as synthesize selective peptide inhibitors, and investigate their activity by *in-vitro* NS2B/NS3 protease inhibition assay. The design of the peptide ligands was based on studying the interactions with the dengue NS2B/NS3 protease using the computational docking technique in the MOE and AutoDock (version 4.2) software. To this end, the researchers designed 26 linear pentapeptides based on previous studies. It was revealed that two linear pentapeptides (i.e., GKRRK and KRRRK) are the best potential inhibitors. Furthermore, based on the findings of the two independent docking programs, the peptide GKRRK was synthesized by solid-phase peptide synthesis and its structure was confirmed. The *in-vitro* protease inhibitor study was conducted for these two peptides to examine their activity against the dengue virus using a protin as a control. It was found that the designed potential peptides possess interesting inhibition against the NS2B/NS3 protease. Additionally, the findings showed that the peptide GKRRK had the highest percentage of inhibition (71.11%) at 100 µM with the IC_{50} of 48.87 µM; therefore, this linear peptide could serve as a good inhibitor for the DENV.

Keywords: Dengue virus, *in-vitro* protease assay, Linear pentapeptides, Molecular docking, NS2B/NS3 protease

1. Introduction

The dengue virus infection (DENV) is one of the fatal diseases that has become a serious problem in the world for the last few decades. To date, there are five dengue virus serotypes (i.e., DENV-1-5), with each serotype showing a similar range of disease manifestations during infection. Dengue fever, dengue hemorrhagic fever, and dengue shock syndrome are caused by DENV (1). So far, there have not been any specific proven therapeutic agents to prevent or cure infections caused by DENV. Therefore, discovering a convenient and effective therapeutic agent to fight this quickly-developing infectious disease is an urgent need (2). The DENV is an enveloped, positive single-stranded-RNA *flavivirus* of the Flaviviridae family. Under the same genus, there are also the west Nile virus, zika virus, yellow fever virus, and Japanese encephalitis virus. The dengue virion itself is spherical with surface proteins, the envelope (E), the precursor (prM)/membrane (M), and capsid (C) proteins located in the lipid bilayer, which encapsulate the genome of the virus (3). The DENV genome encodes three

structural proteins (i.e., C, prM/M, as well as E) and seven non-structural (NS) proteins (i.e., NS1, NS2A, 2B, NS3, NS4A, 4B, as well asNS5). Structural proteins constitute the viral particle, while NS proteins participate in the immune system replication (4). Overall, the most popular modes of action for antiviral medications are targeting the virus itself (virus-targeting antivirals) and host-targeting antivirals (5). The primary targets for developing antiviral medication are NS2B/NS3 protease enzymes since they are essential for viral replication and are considered a great inhibitory target. The NS3 protease is important and necessary for polyprotein cleavage processing, which requires an NS2B cofactor for its efficient catalytic activity (6). The NS2B/NS3 protease is a trypsin-like protease with a polybasic substrate recognition profile that is a serine protease catalytic triad (His51, Asp75, Ser135) (7). Therefore, in this study, the researchers were focusing on dengue NS2B/NS3 protease and the catalytic triad as the target to study the interactions of the proposed linear peptides.

The present study aimed at designing and synthesizing selective peptide inhibitors, as well as investigating their activity by *in-vitro* NS2B/NS3 protease inhibition assay.

2. Materials and Methods

2.1. Design of the Proposed Peptide Ligands

A total of 26 linear tetra peptides were considered in this study based on the review of literature, previous findings concerning substrate specificity, and the analysis of the binding pocket of NS2B/NS3 protease (Supplementary File 1). These linear tetra peptides were further docked against DENV NS2B/NS3 protease using the MOE and AutoDock (version 4.2) software. The potential peptides were chosen for the synthesis based on the results from molecular docking. Afterward, the *in-vitro* NS2B/NS3 protease inhibition assay was performed. The 2D structures of the proposed linear pentapeptides are illustrated in supplementary file 2.

2.2. Molecular Docking by the MOE and AutoDock 4.2 software

The peptide ligand structures were built and converted into their respective 3D structures using PerkinElmer ChemDraw Ultra software (version 16.0). The energy of the proposed peptides was minimized using the same program (MM2 force field minimization). For the MOE, all the designed peptides were optimized using the same platform and were saved in the mdb (mole2) format for further docking studies, while for the AutoDock, the peptides were optimized by using AutoDock 1.5.6 tools. The Gasteiger charges and rotatable numbers were assigned to the peptides, and they were then saved in the pdbqt format for docking. The (Bz-Nle-Lys-Arg-Arg-H) has been chosen in the molecular docking as a control ligand due to its activity and anti-dengue strength (8). This control ligand was retrieved from the PubChem database (National Center for Biotechnology Information, Maryland, U.S.) and was optimized using the same procedures as described previously.

The dengue NS2B/NS3 protease homology model used for docking was downloaded from the literature of Wichapong, Pianwanit (9). The MOE software and AutoDock tools 1.5.6 were utilized to optimize the protease structure geometrically by first eliminating all the water molecules that performed the protonation of the NS2B/NS3 protease and optimizing the partial charges. The optimized protein structure was saved in the pdbqt format for further docking. The docking score of MOE was used to determine the ligand-receptor binding affinity and the refinement to the corresponding force field of the ligand-receptor complex. With the help of the docking algorithm of the MOE software, the proposed peptides were docked onto the catalytic triad of dengue protease, which consists of His51, Asp75, as well asSer135, and other close contact residues, compared to the control ligand. The docking parameters were set according to previous studies by Idrees and Ashfaq (10), as well as Nadeem Abdalsatar Abdalrazaq (11). In this study, the conformation of every peptide was chosen based on the minimum S score, and further

analysis was conducted on their hydrogen bonding, van der Waals, as well as hydrophobic interactions with dengue NS2B/NS3 protease.

For molecular docking of the proposed peptide ligands with dengue NS2B/NS3 protease using AutoDock software (version 4.2), a grid box with a dimension of 60×60×60 points and 0.375 Å of grid spacing was used to cover the entire enzyme binding site in order to accommodate ligands to move freely. All calculation for docking was done using the Lamarckian Genetic Algorithm method, and the parameters were set as default for 100 search runs. After the completion of the docking search, Ki was selected as the best conformation with the lowest estimated binding energy and the estimated inhibition constant. Additionally, the interactions between the enzyme and ligand conformations were evaluated (11, 12). The quantitative docking results were obtained from the binding energies, while the qualitative results of the 2D visualization of the selected best peptides with the NS2B/NS3 protease are presented using LigPlot^{+} v.2.1 programs (13). Meanwhile, the 3D visualization was prepared by using a discovery studio visualizer (version 17.2).

2.3. Chemicals and Reagents

The chemicals and reagents for peptide synthesis included wang resin loaded Fmoc amino acid, coupling reagents, such as 2-(6-Chloro-1h-benzotriazole-1-yl)-1,1,3,3-tetramethylammoniumhexafluorophosphate (HCTU), *N,N*-Diisopropylethylamine (DIPEA), Fmoc-amino acid building blocks, Trifluoroacetic acid (TFA), and Piperidine. In addition, the utilized solvents were *N,N* dimethylformamide (DMF), dichloromethane (DCM), ethanol, and diethyl ether, which were purchased from Sigma-Aldridge (Germany). For protein expression, Luria Bertani (LB) media was purchased from HIMEDIA (Mumbai, India), and Isopropyl β-D-1-thiogalactopyranoside (IPTG), as well as Ampicillin sodium salt, were purchased from Bio Basic Inc. (Toronto, Canada).Furthermore, Bradford reagent was bought from HIMEDIA (Mumbai, India), purified bovine serum albumin from Sigma Aldrich (Missouri, U.S.), TALON Metal affinity resin from Clontech Laboratories Inc.(California, U.S.), and lastly, BOC-GRR-AMCwas purchased from Peptide Institute (Osaka, Japan), which was used as fluorogenic substrate. All the chemical substances were of analytical quality; and thus, they were used without further purification.

2.4. Synthesis of the Linear Pentapeptide GKRRK by Fmoc Solid-phase Peptide Synthesis

Based on the docking results from the two docking programs (i.e., MOE and AutoDock4.2), two of the linear pentapeptides (i.e., KRRRK and GKRRK) were selected as potential peptides. The linear pentapeptide GKRRK was synthesized in the lab by Fmoc solid-phase peptide synthesis (SPPS), while the KRRRK was obtained commercially by Biomatik (Canada). The established method of SPPS for the synthesis of peptides is pioneered and developed by Merrifield (14). The reactions were carried out in Bio-Rad polypropylene columns by shaking and mixing through a vortex mixer (3D shaker) at room temperature under dry conditions. The peptide was synthesized by employing Fmoc-L-amino acid building blocks. The HCTU and DIPEA in the DMF were utilizedas coupling and activating reagents, respectively. The general procedure consisted of swelling 0.1 mmol of amino acid wang resin in the DMF for 60 min, Fmoc deprotection with 20% piperidine for 20 min, and the activation and coupling of the three equivalents of the second amino acid with the three equivalents of HCTU, as well as six equivalents of DIPEA in the DMF for 40 min. Afterward, a bromophenol blue test was performed to confirm the success of the coupling process, which was then continued with washing with the DMF and DCM. The synthesis was continued again with Fmoc deprotection, and activation, as well as coupling of the next amino acids until reaching the desired sequence. The last step was Fmoc deprotection and cleavage of the wang resin and other protection groups by using the cocktail solution of TFA, deionized water, and Triisopropylsilane

in a ratio of 95:2.5:2.5 (v/v) for 3 h. The peptides were then precipitated in the cold diethyl ether and purified. The purification of peptides was carried out by reverse-phase high-performance liquid chromatography (RP-HPLC) Agilent technologies (U.S.) on a Zorbax SB-C18 column (5 µm, 4.6×250 mm) using Solvent A (0.1%TFA) in 100%deionized water and Solvent B (0.1% TFA) in 100% acetonitrile. The gradient was set at 98% of A and 2% of B at 0 min and was then changed into 40% of A and 60% of B for another 20 min. The injection volume was 10 µL with a flow rate of 1.0 mL/min and the wavelength was set at 220 nm. The purity and molecular mass of the synthesized linear tetrapeptides were confirmed using mass spectrometric analysis (Thermo Scientific, U.S.).

2.5. Expression of Dengue-2 NS2B/NS3 Protease in pET-14b Vector

The protocol used in the expression of the DENV-2 NS2B/NS3protease was based on the procedures described by Erbel, Schiering (15). The pET-14b NS2B/NS3protease recombinant plasmid encoding the NS2B/NS3 protease sequence was transformed into the freshly prepared competent cell *E. coli* BL21 (DE3) (16). A starter culture of 5 mL was grown overnight in the LB medium containing 100 µg/mL ampicillin. The cells were incubated at 37˚C, 160 to180 rpm until OD_{600} reached 0.5. A total of 100 µLof 0.5 mM of Isopropyl β-D-1-thiogalactopyranoside (IPTG) was added to the bacterial cells as an inducer of protein expression and the mixture was incubated for 16 to 20 h at 37˚C, 160 to 180 rpm. The cells were harvested by centrifugation at 5,000 rpm for 15 min (Thermo Fisher Scientific, U.S.) and the pellet was stored at-20˚C prior to the purification process.

For protein purification, the cell pellets were thawed in ice and resuspended in 10 mL of lysis buffer and lysed by sonication conducting (6 times, 10-sec pulse, duty cycle 10%, output control no=3) using Ultrasonic Cell Disruptor, TOMY, UD-201 (Germany). The lysate was incubated on ice and then centrifuged at 8,000 rpm for 15min at 15˚C. Buffer-equilibrated TALON resin and the supernatant were mixed and loaded into the purification columns (Bio-Rad columns, 3 cm). The column was initially washed with phosphate buffer, and then with imidazole of increasing concentrations, starting from 10 mM, 20 mM, 40 mM, 60 mM, and 100 mM. The eluted fractions were collected in microcentrifuge tubes. The concentration of the purified protein fractions was estimated using the Bradford assay. The purified protein was stored at-20˚C.

2.6. The *in-vitro* Dengue NS2B/NS3 Protease Inhibition Assay

The DENV NS2B/NS3 protease inhibition assay was conducted in 96-well microtiter black plates, following the protocol proposed by Kiat, Pippen (17), as well as Tomlinson, Malmstrom (18). The assay mixtures with a total volume of 100 µLcontaining 0.5 µm NS2B/NS3 protease in 200 mM Tris-HCl, pH 8.5, and 100 µm inhibitor were pre-incubated at room temperature for 15 min to allow the formation of enzyme-inhibitor complexes. Afterward, 1µL of the fluorogenic substrate BOC-GRR-AMC (10 mM) was added, and the mixture was further incubated for an additional 60 min. The assay condition was validated by using positive control (a mixture of 100µM aprotinin, 0.5µM NS2B-NS3protease, and 100µM peptide substrate) (16, 18), negative control (100µL buffer blank), and no inhibitor control (0.5 µM NS2B-NS3protease and 100 µM peptide substrate). The measurements were taken in triplicates for each data point, and the data were reported as the mean±SEM. The percentage of inhibition of the potential peptides was recorded, and the IC_{50} values were calculated using non-linear regression in GraphPad Prism software (version 8).

3. Results and Discussion

3.1. Docking Results

Two essential criteria were used in choosing the potential peptide ligands after docking. The first criterion was selecting the lowest binding free energy (ΔGbind) of the docked peptide, which represented the quantitative results. After the ligand was docked with the receptor, the ligand-binding affinity was assessed by measuring the free binding energy; thereby demonstrating the interactions between the ligand and

the receptor protein in the lower score for any ligand and indicating a favorable ligand-receptor protein interaction (19) . It has been suggested that the predicted (ΔGbind) possesses a typical error of ±2 kcal/mol (20). The second criterion was the binding interactions of the peptide ligand with the dengue NS2B/NS3 catalytic triad (His51, Asp75, and Ser135), which is considered as qualitative results. Since the catalytic triad residues are significant for viral replication, targeting them at this site could interfere with the viral replication (21). For this reason, the researchers chose the best conformation based on the hydrogen bonding interaction between ligands with a minimum of two interactions with the protease catalytic triad. Taken together, aninteraction may occur between the dengue NS2B/NS3 protease and the peptides proposed in this study, on the one hand, with hydrogen bond interactions, on the other hand. It could also occur with other non-covalent interactions, such as van der Waals and hydrophobic interactions, which may also affect the binding affinity (22).

Based on these criteria, the top peptides with the minimum S score (MOE) and ΔGbind (AutoDock 4.2) were selected and analyzed further for protease interaction. The best-proposed peptides from both docking softwares were chosen based on the lowest energy, as represented in table 1, and the high number of interactions with the dengue protease catalytic triad are shown in table 2. Based on the binding affinity (quantitative) and binding interaction (qualitative) results obtained from the MOE and AutoDock 4.2 software, two linear pentapeptides (i.e., KRRRK and GKRRK) were observed in both independent docking programs. Overall, the S Score values from the MOE for the potentially designed peptides KRRRK (-17.90) and GKRRK (-17.64) were lower than those of the control ligand, Bz-Nle-Lys-Arg-Arg-H (-15.64), and the ΔGbind of the peptide GKRRK (-6.02) from AutoDock was also lower than that of the control ligand (-5.96), while for KRRRK, it was (-5.88). The lower and more negative values of the S score and ΔGbind energies demonstrated a strong favorable binding between peptide ligands and dengue protease. The docked conformations of the control ligand, Bz-Nle-Lys-Arg-Arg-H from both MOE and AutoDock 4.2 programs bound at the same binding region of the protease, as its conformation before docking is illustrated in figure 1. This indicates that the parameters used for docking are in good selection and acceptable to use for docking of the designed linear pentapeptides in this study. The best potential docked conformation of linear tetrapeptide GKRRK from MOE and KRRRK from AutoDock4.2 are depictedin figure 2 (A and B). It was found that the designed ligands formed a network of hydrogen bond interaction and close contact residues with almost the same amino acids shown by the control ligand, Bz-Nle-Lys-Arg-Arg-H. This shows that the computational docked results bind at the same binding region as the control ligand, and it is hoped that these peptides could give a good inhibition activity against dengue protease.

3.2. Synthesis of the Linear Pentapeptide GKRRK

The linear tetrapeptides GKRRK was synthesized in the lab by using the SPPS. The general procedure relies on stepwise synthesis: repeated cycles of covalently coupling a single protected amino acid with the amine functionalized solid support, followed by deprotection of the N-terminus, which allows the next amino acid to be coupled. Once the synthesis is completed, deprotection of the amino acid side chainsis conducted, and the peptide is then cleaved from the solid support (wang resin). In the SPPS, since the peptide stays bound with the solid support during the synthesis, the excess of the reagents, as well as side products, can then be eliminated by washing and filtrating the solvents. This approach bypasses the time-consuming comparative isolation of the product from the solution after each reaction step, which is necessary for the conventional solution phase synthesis (23). The peptide was purified by the RP-HPLC, which is a white powder, with a melting point of 137 to 140°C.Percent of yield (35%) and the molecular mass were confirmed by mass spectrometric analysis (Supplementary Files 3 and 4).

Table 1. S scores and the interactions of the best proposed linear pentapeptides with the NS2B/NS3 protease using the MOE program

Linear peptide	S score (kcal/mol)	Hydrogen bond interaction	Close contact residues
SIRRK	-18.5853	His51, Asp75, Met84, Asp129, Gly153	Gly82, Met84, IIe86, Gly151, Asn152, Val154, Tyr161
KRRRK*	-17.9037	His51, Asp75, Asp81, Ser83, Met84, Ser135, Tyr150, Val155	Ile36, Gly82, Pro132, Gly151, Asn152, Gly153, Tyr161
ALKRR	-17.8881	His51, Asp75, Asp129, Phe130, Asn152	Ser131, Pro132, Thr134, Ser135, Tyr150, Gly151,Tyr161
GKRRK	-17.4681	His51, Asp75, Ser83, Asn152, Gly153	Met84, Val155, Gly82, Asp129, Ser131, Pro132, Gly151, Tyr161
IKRRK	-17.3024	His51, Asp75, Ser83, Met84, Asp129, Phe130, Ser135,Tyr150, Gly153	Val72, Asp81, Gly82, Gly151, Val155, Tyr161
AKRRK	-16.4819	His51, Asp75, Met84, Asp129, Gly151, Tyr161	Gly82, Ser83, Gly153, Val154, Val155, Gly159, Ala160
Control :(Bz-Nle-Lys-Arg-Arg-H)	-15.6426	His51, Asp75, Met84, Ser135,Gly151, Gly153	Ser83,Phe130,Pro132,Tyr150, Val154, Val155 and Tyr161

Table 2. Estimated free binding energy, estimated inhibition constant, and the interaction of potential proposed linear pentapeptides with dengue NS2B/NS3 protease using AutoDock 4.2 program

Peptide	Estimated free energy of binding (kcal/mol)	Estimated inhibition constant (μM)	Hydrogen bond interaction	Close contact residues
GKRRK*	-6.02	38.88	His51, Asp75, Met84, Asp129, Phe130, Ser135, Gly153	Ser83, Gly151, Val154, Val155
KRRRK	-5.88	48.89	His51, Asp75, Asp81, Met84, Phe130, Ser135, Val155	Gly82, Ser83, Ile86, Asp129, Ser131, Pro132, Gly151, Asn152, Gly153,Tyr161
ALRRK	-4.45	544.64	His51, Asp75, Ser83, Asp129, Phe130, Ser135, Asn152, Gly153	Asp81, Gly82, Met84, Pro132, Gly151,Val155, Tyr161
GIKRR	-4.33	669.79	His51, Asp75, Asp81, Asp129, Phe130	Gly82, Ser83, Pro132, Ser135, Gly151,Asn152, Tyr161
Control:(Bz-Nle-Lys-Arg-Arg-H)	-5.96	42.80	His51, Asp75, Gly82, Phe130, Ser135, Tyr161	Met84, Asp129, Tyr150, Gly151, Gly153, Val155

* Bold fonts highlighted indicate the potential peptides that were observed from the two independent programs, and the catalytic triads are highlighted with red color.

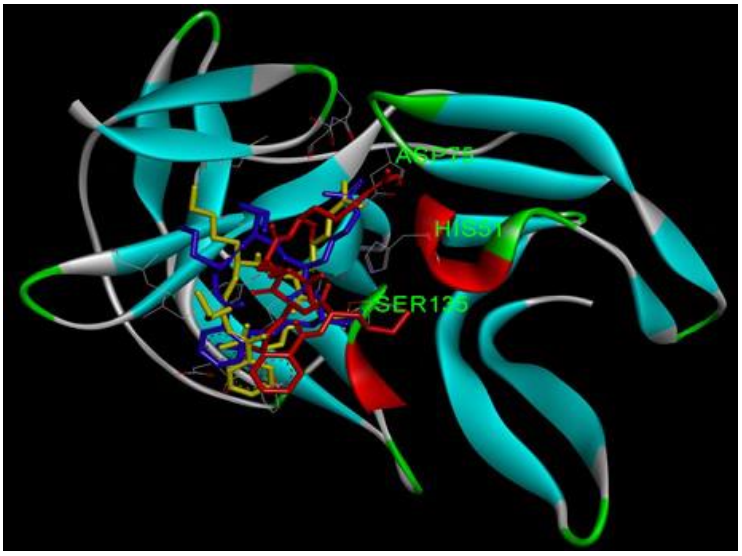

Figure 1. An overview of the control ligand, Bz-Nle-Lys-Arg-Arg-H, before docking (yellow), in comparison with its docked structures from MOE (red) and AutoDock4.2 (blue) at the binding site of NS2B/NS3 dengue protease. The closed contact residues of the enzyme are shown as grey sticks and catalytic triads are highlighted with green colour

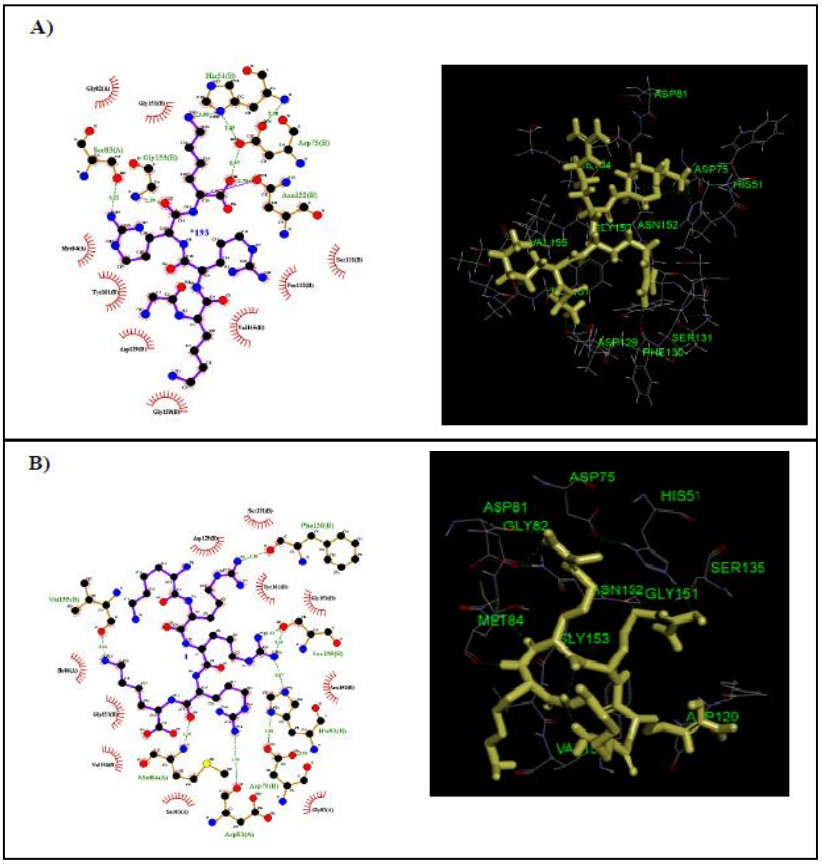

Figure 2. The docked conformations of GKRRK **(A)** and KRRRK **(B)** using MOEand AutoDock4.2, respectively, at the binding site of NS2B/NS3 protease. Left: The 2D interaction using LigPlot+. The green dotted lines represent the hydrogen bond interaction, the purple line represents the covalent bond, and the red half-circle corresponds to the hydrophobic interactions of close contact residues. Right: 3D visualization using BIOVIA Discovery Studio. The close contact residues and catalytic triads of the enzyme are shown as grey sticks

3.3. Dengue NS2B/NS3 Protease Expression, Purification, and Inhibition Assay

The product of dengue NS2B/NS3 protease expression was confirmed by the presence of a thick band on the 12% SDS-PAGE gel with a molecular weight of 33 kDa. The expressed NS2B/NS3protease contains a His-tag that allows it to be immobilized and purified using TALON® Metal Affinity Resin. The size of his-tagged NS2B/NS3protease was as expected at 33 kDa (Figure 3).

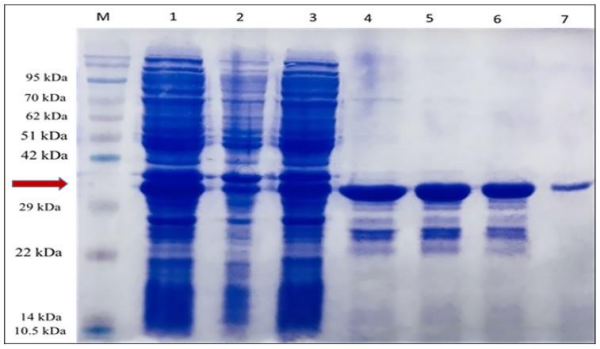

Figure 3. SDS-PAGE analysis of NS2B/NS3protease using TALON® Metal Affinity Resin. M: Protein marker (Chromatin Pre-stained Protein Ladder).Lanes 1, 2, and 3: purified protein soluble, pellet, and flow-through fractions. Lanes 4 to 7 elutions of NS2B/NS3pro with 20, 40, 60, and 100mM imidazole. The red arrow indicates the protein of interest with an MW of approximately 33 kDa

The principle of the protease assay depends on the fluorogenic substrate Boc-Gly-Arg-Arg-AMC whereby the fluorogenic moiety 7-amino-4-methyl coumarin (AMC) is released upon cleavage by the active protease. Measurements of the fluorescence intensity of the released AMC were conducted by fluorometric Glomax® multi-microplate reader (excitation at 365 and emission 410 to 460nm) after 60 min of incubation. Based on the docking results from the two docking programs (i.e., MOE and AutoDock4.2), the two linear pentapeptides, GKRRK and KRRRK, were selected for the *in-vitro* dengue NS2B/NS3 protease inhibition assay, and the protinin was used as a control. The fluorescence intensity was measured and used as an indication of the substrate hydrolysis activity. The percentage of inhibition for the two peptides and the control was calculated, taking the hydrolysis activity of the protease and substrate with no inhibitor (E+S) as 100% activity.

The results revealed that the two linear pentapeptides exhibited inhibition against protease at a concentration of 100µM. The GKRRK had the highest percentage of inhibition (71.11%±1.31%), compared to the other peptide KRRRK with (48.92%±3.6%) of inhibition (Figure 4). The percentage of inhibition for the aprotinin as control was more than 90%. Aprotinin is a peptide known as a serine protease inhibitor but it has limitations for being used as an anti-dengue inhibitor because it is a broad-spectrum and non-selective serine protease inhibitor; however, it can be used as a guide in this study (24). The two peptides, GKRRK and KRRRK, together with aprotinin, were then selected for calculating the IC_{50} using non-linear regression in GraphPad Prism (version 8), the results of which are illustrated in figure 5. A total of eight concentrations in a range of 6.25 to 500 µM were utilized to measure IC_{50} values of GKRRK (48.87 µM) and KRRRK (154.3 µM), compared to aprotinin with IC_{50} of 18.29 µM, though the aprotinin is a non-selective inhibitor toward the dengue NS2B/NS3 protease (Figure 5).

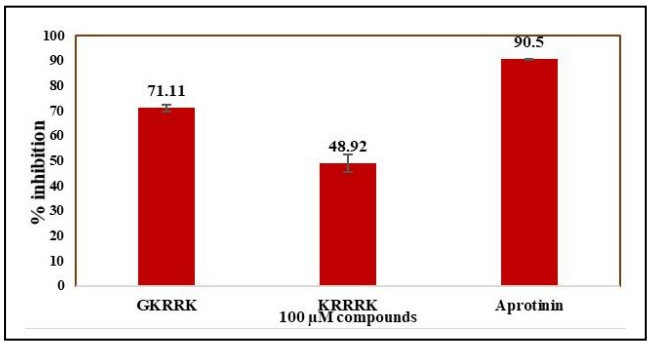

Figure 4. *in-vitro* DENV-2 NS2B/NS3 protease assay of linear pentapeptides (GKRRK and KRRRK) and the aprotinin as control at a concentration of 100µM

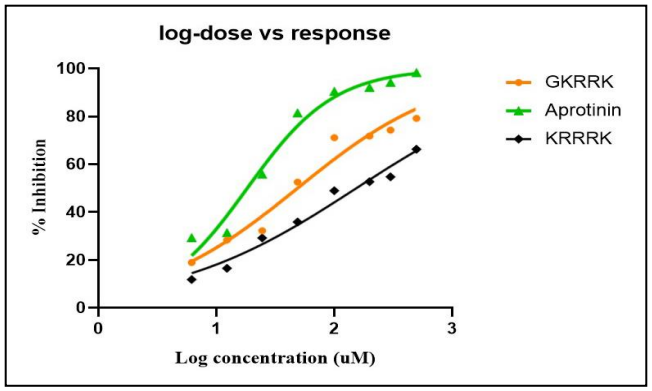

Figure 5. Plot of the percentage of dengue NS2B/NS3 protease inhibition, as opposed to the log concentration of the linear pentapeptides, GKRRK and KRRRK, with the a protinin as a control

In the present study, the proposed peptide ligands were designed, based on the previously mentioned literature, for substrate specificity and analyzing the binding pocket of the NS2B/NS3 protease catalytic site. The proposed peptides were investigated by molecular docking for their interaction with the active site residues and were ranked in terms of the free energy of binding, as well as their interactions with the catalytic triad and other close contact residues. The validation of the designing of the proposed peptides relies on their *in-vitro* dengue NS2B/NS3 protease inhibition assay. The use of two independent docking programs (i.e., MOE and AutoDock 4.2) gave almost the same results based on the criteria that have been demonstrated.

From the *in-vitro* dengue NS2B/NS3 protease inhibition assay, the researchers identified that the two potential peptides have interesting inhibition against the NS2B/NS3 protease. It was found that the GKRRK (16-L5) had the highest percentage of inhibition (71.11%) at 100µM, and the IC_{50} was 48.87 µM. The analysis of molecular docking of the two programs revealed that this peptide ligand had a low free binding energy and formed a network of interaction with the catalytic triad and other close contact residues of the protease leading to the formation of a favorable stable enzyme-ligand complex. Therefore, this linear peptide could serve as a good inhibitor for the dengue virus.

Authors' Contribution

Study concept and design: E. E. B. K. and N. A. A.
Drafting of the manuscript: N. A. A.
Analysis and interpretation of data: E. E. B. K. and N. A. A.

Conflict of Interest

The authors declare that they have no conflict of interest.

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