CHAPTER III

Materials and Methods

3.1 Materials

3.1.1 Chemicals

3.1.1.1 Analytical grade

Reagents for buffer preparation such as Tris base, phosphate-buffered saline (PBS), glycine, copper sulfate, sodium carbonate, sodium hydroxide, sodium potassium tartrate, sodium chloride, etc; reagents for SDS-PAGE analysis such as acrylamide, bis-acrylamide, ammonium persulphate (APS), sodium dodecyl sulfate (SDS), tetramethylethylenediamine (TEMED), β-mercaptoethanol, dithiothreitol (DTT), bromophenol blue, glycerol, Coomassie brilliant blue R-250, etc; solvents such as ethanol, methanol, acetic acid, etc.; and all other analytical grade reagents were purchased from HiMedia, India and Merck, Germany. Coagulation proteins such as human fibrinogen and thrombin were purchased from Sigma-Aldrich, USA. Thromboplastin reagent for prothrombin time test (Liquiplastin®) and cephaloplastin reagent for activated partial prothrombin time test (Liquicelin-E) were obtained from Tulip Diagnostics, India.

3.1.1.2 Microbiological grade culture media/chemicals

Reagents for media preparation such as nutrient broth, agar, fibrin, skim milk, yeast extract, glucose, Luria bertani, peptone, dextrose, beef extract, tryptone, potassium phosphate, sodium citrate, magnesium sulfate, dipotassium phosphate, ammonium dihydrogen phosphate, etc were purchased from HiMedia, India.

3.1.1.3 Molecular cloning and expression

The protease gene encoding sequence was codon optimized and inserted into the pET-26b(+) expression vector between the NcoI and XhoI sites by GenScript Biotech (Life Sciences Company, USA). The restriction enzymes (NcoI and XhoI), proteinase K, RNase A solution, agarose, 6X gel loading dye, DreamTaq PCR master mix, nuclease-free water, 1 kb DNA ladder, Sybr safe DNA gel stain, Kanamycin, Isopropyl β-D-1-thiogalactopyranoside (IPTG), Ni-NTA agarose resin, urea, imidazole were purchased from Thermo Fisher Scientific (MA, USA) and Himedia (India). The GeneJET genomic DNA purification kit, gel extraction kit, and plasmid miniprep kit were purchased from Thermo Fisher Scientific (MA, USA).

3.1.1.4 Cell culture reagents

Dulbecco's Modified Eagle Medium (DMEM), Fetal Bovine Serum (FBS), Penicillin-Streptomycin, 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) dye, dimethylsulfoxide (DMSO) and trypsin were purchased from GibcoTM (USA).

3.1.1.5 Serum profiling

All diagnostic kits used for the analysis of biochemical parameters of plasma, such as Serum Glutamic Pyruvic Transaminase (SGPT), Serum Glutamic Oxaloacetic Transaminase (SGOT), blood urea nitrogen, low-density lipoprotein (LDL), high-density lipoprotein (HDL), total protein (TP), triglycerides (TRIG), creatinine (CR), and cholesterol (CH), were purchased from Sirus Biocare Pvt. Ltd., Kolkata.

3.2 Methods

3.2.1 Computational (*in silico*) analysis to study bacterial fibrinolytic proteases' structure-function relationship

3.2.1.1 Sequence retrieval and alignment

The keyword '*Bacillus* fibrinolytic enzyme', was used to search for submitted nucleotide sequences of fibrinolytic enzymes produced by the genus *Bacillus* from the NCBI database (https://www.ncbi.nlm.nih.gov/) covering the period from 2002 to May 31, 2022 (approximately 20 years). The selected fibrinolytic enzyme nucleotide sequences' corresponding protein sequences were retrieved from the NCBI database. The obtained protein sequences were aligned using multiple sequence alignments using PRALINE programme (https://www.ibi.vu.nl/programs/pralinewww/), and the alignments were examined using the CLC sequence viewer 8.0 (http://www.clcbio.com).

3.2.1.2 Determination of physiochemical characteristics of the proteins

The ExPASy-ProtParam tool (https://web.expasy.org/protparam) was used to calculate the varied physiochemical parameters of the fibrinolytic enzymes [1]. The computed parameters in the ProtParam data include- molecular mass, isoelectric point (pI), extinction coefficient (EC), aliphatic index (AI), instability index (II), and Grand Average of Hydropathicities (GRAVY).

3.2.1.3 Structure analyses of fibrinolytic proteases

The ExPASy-ProtParam tool analysed the primary structure, i.e., the number of amino acids in the polypeptide chain. The Pfam site was used for its domain search, and the MEME server was used for motif analysis [2]. Protein BLAST was used to conduct a functional biological study of MEME-deduced conserved protein motifs. The domains were predicted using the InterPro scan, which identified the best match based on the highest resemblance score.

SOPMA from the Network Protein Sequence Analysis (NPS@) server (https://npsa-prabi.ibcp.fr/cgi-bin/npsa_automat.pl?page=/NPSA/npsa_sopma.html) performed secondary structure analysis on the retrieved fibrinolytic enzymes, which comprised the number of α-helices, β-turn, extended strand, β-sheet, and random coils [1]. The PDBsum tool (https://www.ebi.ac.uk/thornton-srv/databases/cgi-bin/pdbsum/GetPage.pl?pdbcode=index.html) was used to generate the secondary motif map and topology diagram [3]. Each protein's anticipated disulfide patterns and consensus secondary structure contents were tallied. The CYS-REC tool (http://www.softberry.com/berry.phtml?topic=cys_rec) was used to determine the presence of disulfide bridges by predicting the most likely bonding patterns between accessible cysteine residues.

The SWISS-MODEL 3.1.0 (https://swissmodel.expasy.ord/) was used to create 3D models of all the obtained fibrinolytic enzymes for the tertiary structure analysis and validation [4]. The most crucial aspect of structure prediction was structure evaluation. The QMEAN (https://swissmodel.expasy.org/qmean/) and SAVES servers (https://saves.mbi.ucla.edu/) were used to test and verify the predicted protein models for all of the bacterial fibrinolytic enzymes. SAVES server was used to analyse Verify3D [5] and ERRAT [6]. Verify3D software testifies whether an atomic model (3D) was well-matched with its amino acid sequence (1D) [7], whereas ERRAT verifies the crystallographic structure of proteins.

3.2.1.4 Functional analysis

The protein (protease) sequences were examined for any areas of globularity and disorder using the GlobPlot 2.3 (http://globplot.embl.de/). This online bioinformatics tool searches the domain databases and sets of disordered proteins for order/globularity or disorder tendency in the query protein based on a running total of the propensity for an amino acid [8]. Proteolytic cleavage sites in the fibrinolytic enzymes were predicted using peptide cutter software

(http://web.expasy.org/peptide_cutter) [1]. This web-based application indicates proteolytic cleavage and chemically cleaved sites in a given protein sequence.

3.2.1.5 Validation of the *in-silico* data analysis

To validate the *in-silico work*, published research articles on the selected fibrinolytic enzyme sequences were downloaded, and the experimental results were compared with the assessed *in-silico* analysis data.

3.2.2 Isolation and characterization of fibrinolytic enzyme producing bacteria from few fermented food/ starter cuture samples of Northeastern, India

3.2.2.1 Collection of fermented food/starter culture samples

Northeast India was the source of the traditional fermented food/ starter culture samples (Fermented fish and alcohol starter culture), which were gathered from Tezpur (Assam), Guwahati (Assam), and Dimapur (Nagaland).

3.2.2.2 Screening of protease-producing bacteria

1 g of the fermented food/starter culture sample was mixed with 9.0 ml of 0.9% (w/v) sterile saline, and the mixture was serially diluted up to 10^{-4} with 0.9% w/v saline to screen for protease-producing bacteria. Each dilution was spread onto nutrient agar plates (Table 3.1) with a pH of 7.4. The plates were then incubated for 24 h at 37°C in an inverted position under static conditions.

Table 3.1: Composition of nutrient agar plate

Composition	**Concentration (g/L)**
Nutrient broth	13.0
Agar	20.0
pH	7.4

3.2.2.3 Fibrinolytic protease production

To evaluate fibrinolytic protease production, bacteria were cultured in 100 ml of M9 media (Table 3.2) at 37°C for 24 hr in shaking condition. The cell-free culture supernatant (CFC) was

gained by spinning by centrifuging the bacterial culture at 5000 g for 15 min at 4°C, followed by its protein estimation and fibrinolytic activity assay.

Table 3.2: Composition of M9 media for protease production

M9 media composition	
Composition	**Concentration (g/L)**
Na_2HPO_4	6.0
KH_2PO_4	3.0
NH_4Cl	1.0
NaCl	0.5
$MgSO_4.H_2O$	0.246
$CaCl_2.7H_2O$	0.014
Carbon source	1.0
Macro-nutrient composition	
Composition	**Concentration**
$FeSO_4.7H_2O$	1.0 mg L^{-1}
$CuSO_4.5H_2O$	50.0 µg L^{-1}
H_3BO_3	10.0 µg L^{-1}
$MgSO_4.5H_2O$	10.0 µg L^{-1}
$ZnSO_4.7H_2O$	70.0 µg L^{-1}
MoO_3	10.0 µg L^{-1}

Note: Add 1 ml of macro nutrient in 1000 ml of M9 production media

3.2.2.3.1 Protein estimation

The protein content of the CFC was calculated using the Lowry et. al. [9] technique. A serial dilution of 0.5, 1.0, 2.5, 5.0, 7.5, and 10 µg per well of bovine serum albumin (BSA, 1 mg/ml stock solution) in 100 µl of distilled water was prepared in a microtitre plate. After that, 200 µl of alkaline copper sulfate solution (0.5% $CuSO_4.5H_2O$ in 1% sodium-potassium tartrate and 2% Na_2CO_3 in 0.1 N NaOH in a 1:49 ratio) was added to the well and allowed to sit at room temperature (~23°C) for 10 min. Then, 30 min of room temperature incubation was spent after adding 20 µl of Folin-Ciocalteau Phenol reagent, diluted to a 1:2 ratio (Folin:dH_2O). A standard

protein calibration curve was established by plotting the absorbance at 660 nm vs. the concentration of BSA to calculate the unknown sample's protein content.

3.2.2.3.2 Measurement of zone of hydrolysis on fibrin agar plate

The zone of fibrin hydrolysis surrounding the well containing the bacterial inoculum was used to measure the production of fibrinolytic enzymes. 50 µg of the CFC was inoculated into the well using a sterile borer on plasminogen-free fibrin agar plates followed by incubation of 24 hr at 37°C in static condition. These plates also contained fluconazole as an antifungal agent (1 µl of a 50 mg/ml stock solution was supplemented per ml of culture media) [10]. Using the antibiotic zone scale, the hydrolysis zone around the well was measured (in mm).

Table 3.3: Composition of plasminogen-free fibrin agar plate

Composition	**Concentration (g/L)**
Fibrin	4.0
Skim milk	1.0
Yeast extract	2.5
Glucose	1.0
Agar	20.0
pH	7.4

Note: Adjust the media pH separately, followed by adding skim milk, agar, and yeast extract.

3.2.2.3.3 Fibrinolytic assay

In order to analyse the fibrinolytic assay, 40 µl of 2.5% (w/v) fibrinogen and 3 µl of thrombin (10 NIH U/ml) were combined, and the mixture was incubated for 30 minutes at room temperature to produce a clot. Subsequently, a specific amount of the CFC/Nattokinase (positive control) /1X PBS (control) was supplemented to the mixture. The final reaction mixture was made of 60 µl that had been adjusted with 1X PBS, pH 7.4, and then incubated for 90 min at 37°C to study fibrin degradation, if any. To stop the reaction, 15 µl of reducing dye was applied after incubating. The reaction solution was heated at 95°C for 5 min, and the products of fibrin breakdown had been separated by 12.5% SDS-PAGE. The gel was scanned using ImageJ, and the band intensities were examined.

3.2.2.3.3.1 Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

In accordance to the technique developed by Laemmli [11], SDS-PAGE was done with slight modification to separate the fibrin degradation products. Polyacrylamide gels (12.5%) were prepared and cast following Laemmli's instructions. Table 3.4 displays the composition of the 12.5% resolving gels and their 4% stacking gel. Tables 3.5 and 3.6, on the other hand, display the content of the running buffer and 3X loading dye, respectively. Fresh loading dye and buffers were made for the SDS-PAGE analysis. 15 mA of constant current was used during electrophoresis until the dye front touched the gel's bottom. Coomassie Brilliant Blue R250 staining was used to visualize protein bands. Gels were immersed in a Coomassie Brilliant Blue R-250 stain solution diluted in methanol, acetic acid, and distilled water (4:1:5, v/v/v) overnight. The gels were washed until the background of the gels was clear of stain using a destaining solution (distilled water containing 40% methanol (v/v) and 10% acetic acid (v/v).

Table 3.4: Composition of Tris-glycine SDS-PAGE

Composition	**4% stacking gel (5 ml)**	**12.5% resolving gel (10 ml)**
30% Polyacrylamide (ml)	0.6	4.0
0.5 M Tris-HCl, pH 6.8 (ml)	2.5	-
1.5 M Tris-HCl, pH 8.8 (ml)	-	2.5
10% APS (ml)	0.05	0.1
10% SDS (ml)	0.05	0.1
Distil water (ml)	1.5	3.3
TEMED (ml)	0.01	0.01

Table 3.5: Composition of running buffer

Composition	**Concentration (g/L)**
Tris-Cl	15.1
Glycine	72.0
SDS	5.0

Table 3.6: Composition of 3X loading dye

Composition	**Volume (10 mL)**
Bromophenol blue	10 µL
SDS	0.8 g
Glycerol	4 mL
0.5 M Tris-Cl	6 mL

3.2.2.4 Pure culture of fibrinolytic protease-secreting bacterial isolates

A loopful of bacterial culture exhibiting fibrinolytic activity was inoculated in nutrient broth media (pH 7.4) to obtain a pure culture of the bacteria (Table 3.7) and was allowed to grow until the cell density was 0.5 OD at 600 nm. 0.9% (w/v) of sterile normal saline was mixed with 0.1 ml of culture and successively diluted up to 10^{-4} to reach a final amount of 10.0 ml. To obtain a single distinct colony, 0.1 ml aliquot from the serially diluted mixture was spread over premade sterile nutrient agar plates, and the plates were incubated at 37°C for 24 h. The streak-plate method was used to isolate a single pure colony of the bacteria.

The streak-plate method is the most practical method of obtaining pure cultures and discrete colonies. This approach involves streaking a sufficiently diluted suspension of organisms across the surface of a premade agar plate to create a sequence of parallel, non-overlapping streaks using a transfer needle or sterilized loop.

Table 3.7: Composition of nutrient broth media

Composition	**Concentration (g/L)**
Nutrient Broth	13.0

3.2.2.5 Identification of bacterial strain

The fibrinolytic enzyme-producing bacterial strain was taxonomically identified using the following methods: i) Morphological traits, ii) Biochemical tests, iii) 16s rDNA sequencing, followed by phylogenetic analysis.

3.2.2.5.1 Morphological traits

3.2.2.5.1.1 Gram's staining

Scrap a sterile inoculating loop on a 24-hour bacterial culture, place it onto a sterile glass slide, and air dry the component by keeping it for 5-10 min, followed by heat fixing. Flooded the bacterial culture with a few drops of primary stain, let it sit for a minute, and wash the slide under tap water. After the initial staining, Gram's iodine was flooded over it and was allowed to sit at room temperature for 1 min. Wash the slide under tap water to remove the Gram's iodine mordant, then add a few drops of 95% ethyl alcohol (decolourizing agent). Remove the decolorizer with another wash under tap water, add the counterstain Safranin, and keep it for 45 s. The counterstain was then cleaned with tap water, and the slide was left to air dry completely at room temperature. The microorganisms were observed using a light microscope (BA210, Motic Asia).

3.2.2.5.2 Biochemical test

3.2.2.5.2.1 Carbohydrate fermentation test

A sugar-containing fermentation medium was made, as shown in Table 3.8. Each tube supplemented with the appropriate sugars was inoculated with a loopful of a 24-hour bacterial culture, which was then incubated for 48 hr at 45°C. Following a 48 hr incubation period, changes in the colour of the medium and any gas production were noted. Also, a control was established.

Table 3.8: Composition of carbohydrate fermentation media (Phenol red carbohydrate broth).

Composition	**Concentration (g/L)**
Peptone	10.0
Sodium chloride	5.0
Beef extract	1.0
Phenol red	0.018
Carbohydrate	10.0

3.2.2.5.2.2 Triple sugar iron (TSI) agar test

A loopful of 24-hr bacterial culture loop was streaked over TSI agar slants (Table 3.9) and incubated for 48 hr at 37°C. The change in the colour of the medium was noted on the TSI agar slants. Also, a control was established.

Table 3.9: Composition of triple sugar iron agar media.

Composition	Concentration (g/L)
Triple sugar iron agar	64.52

3.2.2.5.2.3 Catalase test

A microscope slide was placed inside a petri dish, and using a sterile inoculating loop, a 24 hr bacterial culture was taken and placed onto the microscope. The slide was flooded with 3.0% (v/v) hydrogen peroxide (H_2O_2) to verify catalase activity. The bacterium was considered catalase-positive (catalase-producing bacteria) if bubble formation was noticed.

3.2.2.5.2.4 Citrate utilization test

24 hr bacterial culture was streaked onto premade Simmons citrate agar slant medium (Table 3.10) followed by incubation at 37°C for 48 hr. Developing a blue colour complex was interpreted as a sign that the citrate utilisation test had succeeded.

Table 3.10: Composition of Simmons citrate agar media.

Composition	Concentration (g/L)
Magnesium sulfate (heptahydrate)	2.0
Ammonium dihydrogen phosphate	1.0
Dipotassium phosphate	1.0
Sodium citrate (dehydrate)	2.0
Sodium chloride	5.0
Agar	15.0
Bromothymol blue	0.08

3.2.2.5.2.5 Indole production test

A 24-hour bacterial culture was inoculated in tubes containing tryptone broth media (Table 3.11) and incubated for 48 hr at 37°C. After incubation, five drops of Kovac's reagent were added directly to the tubes and observed for any change in the colour of the medium.

Table 3.11: Composition of tryptone broth media.

Composition	Concentration (g/L)
Tryptone	10.0
Sodium chloride	5.0

3.2.2.5.2.6 Oxidase test

A 24-hour bacterial culture was rubbed onto a small piece of filter paper, and 1-2 drops of 1% Kovac's oxidase reagent were added to the rubbed culture to verify the existence of oxidase activity. The presence of oxidase was indicated by the development of dark purple colour within 5-10 sec.

3.2.2.5.2.7 Urease test

24-hour bacterial culture was streaked over the entire surface of the premade urea agar slant (Table 3.12), and the tubes were incubated for 48 h at 37°C. The presence of urease activity was indicated by the change in the colour of the medium to pink.

Table 3.12: Composition of urea agar media.

Composition	Concentration (g/L)
Peptone	1.0
Dextrose	1.0
Sodium chloride	5.0
Potassium phosphate, monobasic	2.0
Urea	20.0
Phenol red	0.012
Agar	15.0

3.2.2.5.2.7 Methyl red--Voges-Proskaurer (MR-VP) test

A 24-hour bacterial culture was added to sterile methyl red-Voges-Proskauer (MR-VP) broth (Table 3.13), and the mixture was incubated for 48 hr at 37°C. The incubated medium was separated into two parts: A and B. A few drops of methyl red indicator were added to component A to confirm the MR test. Part B involved adding 12 drops of Barritt's reagent A and 4 drops of Barritt's reagent B, then carefully shaking the mixture and allowing it to stand for at least 30 minutes. For the MR test, the formation of red indicates a positive test. In the case of the VP test, the formation of red colouration on top of the culture indicates a positive test.

Table 3.13: Composition of MR-VP broth.

Composition	Concentration (g/L)
Peptone	7.0
Dextrose	5.0
Dipotassium phosphate	5.0

3.2.2.5.3 16s rDNA gene sequencing

3.2.2.5.3.1 Genomic DNA isolation

Genomic DNA was isolated using the GeneJET Genomic DNA Purification Kit (Thermo Fisher Scientific, MA, USA) and following the manufacturer's protocol. The eluted genomic DNA was used for further processes.

3.2.2.5.3.2 Agarose gel electrophoresis

A 1% (w/v) agarose gel (Table 3.14) was prepared using 1X Tris-acetate-EDTA (TAE) electrophoresis buffer (Table 3.15) to separate the DNA fragments. The agarose was then heated in a microwave for 1 min or until the agarose was entirely dissolved. After letting the solution cool to about 50°C, Sybr safe DNA gel stain was added, and the gel solution was then put onto a gel tray that had already been pre-casted, and the comb was inserted. The well comb was removed after letting it sit for almost an hour. The electrophoretic chamber, filled with 1X TAE electrophoresis buffer, anchored the gel tray. 1 µl of 6X gel loading dye (Thermo Fisher Scientific, MA, USA) was mixed with 5 µl of the DNA sample and was loaded to the wells,

along with a 1 kb Plus DNA molecular weight marker (Thermo Fisher Scientific, MA, USA). After the gel was electrophoresed for 40 min at 75 volts, or until the sample had reached ¾th of the gel, it was then visualized in a GelDoc go imaging system (BioRad) for documentation.

Table 3.14: Composition of 1% agarose gel.

Composition	**Concentration**
Agarose	0.3 g
Sybr safe DNA gel stain	3.0 µL
1X TAE buffer	30.0 mL

Table 3.15: Composition of 1X TAE buffer.

Composition	**Concentration**
Tris base	4.84 g
Acetic acid	1.21 mL
EDTA disodium salt dihydrate	0.372 g
Distilled water	1000 ml
pH	8.0

3.2.2.5.3.3 PCR amplification of 16s rDNA gene

Polymerase chain reaction (PCR) amplification of a 1.5 kbps conserved segment of 16s rDNA gene was obtained by means of the universal forward and reverse primers set, i.e., 5'-AGAGTTTGATCCTGGCTCAG-3' and 5'- AAGGAGGTGATCCAGCCGCA-3', respectively which was performed on a Proflex PCR system (Thermo Fisher Scientific, MA, USA). The PCR amplified fragment (~1.5 kb) was separated using 1% (w/v) agarose gel electrophoresis as described in section **3.2.2.5.3.2.**

Table 3.16: Optimal PCR reaction conditions for amplification of conserved region of 16s rDNA gene.

PCR condition	**Temperature**	**Time**	**Cycle**
Initial denaturation	94°C	05:00 min	1
Denaturation	94°C	01:00 min	35
Annealing	55°C	00:30 min	
Extension	72°C	02:00 min	
Final extension	72°C	07:00 min	1
Hold	4°C	∞	-

3.2.2.5.3.4 Gel extraction of PCR product

The amplified PCR product was extracted from the gel using a GeneJET gel extraction kit (Thermo Fisher Scientific. MA, USA) following the manufacturer's instructions. The purified PCR product was quantified and used for Sanger chain sequencing.

3.2.2.5.3.5 Phylogenetic analysis

Using the MEGA4 multiple sequence alignment algorithm, the 16s rDNA gene sequences of bacteria were aligned with reference sequences exhibiting sequence homology from the NCBI database (http://blast.ncbi.nlm.nih.gov) [12]. Using distance matrix-based cluster techniques, such as neighbor-joining [13] analysis and the unweighted pair group method with an average (UPGMA), phylogenetic trees were built [14]. Every point in the dataset with gaps and missing data was removed (full deletion). A phylogenetic tree was constructed to illustrate the unique position of each *Bacillus* species within the genus among closely related *Bacillus* species.

3.2.2.6 PCR amplification of fibrinolytic protease encoding gene

The fibrinolytic protease encoding gene was amplified using the forward primer F_1 5´-AGACCATGGGATGGCAGGGAAATC -3' (NcoI site is highlighted) and reverse primer R_1 5´-AGACTCGAGCTGAGCTGCCGCCTG -3' (XhoI site is highlighted) specific for *Bacillus sp*. A 25 µl reaction mixture was prepared, containing 0.88 µM of each forward and reverse primer, 2X DreamTaq PCR master mix (Thermo Fisher Scientific, MA, USA), and ~200 ng of template DNA. PCR was set up in a thermal cycler with the thermocycling conditions listed in

Table 3.17 to amplify the gene. Following the thermal cycling, the PCR mixture was subjected to 1% agarose gel electrophoresis as described in section **3.2.2.5.3.2,** followed by its gel extraction and sequencing as described in section **3.2.2.5.3.4**

Table 3.17: PCR reaction conditions for amplification of fibrinolytic protease encoding gene.

PCR condition	**Temperature**	**Time**	**Cycle**
Initial denaturation	94°C	05:00 min	1
Denaturation	94°C	01:00 min	30
Annealing	52°C	01:00 min	
Extension	72°C	02:00 min	
Final extension	72°C	10:00 min	1
Hold	4°C	∞	

3.2.2.7 Computational analysis of amplified fibrinolytic encoding gene sequence

The ExPASy-ProtParam tool (https://web.expasy.org/protparam) was used to calculate the varied physiochemical parameters of the amplified fibrinolytic protease encoding gene [1]. The computed parameters in the ProtParam data include- molecular mass, isoelectric point (pI), extinction coefficient (EC), aliphatic index (AI), instability index (II), and Grand Average of Hydropathicities (GRAVY). Further, the amplified fibrinolytic encoding gene's protein sequence was aligned using multiple sequence alignments using PRALINE programme (https://www.ibi.vu.nl/programs/pralinewww/) with other homologous fibrinolytic serine protease sequence, and the alignments were examined using the CLC sequence viewer 8.0 (http://www.clcbio.com).

3.2.3 Cloning, expression, purification, and characterization of a recombinant bacterial fibrinolytic serine protease enzyme

3.2.3.1 Cloning of protease gene

3.2.3.1.1 Codon optimization and synthesis of protease gene

The gene sequence encoding for fibrinolytic enzyme derived from *Bacillus subtilis* was selected from the *in-silico* study conducted in the 1st objective [15]. The sequence was codon optimized for the expression of recombinant protease gene by *E. coli* BL21 (DE3) cells and synthesized by GenScript Biotech (NJ, USA). The optimized gene sequence, deprived of its signal sequence, was inserted downstream of the pelB leader sequence (which helps the expression of protein without its signal sequence) in the middle of the NcoI and XhoI restriction sites of the pET-26b(+) expression vector and also included an additional set of six His codons at the 3' end.

3.2.3.1.2 Preparation of BL21 competent cells

Glycerol stock of BL21 cells was revived on Luria Bertani (LB) agar plate (Table 3.18) by streaking method and incubated overnight at 37°C. After selecting a single colony from the overnight culture plate, it was inoculated in 5 ml of LB broth media (Table 3.19) and cultured for 18 h at 37°C under shaking condition (220 rpm). This primary culture was inoculated into 50 ml of LB broth media and allowed to grow at 37°C until the culture reached 0.4 absorbance at 600 nm. The whole culture flask was then incubated at 4°C for an hour, transferred to a pre-cooled 50 ml centrifuge tube, and spinned at 5000 rpm for 10 min at 4°C. The supernatant was castoff carefully, and the cell pellet was put back in 25 ml of 0.1 M $CaCl_2$ (autoclaved and ice-cold). The cells were mixed gently and incubated on ice for 1 hr. The cell lysate was spinned at 2500 rpm for 25 min at 4°C, and the supernatant was castoff carefully. The pellet was put back in 2 ml of 0.1 M $CaCl_2$ containing glycerol and kept overnight in the tube at 4°C. 100 µl of the cells were aliquot in pre-cooled 1.5 ml Eppendorf tubes and stored at -80°C for further use.

Table 3.18: Composition of luria bertani agar media.

Composition	Concentration (g/L)
Luria Bertani	20.0
Agar	20.0

Table 3.19: Composition of luria bertani broth media.

Composition	**Concentration (g/L)**
Luria bertani	20.0

3.2.3.1.3 Transformation of synthetic plasmid into BL21 competent cells

2 µl of the synthetic plasmid (~200 ng) was added to 100 µl of BL21 competent cells and mixed by gentle tapping. The tubes underwent 30 min incubation on ice followed by a 42°C heat shock treatment for precisely 90 sec and 5 min on ice afterward. 1 ml of LB broth (autoclaved) was added to this heat shock product and incubated for an hour at 37°C in shaking condition. The mixture was centrifuged at 6000 rpm for 10 min, and then 700 µl of the media was discarded. The remaining media were appropriately mixed by pipetting and then plated over premade LB agar plates supplemented with Kanamycin (50 µg/µl). The plates were then incubated in static conditions at 37°C for 16 hr in an inverted position, and the next day, the plates were checked for single colonies.

3.2.3.1.4 Screening of transformed recombinant colony

The colonies obtained post-transformation were screened for any recombinant colonies by isolating the plasmid using the GeneJET plasmid miniprep kit (Thermo Fisher Scientific, MA, USA), followed by double digesting the isolated plasmid with restriction enzymes and analysing the result by gel electrophoresis. For this, a single colony was selected and inoculated in 5 ml of LB broth supplemented with Kanamycin (50 µg/µl) and incubated for 18 hr at 37°C in shaking. 1 ml of this culture was transferred to a sterile 1.5 ml Eppendorf tube and centrifuged at 10,000 rpm for 1 min. The supernatant obtained was discarded, and the step was repeated thrice. The leftover media was discarded using a pipette, and the cell pellet was resuspended in 250 µl of re-suspension buffer and vortexed vigorously. To this mixture, 250 µl of lysis solution was added, and the tube was gently inverted ten times, followed by incubation for 3 min at room temperature. 350 µl of neutralization solution was added to the lysate and gently inverted the tubes 10 times. The mixture was then centrifuged at 13,000 rpm for 10 min, and the obtained supernatant was transferred to GeneJET purification column. The purification column was centrifuged at 13,000 rpm for 1 min, and the flow throw was discarded. 500 µl of wash solution was added to the purification column and centrifuged at 13,000 rpm

for 1 min. The flow through was discarded, and 500 µl of wash solution was again added to the purification column, followed by centrifugation at 13,000 rpm for 1 min. The flow through was discarded, and the empty column was centrifuged at 13,000 rpm for 2 min. The purification column was placed onto a new sterile 1.5 ml Eppendorf tube, and then 50 µl of elution buffer was added to the column followed by incubation for 2 min. The column was then centrifuged at 13,000 rpm for 2 min, and the eluted fraction was used to process the process further.

The isolated plasmid was quantified using NanoDrop 2000 Spectrophotometer (Thermo Fisher Scientific, MA, USA) and then double digested with NcoI and XhoI restriction enzymes within 40 min of incubation at 37°C followed by its 1% agarose gel electrophoresis analysis as described in section **3.2.2.5.3.2.**

3.2.3.2 Expression of recombinant protease

Three different approaches were analysed for suitable expression of the recombinant protease gene and renaturation of the recombinant enzyme.

3.2.3.2.1 Renaturation by dialysis

In the first approach, the transformed recombinant colonies were grown for 12 hours in 5 mL of LB broth supplemented with 50 µg/ml of Kanamycin. After that, the bacterial culture was incubated at 37°C after being inoculated in 50 ml of LB broth containing 50 µg/ml of Kanamycin. Thermo Fisher Scientific's IPTG (isopropyl-D-1-thiogalactopyranoside) at 1 mM induced protein expression in the cell culture once its absorbance at 600 nm reached 0.6. This step was followed by a 24-hour incubation period at 37°C. The induced cells were gathered by centrifugation at 10,000 g X 20 min (4°C), resuspended the pellet in bacterial cell lysis buffer (pH 8.0), and subjected to sonication for 20 min with 20 s intervals in between for cell lysis.

The cell lysate was centrifugated for 15 min X 12,000 rpm at 4°C, after which the supernatant was disposed of. The cell pellet (insoluble fraction containing inclusion bodies) was further processed by re-suspending it in 20 mM Tris-HCl buffer (pH 8.0) comprising 8 M urea and dialysed against 20 mM Tris-HCl buffer (pH 8.0) containing 6 M urea using dialysis sacks having flat width 30 mm, 12000 Da at 4°C for 18 h. The urea concentration was reduced gradually from 6 M to 0 M (distilled water) by changing the buffer at an interval of 2 h. Final dialysis with 0 M urea concentration (distilled water) was continued for 10 h at 4°C. After dialysis, the cells were collected by centrifugation at 10,000 g x 15 min, 4°C, and the supernatant was taken. The protein content of the expressed protein was measured as described

in section **3.2.2.3.1,** and expression of the recombinant protein was analysed by 12.5% SDS-PAGE as described in section **3.2.2.3.3.1** by mixing ~50 µg of supernatant with reducing dye.

3.2.3.2.2 Renaturation by direct mixing with refolding solution

The transformed recombinant colonies were grown for 12 hours in 5 mL of LB broth supplemented with 50 µg/ml of Kanamycin in the second method. After that, the bacterial culture was incubated at 37°C after being inoculated in 50 ml of LB broth containing 50 µg/ml of Kanamycin. Following the mid-logarithmic phase, when the cell absorbance (OD_{600}) reached 0.8, 1 mM IPTG was added to the culture to induce protein expression and incubated for 20 h at 30 °C. The recombinant *E. coli* cell suspension was centrifuged at 5,000 g X 15 min (4 °C), followed by resuspending the precipitate in 50 mM Tris-HCl buffer (pH 7.5) comprising 0.5 M NaCl, 10 mM EDTA, 1% Triton X-100 and 0.1 mg/ml lysozyme and the cells were then allowed to lysate by incubating at room temperature for 15 min [16].

Further, the cell lysate underwent 30 cycles of ultrasonication for 5 s each, with cooling for 5 s between each cycle on ice. The disrupted cell lysate was subjected to centrifugation at 10,000 g X 10 min (4 °C), and the cell pellet (containing the inclusion bodies) was resuspended twice in 2 ml of 20 mM Tris-HCl buffer (pH 7.5) comprising 1% Triton X-100 for 10 min, and once in 2 ml of 50 mM Tris-HCl pH 8.0 for washing followed by centrifugation at 10,000 g X 10 min (4°C) at each step. The cell residue was dissolved in water of pH 12.0 and ultrafiltrated using the Amicon Ultra 30 kDa molecular weight cut-off centrifugal tubes (Millipore, Bedford, MA, USA) by centrifugation at 4,000 g X 30 min (4°C). The protein fraction, the mass of which was greater than 30 kDa, was collected for protein refolding.

The direct mixing method for protein folding was used to refold the recombinant protein. An equal volume of the refolding and recombinant protein solution was mixed directly within 5 minutes with magnetic stirring, followed by an additional 1 h of stirring. The protein content of the solution was determined as described in section **3.2.2.3.1** and he protein expression was analyzed by 12.5% SDS-PAGE as described in section **3.2.2.3.3.1**.

3.2.3.2.3 Renaturation using urea

The transformed recombinant colonies were grown for 12 hours in 5 mL of LB broth supplemented with 50 µg/ml of Kanamycin in the third approach. After that, the bacterial culture was incubated at 37°C after being inoculated in 50 ml of LB broth containing 50 µg/ml of Kanamycin. 1 mM of IPTG was used to induce protein expression in the recombinant

transformed colony culture once its absorbance at 600 nm reached 0.6. They were then incubated for an additional 6 h at 37°C. The induced culture was gathered by centrifuging at 8000 rpm x 10 min (4°C), and the cell pellet was resuspended in 1X PBS (pH 7.4) containing 8 M urea. The cell lysate was sonicated for 5 min with 40 pulse duration to break down the cells, followed by centrifuging the cell lysate at 8000 rpm x 30 min (4°C). The obtained supernatant was examined for its protein content as described in section **3.2.2.3.1,** and 12.5% SDS-PAGE analysis was performed to observe the protein expression as described in section **3.2.2.3.3.1**.

3.2.3.3 Purification of recombinant protease by Ni-NTA affinity chromatography

The affinity chromatography used nickelnitriloacetic acid (Ni-NTA) agarose resin (Thermo Fisher Scientific, MA, USA) to purify the recombinant protease. The recombinant protease that was expressed and renatured by the first two approaches i.e., renaturation by dialysis and renaturation by direct mixing method by refolding solution, was run through Ni-NTA affinity column previously equilibrated with 20 mM Tris-HCl to purify the expressed protein. In brief, the outlet of the empty PD10 column was closed, and 2 ml of resin was added, allowing the resin to settle. The column was washed twice with distilled water to remove ethanol and equilibrated with 20 mM Tris-HCl (pH 8.0). The unbound protein fractions were allowed to flow through by washing with 20 mM Tris-HCl (pH 8.0), and the target protein bound in the column was eluted by gradually increasing the elution buffer's imidazole concentration from 100, 200, 250, 300, 500, and 750 mM. To make sure the target protein was eluted, a 12.5% SDS-PAGE was performed as described in section **3.2.2.3.3.1**, and the eluted protein's enzyme activity was examined (see below).

The recombinant protease enzyme expressed by the third approach, i.e., renaturation using urea, was purified using the Ni-NTA column using a different method. To purify the recombinant protease, 2 ml of resin was loaded into an empty PD10 column and allowed to settle down. The column was washed twice with distilled water to remove the ethanol and equilibrated with 1X PBS (pH 7.4) containing 8 M urea. Then, the supernatant containing the expressed protein was mixed with the column matrix and incubated for 40 min. After the incubation, the column was washed with a decreasing urea concentration from 7 to 1 M. The protein of interest bound in the column was eluted by gradually increasing the elution buffer's imidazole concentration starting from 20 mM, 40 mM, 60 mM, and 300 mM. To verify that

the target protein was eluted, a 12.5% SDS-PAGE was performed as defined in section **3.2.2.3.3.1**, and its enzyme activity was analysed (see below).

3.2.3.4 Biochemical characterization of recombinant fibrinolytic protease

3.2.3.4.1 Protein estimation

The protein content of the purified eluted fractions of recombinant protease was analysed as described in section **3.2.2.3.1.**

3.2.3.4.2 Determination of molecular weight and purity of recombinant protease by SDS-PAGE analysis

The expression and molecular mass of the recombinant protease (20 µg) were determined by SDS-PAGE analysis as described in section **3.2.2.3.3.1** under reducing conditions. 5 µl of the loading dye containing β-mercaptoethanol (30.0 µl ml^{-1} of loading dye) and DTT (2.0 mM) was added to the sample and heated at 95°C for 5 min before loading in the gel to analyse the sample under reducing condition.

3.2.3.4.3 Fibrinolytic assay

3.2.3.4.3.1 Colorimetric assay

The purified recombinant protease's dose-dependent and time-dependent fibrinolytic activity was assessed against fibrin in a 20 mM K-phosphate buffer (pH 7.4) [17]. In brief, a fibrin clot was formed by combining 40 µl of 2.5 mg/ml bovine fibrinogen (dissolved in 20 mM K-phosphate buffer, pH 7.4) with 3 µl of thrombin (10 NIH U/ml) and incubated for 30 minutes at 37°C. For analysing the dose-dependent fibrinolytic activity, different concentrations of the recombinant protease (6.25 µM, 12.5 µM, 25 µM, and 50 µM) were added to the clot, and the reaction was left to incubate at 37°C for 90 min. For analysing the time-dependent fibrinolytic activity, 12.5 µM of recombinant protease was added to the clot, and the reaction was left to incubate at 37°C for different time intervals (60 min, 90 min, 120 min, and 180 min). The reaction was stopped by adding 10 µl of ice-cold 10% (v/v) trichloroacetic acid (TCA), and Folin-Ciocalteu's reagent was used to measure the amount of free amino acids (tyrosine) released at 660 nm in the supernatant. One unit (U) of fibrinolytic activity has been defined as n mole equivalent of tyrosine formed per min [18]. The fibrin plate method also evaluated the fibrinolytic activity to analyze the fibrin degradation pattern.

3.2.3.4.3.2 SDS-PAGE analysis

In brief, 40 µl of 2.5 mg/ml bovine fibrinogen (dissolved in 1X PBS, pH 7.4) was combined with 3 µl of thrombin (10 NIH U/ml) and incubated for 30 minutes at 37°C to form a fibrin clot. Subsequently, the fibrin clot was treated with 50 µg of the purified recombinant protease for 90 min at 37°C. The fibrin degradation products were then separated by 12.5% SDS-PAGE. The gel was stained using Coomassie Brilliant Blue R-250 (section **3.2.2.3.3.1)**, allowing the protein bands to be seen. For control, instead of adding the purified recombinant protein, 0.1 ml of PBS (1X, pH 7.4) was added, and the experiment was then performed under the same conditions. The ImageJ software (version 1.47; Wayne Rasband, NIH, USA) was used to scan and analyze the gel, and ImageQuant TL 8.1 software (Ge Healthcare, Sweden) was used to determine the band intensities, where the percent degradation of the fibrin's α- and β-chains was determined, taking into account that the untreated (control) fibrin's band intensity was 100% [19].

3.2.3.4.4 Effect of metal ions on protease activity by colourimetric method

The impact of distinct divalent cations, namely Ca^{2+}, Mg^{2+}, Fe^{2+}, Co^{2+}, and Hg^{2+}, on enzyme activity was ascertained through the incubation of 12.5 µM of recombinant protease in 20 mM K-phosphate buffer pH 7.4 for 30 minutes at 37 ºC, along with varying cations (final concentration 4 mM). The assay employed fibrin (7.35 µM) as a substrate. The recombinant protease's activity was determined by comparing it to the recombinant protease incubated in identical conditions without metal ions (control).

3.2.3.4.5 Determination of interaction between the recombinant protease and thrombin by spectrofluorometric analysis

Briefly, thrombin dissolved in 1X PBS, pH 7.4 was incubated with varying concentrations of the recombinant protease for 60 seconds at room temperature (approximately 23 ºC) [20]. The reaction mixture was excited at a fluorescence intensity of 280 nm, and the emission spectrum was captured between 300 nm and 425 nm using a fluorescence spectrometer (LS55, Perkin Elmer) [20,21]. All binding experiments were conducted in triplicate to verify their consistency. The determination of the dissociation constant (Kd) involved employing the one-site binding model equation [21,22], i.e.,

$$\Delta F = \frac{\Delta Fmax \times C}{Kd + C}$$

In this context, ΔF represents the alteration in the fluorescence intensity of thrombin in the presence of the examined protease, ΔFmax denotes the maximum fluorescence intensity change of thrombin upon saturation with the corresponding protease, and C stands for the enzyme concentration. The dissociation constant (Kd) for the interaction between recombinant protease and thrombin was calculated using Graph Pad Prism 5 software from GraphPad Software, CA, USA.

3.2.3.5 Pharmacological characterization of recombinant fibrinolytic protease

3.2.3.5.1 Hemolytic assay

The method outlined by Mukherjee et. al. [23] was used to determine the hemolytic activity. In brief, blood from a healthy goat (9 ml) was collected in a sterile tube containing 3.8% trisodium citrate (an anticoagulant), and the tubes were centrifuged at 4300 rpm for 15 min. The pellet was resuspended in 1X PBS and washed with 1X PBS (pH 7.4) thrice and then diluted to 0.5% (v/v) in 1X PBS (pH 7.4) after discarding the platelet-poor plasma (PPP). Afterward, 2 ml of erythrocyte suspension was added to each tube containing the recombinant protease to be evaluated. The tubes were then gently inverted and incubated at 37°C for 90 min.

3.2.3.5.2 Anticoagulant assay

The plasma clotting activity of recombinant protease was determined by the method of Angulo et. al. [24] with some modifications as described by Mukherjee & Maity [25]. In brief, blood was obtained from the goat in a sterile container containing 3.8% trisodium citrate (9:1, Blood: Sodium citrate), and the platelet-poor plasma (PPP) was obtained by centrifuging the blood at 4300 rpm for 15 min at 4°C twice. The obtained PPP was used within 4 h of collection. The specific amount of recombinant protease (in a final volume of 20.0 µl) was added to 300.0 µl of prewarmed PPP at 37° C, and the mixture was incubated for 3.0 min at 37°C to analyse the plasma re-calcification time. 40.0 µl of 250 mM $CaCl_2$ was added to the mixture, and with the help of a stopwatch, the time taken for the appearance of the first visible fibrin thread/clot formation was recorded. Instead of the recombinant protease, 20 µl of 20 mM potassium phosphate buffer, pH 7.4, was incubated with plasma aliquot to be treated as a control.

The coagulation time was recorded identically. One unit of anticoagulant activity has been defined as 1.0 s increase in the clotting time of PPP as compared to the clotting time of average PPP (control) under identical assay conditions [17,22,26].

3.2.3.5.3 Determination of activated partial thromboplastin time (APTT)

A commercial Liquicelin-E-kit determined the activated partial thromboplastin time (APTT). In brief, 0.1 ml of PPP was taken and heated to 37°C in a water bath for 3-5 min. With the addition of a predetermined quantity of recombinant protease to the PPP, the mixture was incubated for 3 min at 37°C in a water bath. 0.1 ml of Liquicelin-E reagent was added to the tube, which had been pre-warmed for 3 min at 37°C. After the incubation, 0.1 ml of 25 mM $CaCl_2$ (pre-warmed) was added to the plasma-Liquicelin mixture. A stopwatch recorded the first evident appearance of fibrin thread/clot development. Instead of the recombinant protease, 20 µl of 20 mM potassium phosphate buffer, pH 7.4, was incubated with plasma aliquot to be treated as a control, and the coagulation time was recorded identically [27]. One unit of anticoagulant activity has been defined as 1.0 s increase in the clotting time of PPP compared to average PPP (control) under identical assay conditions.

3.2.3.5.4 Determination of prothrombin time (PT)

A commercial Liquiplastin kit determined goat PPP's prothrombin time (PT) [22,27]. In brief, 0.1 ml of PPP was taken and heated to 37°C in a water bath for 3-5 min. With the addition of a predetermined quantity of recombinant protease to the PPP, the mixture was incubated for 3 min at 37°C in a water bath. To the tube, 0.2 ml of the Liquiplastin reagent was added, which had been pre-warmed for 3 min at 37°C, and the contents were gently mixed by shaking the tube. A stopwatch recorded the first evident appearance of fibrin thread/clot development. Instead of the recombinant protease, 20 µl of 20 mM potassium phosphate buffer, pH 7.4, was incubated with plasma aliquot to be treated as a control, and the coagulation time was recorded identically. One unit of anticoagulant activity has been defined as 1.0 s increase in the clotting time of PPP compared to average PPP (control) under identical assay conditions.

3.2.3.5.5 *In vitro* thrombolytic activity

The in vitro thrombolytic efficacy was assessed following the method previously outlined by Majumdar et al. [28]. In brief, blood of goat (1.0 ml) was obtained in 3.8% trisodium citrate, was left to form a clot at room temperature with 100 µl of 250 mM $CaCl_2$. The clot's weight was recorded, and various concentrations (ranging from 0.42 µM to 12.5 µM) of recombinant protease were introduced and incubated for 60 minutes at 37 °C. As a control, the blood clot underwent incubation with 1X PBS under the same experimental setup. The in vitro blood clot lysis efficiency (thrombolytic activity) was quantified as 1 mg of blood clump (thrombus) lysed

per µg of the test sample/enzyme, in comparison to the control. The volume of blood clot that was lysed was calculated by comparing the weight of the clot before and after the thrombolytic reagent was added. In an additional set of assessment, the blood clump had been heated to 80°C for 30 min in order to breakdown the plasmin, plasminogen, and t-PA, which are endogenous fibrinolytic components, before measuring the thrombolytic activity.

3.2.3.5.6 Effect on inhibition of fibrinogen clotting time of thrombin

For 30 minutes at 37 °C, different doses of recombinant protease (ranging from from 0.42 µM to 6.25 µM) were first incubated with thrombin (3 µl, 10 NIH U/ml in 1X PBS, pH 7.4). In the control, thrombin was incubated with 1 PBS under the same identical conditions. 40.0 µl of 0.25% (w/v) human plasma fibrinogen was added to initiate the reaction. Visual examination was used to determine whether any fibrin clots formed [17]. The activity of thrombin against its substrate in the control group was considered 100% activity, and other datas were assessed accordingly.

3.2.3.5.7 Platelet modulating activity of recombinant protease

Platelet modulating activity of recombinant protease on platelet-rich plasma (PRP) made from goat blood was ascertained by Horn et. al., [29] and as reformed by Dutta et. al., [22]. In brief, 100 µl of PRP suspension was mixed with varying doses of recombinant protease (6.25-50 µM) in a 96-well plate. The absorbance was then continuously recorded at 540 nm for 5 min at 15-sec breaks in a microplate reader (MultiskanGO, Thermo Scientific, USA). PRP's absorbance was determined at 540 nm as a control. The following formula was used to compute the percentage of platelet aggregation, which expressed the degree of aggregation as a rise in light transmission.

$$\% \text{ Aggregation} = \frac{\text{A540 of PRP before the addition of rBariumfibrase} - \text{A540 of PRP after the addition of rBariumfibrase}}{\text{A540 of PRP before the addition of rBariumfibrase}} \text{X } 100$$

3.2.3.6 Cell cytotoxicity assay of recombinant fibrinolytic protease

NRK-52^{E} (Normal Rat Kidney) cells were cultivated in 75-cm^2 flasks containing Dulbecco's Modified Eagle Medium (DMEM), enriched with 10% fetal bovine serum, 0.25% of Penicillin-Streptomycin on a 96-well cell culture plate. For the cell viability evaluation, 3-(4,5-

dimethylthiazole-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) dye assay was performed on NRK-52^{E} (Normal Rat Kidney) cells (0.1 x 10^5) as previously described by us [30,31].

Briefly, a 96-well plate was filled with cells at a density of 1×10^4 cells/well, and they were incubated in 5% CO_2 to promote cell adherence. After 24 h, the cultured cells were treated with various doses (5-80 µg/ml) of recombinant protease or growth medium (control). A positive control was also considered for comparative analysis, where the cells were treated with triton-X. After incubation, the wells were filled with new medium and MTT dye and incubated for four hours. After dissolving the blue formazan precipitate with 100µL of dimethylsulfoxide (DMSO), the absorbance at 570 nm was measured. The viability of the cells was measured using the following equation.

$$\text{Percentage of Viability} = \left(\frac{\text{Absorbance of the treatment at 570 nm}}{\text{The absorbance of the control at 570 nm}}\right) \times 100$$

3.2.3.7 *In vivo* toxicity assessment

Swiss albino mice (30-35 g) and Wistar strain rats (190-220 g) of both sexes were obtained from the Institute of Advance Study in Science and Technology (IASST), Guwahati, Assam, Animal House Experimentation Facility. Every animal was clear of pathogens. The overall surrounding of captivity was retained at 25-32°C with a humidity level of at least 70%, simulating an atmospheric environment. The animals were expected to have a stable physiological state. The animals had been placed in social groups before the studies, and the standard conditions of captivity were followed, as previously mentioned. Animal experiments were conducted following the OECD/OCED guidelines 425 methodology at IASST, Guwahati, Assam. The Institutional Ethical Committee of IASST, Guwahati, Assam, approved the protocols for the animal experiments (Approval No. IASST/IAEC/2024/05).

3.2.3.7.1 *In vivo* anticoagulant potency of recombinant protease in swiss albino mice

Different doses of recombinant protease (0.25 mg/kg and 0.5 mg/kg in 100 µl of 1X PBS) were administered via i.v. injection to a six Swiss albino mice group to determine the *in vivo* anticoagulant properties. After 6 h of treatment, blood was collected via retro-orbital piercing and gathered in microfuge tubes filled with 3.8% sodium citrate solution (1:9, v/v). The Ca-

clotting time was then measured using the PPP extracted from the mice in the treated and control groups as described in section **3.2.3.4.3.2.**

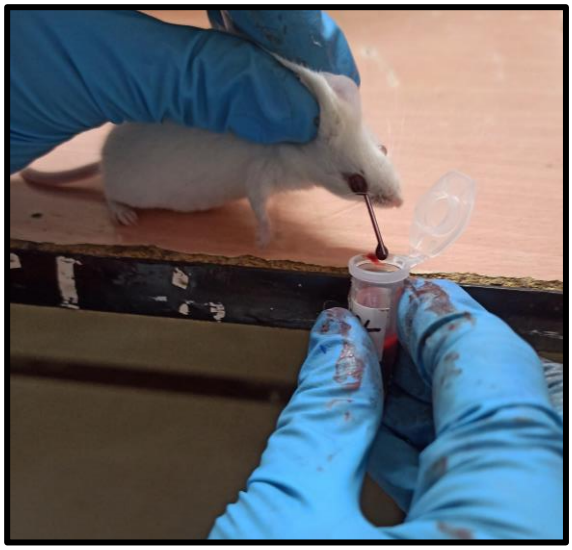

Figure 3.1: Collection of blood sample from mice by mechanically disrupting the retro orbital sinus.

3.2.3.7.2 *In vivo* toxicity assessment in rat model

Recombinant protease was injected intravenously at a dosage of 4 mg/kg body weight of rat (n=5) in a overall volume of 200 µl 1X PBS, pH 7.4. The rats in the control group (placebo) were given an equivalent amount of 1X PBS, pH 7.4. For a period of 72 hr after the injection, the animals were monitored for behavioural changes and physical characteristics, including body weight, food and water intake, grip strength, rectal Temperature, and death, at regular intervals for up to 72 hours after the injection.

Blood specimens were drawn from the mice in the control and treatment groups via retro-orbital sinus venipuncture using a hematocrit capillary tube in an anticoagulant-free tube. Within an hour of the sample collection, the serum was separated by spinning at 3000 rpm for 10 minutes, 4 °C. Using an automatic biochemical analyzer (BeneSphera C61), the following biochemical characteristics of the serum were examined using commercial diagnostic kits following the instructions provide by the maker: Serum Glutamic Pyruvic Transaminase (SGPT), Serum Glutamic Oxaloacetic Transaminase (SGOT), Blood Urea Nitrogen, low-density lipoprotein (LDL), high-density lipoprotein (HDL), total protein (TP), triglycerides (TRIG), creatinine (CR) and cholesterol (CH).

Figure 3.2: Intravenous administration of recombinant protease at a dose of 4 mg/kg in tail vein of rats

3.2.3.7.3 Histopathological study

After 72 h of treatment, the rats in the treated and control groups were killed by sodium pentobarbital overdose. The tissues of several organs, including the liver, kidney, heart, lung, and ovaries, were taken, and any adhesive blood was removed by washing them with PBS (1X, pH 7.4). Fixation was done in 10.0% neutral buffered formalin, and the tissues were dehydrated using progressively higher ethanol grades embedded in paraffin (ParaplastTM resin). Hematoxylin and eosin staining were used to prepare paraffin slices (5.0 µm) for light microscopic examination (Leica DM 3000) [32].

3.2.4 Statistical analysis

All of the data were represented using the mean ± standard deviation (SD) of independent triplicates. Sigma Plot 11.0 for Windows (version 10.0) was used to analyse significant differences between the test and control using the student's t-test. With GraphPad Prism software, one-way variance analysis (ANOVA) was used to analyse the significance of differences for more than two groups. Statistical significance was established when the p-value was <0.05.

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