

CHAPTER III

Materials and Methods

CHAPTER 3

MATERIALS AND METHODS

3.1 Materials

3.1.1 Columns

Hi Prep™ Phenyl 16/10 FF column and Hi Load™ 16/60 Superdex75 Pg were purchased from GE Healthcare Biosciences (Wipro-GE Healthcare Biosciences, Uppasala, Sweden). RP-UHPLC Acclaim® 300 C₁₈ column (2.1 mm x 150 mm, 3 μm, 300 Å) and Acclaim RSLC 120, C₁₈ RP-UPLC column (2.1 X 150mm, 3μm, 300 Å) were purchased from (Dionex Ultimate Mate 3000RSLC, Dreieich, Germany).

3.1.2 Chemicals

3.1.2.1 Analytical grade

All analytical grade reagents required such as phenylmethylsulfonyl fluoride (PMSF), p-bromophenacyl bromide (pBPP), N-p-Tosyl-phenylalanyl chloromethyl ketone hydrochloride (TPCK), N α -Tosyl-L-lysine chloromethyl ketone hydrochloride (TLCK), disodium- ethylene diamine tetra acetic acid (diNa-EDTA), dithiothreitol (DTT) and iodoacetamide (IAA) were purchased from Sigma-Aldrich, USA. Methanol, 2-propanol, ethanol, glycine, Tween-20, Triton-X-100, sodium dodecyl sulphate (SDS), Tris-HCl, sodium hydroxide, Sodium carbonate, potassium dihydrogen phosphate, coomassie brilliant blue R-250, glycine, casein, Tween-20, di-sodium hydrogen phosphate, di-potassium hydrogen phosphate, Folin-Ciocalteu's reagent, D-glucose, fructose, sucrose, galactose, maltose, lactose, cellulose, ammonium sulphate, magnesium chloride, mercuric chloride, copper chloride, zinc sulphate, manganese chloride, calcium chloride, cadmium chloride, cobalt chloride, nickel chloride, sodium nitrate, HPLC grade solvents like acetonitrile and trifluoroacetic acid (TFA) were purchased from Merck, India. For cell culture study Dulbecco's Modified Eagle's Medium (DMEM), 2,3-Bis-(2-Methoxy-4-Nitro-5-Sulfophenyl)-2H-Tetrazolium-5-Carboxanilide (XTT), Trypsin, 100 units/ml Penicillin and 100 μg/ml Streptomycin were purchased from Sigma Aldrich, USA and Fetal bovine serum (FBS) was purchased from PAA laboratory, Austria.

3.1.2.2 Microbiological grade culture media/chemicals

Nutrient agar, nutrient broth, skim-milk agar, peptone, urea broth, litmus milk broth, phenol red dextrose broth, phenol red lactose broth, phenol red sucrose broth, phenol red mannitol broth, tryptone broth, nitrate broth, indole nitrate broth, methyl red (MR) voges proskaure (VP) broth, triple sugar ,microbiological grade agar were obtained from Himedia, India.

3.1.2.3 Peptide mass finger printing analysis kit

“In Gel Tryptic Digestion Kit” lot no. 89871 for peptide mass fingerprinting was procured from Pierce, USA.

3.1.2.4 Cell lines used cell cytotoxicity study

The human colon adenocarcinoma (HT-29) and cervical carcinoma (HeLa) cell lines were procured from National Centre for Cell Sciences (NCCS), Pune, India.

3.1.2.5 Serum profiling kits

The kits for the estimation of low density lipoprotein (LDL), high density lipoprotein (HDL), glucose (GLU), urea (U), total protein (TP), uric acid (UA), triglycerides (TRIG), cholesterol (CH), creatinine (CR), alkaline phosphatase (ALP), serum glutamic oxaloacetic transaminase (SGOT) and serum glutamic pyruvic transaminase (SGPT) were procured from Coral Clinical Systems (Goa, India) whereas measurement of serum lactate dehydrogenase (LDH) was obtained from Fisher scientific (Mumbai, India). The animal food was obtained from Pranav Agrotech, Delhi, India. Carrageenan was procured from Sigma Aldrich, USA. Bovine fibrinogen and thrombin were purchased from Himedia. Liquiplastin kit (for PT determination) was purchased from Tulip Diagnostics (P) Ltd, India. C.K.Prest (for APTT determination), FIBRI-PREST (for quantitative estimation of fibrinogen), Thrombin kit (for determination of thrombin time) were procured from Stago Diagnostic, France. Animal foods were obtained from Pranav Agrotech, Delhi, India.

3.2 Methods

3.2.1 Collection of fermented food samples

Fermented food samples were collected from various regions of North-East India [Kukurmati(Assam), Mishing (Arunachal Pradesh), Nopaam (Assam), Dibrugarh (Assam), Kohima (Nagaland)].

3.2.2 Screening of fibrinolytic /fibrin(ogen)olytic enzyme producing bacteria

For screening of fibrin(ogen)olytic bacteria contained in fermented food sample therein, 1.0 gm of sample was mixed with 9.0 ml of 0.9% (w/v) sterile saline and serially diluted up to 10^{-4} with 0.9% w/v saline. Each dilution was spread on plasminogen-free fibrin agar plates, pH 7.4, containing (1 μ l of 50 mg/ml fluconazole stock solution was added per ml of culture media) fluconazole as antifungal agent ^[118] and plates were incubated at 37 °C for 48 h under static condition in an inverted position.

3.2.2.1 Measurement of zone of hydrolysis on fibrin agar plate

The plates were incubated at 37 °C for 48 h and fibrinolytic enzyme production was evaluated by the zone of hydrolysis of fibrin around the bacterial colonies. The fibrinolytic protease producing bacteria were counted in a Cubek colony counter and the zone of hydrolysis around the colonies was measured (in mm). Based on visible zone of clearance (secretion of proteolytic enzymes) measurement around the colonies, a few extracellular protease producing promising bacterial colonies were isolated and pure culture of such colonies were obtained.

3.2.2.2 Growth kinetics and fibrinolytic/ fibrin(ogen)olytic protease production

For study the bacterial growth and fibrinolytic/ fibrin(ogen)olytic protease production, bacteria were propagated at 37 °C and pH 7.4 for different time intervals (24–120 h) in 100 ml M9 media [Appendix I-B(i)] placed in a 0.5 l flask with constant shaking (200 rev /min) on a rotary shaker. At a regular time interval samples (5.0 ml aliquots) were withdrawn aseptically, followed by assay of fibrinolytic/ fibrin(ogen)olytic activity and protein estimation of cell free culture

supernatant (CFC) obtained by centrifuging the culture at 5000 x g for 15 min at 4 °C.

3.2.2.3 Fibrinolytic to caseinolytic (F/C) ratio

Culture supernatants were withdrawn at 12 h interval by centrifugation at 5000 x g for 15 min and CFC assayed for fibrinolytic and caseinolytic activity at 37 °C with pH 7.4 to determine fibrinolytic to caseinolytic (F/C) ratio. The potential strains were selected based on F/C ratio; higher is the F/C ratio, more potent is the strain for fibrinogenolytic enzyme production.

3.2.2.4 Thrombolytic activity

One ml of 3.8% sodium citrate containing goat blood was allowed to clot at room temperature for an hour after the addition of 100 µl of 250 mM CaCl₂. The clot was weighted and thereafter, 5.0 µg of crude enzyme (CFC) or commercial drugs (plasmin, or streptokinase) was added and incubated for 60 min at 37 °C. As a control, blood clot was incubated with phosphate buffered saline (1X PBS) under identical experimental conditions. The *in vitro* blood clot lysis activity (thrombolytic activity) was measured as 1 mg of blood clot (thrombus) lysed per µg of test sample as compared to the control. In another set of experiments, blood clot was heated at 80 °C for 30 min to denature the endogenous fibrinolytic factors (plasmin, plasminogen, t-PA) before the thrombolytic activity determination. To ensure reproducibility, each experiment was repeated in triplicate.

3.2.2.5 Substrate specificity

The activity of crude enzymes against other protein substrates (casein, bovine serum albumin, bovine serum γ-globulin, bovine plasma fibrinogen) was determined by incubating 0.1 µM of enzyme with 1.0 mg/ml of substrate and the final volume of the reaction mixture was adjusted to 1.0 ml with 50 mM sodium phosphate buffer at pH 7.4. After incubation at 37 °C for 30 min, the reaction was terminated by adding 10.0 µl of ice-cold 100 g/l trichloroacetic acid. One unit of protease activity is defined as 1 µg of tyrosine liberated per min per ml of enzyme.

3.2.3 Pure culture of fibrinolytic/ fibrin(ogen)olytic protease secreting bacterial isolates

In order to obtain a pure culture a loopful of bacterial culture was inoculated in nutrient broth with pH adjusted to 7.4 (Appendix I-M). With a 0.5 growth OD at 600 nm, 100 µl of culture was mixed in 0.9 % (w/v) sterile normal saline and serially diluted up to 10^{-4} with a final volume of 2.0 ml. 100 µl aliquot were spread over sterile nutrient agar plates (Appendix I-D), and kept for 24 h at 37 °C in order to obtain single distinct colonies. For the isolation of single pure colony of bacteria, the following techniques were used: (i) spread-plate method and (ii) streak-plate method.

3.2.3.1 Spread plate technique

The spread-plate technique is used for the separation of a dilute, mixed population of micro-organisms so that individual colonies can be isolated. In this technique microorganisms were spread over the solidified agar medium with a sterile L-shaped glass rod while the Petridis was spread on a turn table. The advantage of this technique is to allow cells to be separated from each other by a distance sufficient to allow the colonies that develop to be free from each other.

3.2.3.2 Streak-plate technique

The streak-plate method offers a most practical method of obtaining discrete colonies and pure cultures. In this method, a sterilized loop or transfer needle was dipped into a suitable diluted suspension of organisms which was then streaked on the surface of an already solidified agar plate to make a series of parallel, non-overlapping streaks.

3.2.3.3 Routine maintenance and preservation of microorganisms

Pure cultures of bacteria were preserved at 4 °C in nutrient agar slants and transferred to fresh slants at an interval of one month. Isolates were also stored in 15% (v/v) glycerol in nutrient broth and kept at -80 °C for long term storage.

3.2.3.4 Taxonomic identification of fibrinolytic/ fibrin(ogen)olytic producing bacteria

Taxonomic identification of fibrinolytic/ fibrin(ogen)olytic enzyme producing bacterial strain was done by (a) morphological characteristics, (b) biochemical tests, (c) sequencing of 16S rDNA as well as 16S-23S ISR followed by phylogenetic analysis.

3.2.3.5 Morphological tests

3.2.3.5.1 Simple staining

Two drops of bacterial culture were heat fixed over a clean glass slide and flooded by 5 drops of methylene blue stain for about 5 min. The stain was removed gently by placing the slide under running tap water. The slide was then dried by gently tapping with tissue paper and bacteria were observed under oil-immersion objective lens (1000 X).

3.2.3.5.2 Negative staining

One drop of nigrosine or Indian ink stain was placed at one end of clean glass slide with the help of Pasteur pipette. Two drops of liquid culture was placed on it, and with the help of clean slide held at an angle of 30 °C the mixture was spread throughout the slide and kept at room temperature for drying. This was followed by visualization of bacteria under oil-immersion objective lens (X 1000).

3.2.3.5.3 Gram's staining

Over a clean glass slide using sterile inoculating loop, a loop full of 24 h bacterial culture was spread over sterile glass slide and kept for 5-10 min for air drying followed by heat fixing. Few drops of primary stain were flooded over bacterial culture and kept for 1 min followed by washing the slide under tap water. Gram's iodine mordant was flooded over this after primary staining and kept at room temperature for 1 min. Removal of Gram's iodine mordant by washing under tap water was followed by addition of few drops of decolorizing agent (95 % ethyl alcohol) over bacterial culture. It was washed again with tap water to remove the decolorizer, and followed by addition of counter stain Safranin for 45 s. After this step counterstain was washed with tap water and slide was kept at room temperature

for complete air drying. The bacteria were visualized under a light microscope (X1000).

3.2.3.5.4 Spore staining

Using clean glass slide, loopful of 24 h bacterial culture was air dry followed by heat fixing, Then few drops of malachite green were added and the slide was placed on a warm hot plate, allowing the preparation to steam for 2-3 min. Slides from hot plate were removed and kept at room temperature for cooling and then washed under running tap water. Few drops of counter stain (safranin) were added over bacterial culture and kept for 30 s. This was followed by washing with tap water. Remaining water drops were absorbed by blotting paper and bacteria were examined under oil immersion objective lens (X1000).

3.2.4 Biochemical tests for strain identification

3.2.4.1 Hydrolysis test for casein, starch, lipid and gelatin

Sterile nutrient agar plates (pH 8.0) containing with 1% (w/v) of casein, starch or triglyceride tributyrin (as a lipid substrate) were used for hydrolysis tests. 24 h bacterial cultures after 24 h incubation at 37 °C were streaked with into plates using inoculating loop and kept in an inverted position for 24-36 h at 45 °C. In case of gelatin hydrolysis, 12% (w/v) of gelatin was supplemented in nutrient broth tubes and inoculated with a loopful of bacterial culture. The tubes were kept at 45 °C for 48h followed by placing the tubes in refrigerator at 4 °C for 30 min. The hydrolysis result was considered as positive on the basis of zone of hydrolysis around the colonies.

3.2.4.2 Carbohydrate fermentation test

Fermentation medium containing sugars was prepared as shown in appendix I-C(i). A loopful of 24 h bacterial culture was inoculated into all tubes supplemented with respective sugars and incubated for 48 h at 45 °C. Change in medium colour and gas formation (if any) post 48 h incubation were recorded. A control was also set up.

3.2.4.3 Triple sugar iron (TSI) agar test

Sucrose, lactose and glucose (0.1%, w/v) were supplemented in nutrient agar slants (Appendix I-L), and a loopful of 24 h bacterial culture was streak over TSI agar

slants. The slants were kept at 37 °C for 48 h. Change in medium colour was observed over TSI agar slants. A control was also set up.

3.2.4.4 IMViC test

3.2.4.4.1 Indole production test

Sterile sulfur indole motility (SIM) agar deep tubes were streaked with 24 h bacterial culture over agar surface and kept at 37 °C for 48 h. Change in medium colour after addition of Kovac's reagent was observed and recorded.

3.2.4.4.2 Methyl red - Voges-Proskauer (MR-VP) test

100 ml of sterile methyl red- Voges-Proskauer (MR-VP) broth was inoculated with 1.0 ml of 24h bacterial culture and kept at 37 °C for 48 h. MR-VP medium was separated into parts A and B. In part A, few drops of methyl red indicator was added for conformation of MR test. In part B, mixtures of Barritt A and B solutions were added in VP broth. In MR test, red colour formation after addition of methyl red indicator showed positive test and formation of yellow colour indicated negative test. In case of VP test, formation of pink colour complex indicated positive test.

3.2.4.4.3 Citrate Utilization test

10.0 ml of sterile Simmons citrate agar slants were prepared in streaked hard tubes with 24 h bacterial cultures and kept at 37 °C for 48 h, followed by addition of bromo-thymol blue indicator over surface. Formation of blue colour complex was taken as a positive confirmation test for citrate utilization.

3.2.4.4.4 Hydrogen sulfide test

10 ml of sterile sulfur indole motility (SIM) agar tubes was inoculated with a loopful of 24-h bacterial culture inside agar slants, and kept at 37 °C for 48 h. Formation of black colour indicated the production of hydrogen sulfide by bacterial culture.

3.2.4.4.5 Urease test

10.0 ml of sterile urea broth was inoculated with 0.1 ml of 24 h bacterial culture and kept at 37 °C for 48 h. Change in medium color from red to pink indicated the presence of urease activity.

3.2.4.4.6 Litmus-milk reactions test

10.0 ml of sterile litmus milk broth was inoculated with 0.1 ml of 24 h bacterial culture and kept at 37 °C for 48 h. Change in medium color, lactose fermentation, gas formation, curd formation, litmus reduction, peptonization and alkaline reaction if any, were observed post 48 h of incubation.

3.2.4.4.7 Nitrate reduction test

100.0 ml of sterile trypticase nitrate broth was inoculated with 1.0 ml of 24 h bacterial culture and kept at 37 °C for 48 h. To confirm the nitrate reduction capability of bacterial culture post 48 h of incubation, solution A (sulfanilic acid) and solution B (alpha-naphthylamine) and amount of zinc powder were mixed with bacterial culture. Formation of cherry red colour indicated the reduction of nitrate (ability of bacterial cells to reduce nitrate).

3.2.4.4.8 Catalase test

10.0 ml of trypticase soy agar slant was streaked with a loopful of 24 h bacterial culture and kept at 37 °C for 48 h. To confirm the presence of catalase activity, 3.0% (v/v) hydrogen peroxide (H₂O₂) was flooded over it. If bubble formation was observed, it indicated the catalase positive test (production of catalase by bacteria).

3.2.4.4.9 Oxidase test

10.0 ml of trypticase soy agar slant was streak with a loopful of 24 h bacterial culture and kept at 37 °C for 48 h. To confirm the presence of oxidase activity, p-aminodimethylaniline oxalate was added over the surface of bacterial culture. Development of pink color which finally turned on colony surface indicated the presence of oxidase.

3.2.5 Identification of bacterial strains

Taxonomic identification of FF01 strain was done by (a) morphological characteristics, (b) biochemical tests, (c) sequencing of 16S rDNA as well as 16S-23S ISR followed by phylogenetic analysis.

3.2.5.1 Morphological and biochemical properties

The bacterial isolate was identified by investigating its morphological, physiological and biochemical properties. The isolate was taxonomically identified up to genus level according to Bergey's Manual of Systematic Bacteriology^[119].

3.2.5.2 16S rRNA gene sequencing

Genomic DNA from strain FF01 was isolated according to our previously described procedures described by us^[120]. Universal primers were designed to amplify a 1.5Kbps conserved segment of 16S rDNA by polymerase chain reaction (PCR) (Table 3.1). The forward and reverse primers were 5'-AGAGTTTGATCCTGGCTCAG-3' and 5'-AAGGAGGTGATCCAGCCGCA-3', respectively^[121]. The PCR was performed on a PCR system 9700 (Applied Biosystems) as described by us^[87, 120]. Separation of PCR amplified DNA fragment (1.5 kb) was done on 1% (w /v) agarose gel electrophoresis. The DNA was stained with ethidium bromide and purified from gel using the Q1Aquick Gel Extract ion Kit (Qiagen, Germany) by following the instructions of the manufacturer. This purified product was directly subject to automated DNA sequencing using 3130 Genetic Analyzer (Applied Biosystem, Switzerland) from Department of Molecular Biology and Biotechnology, Tezpur University. To retrieve the homologous sequences in GenBank, the deduced sequence was analyzed by BLAST search programme of NCBI (<http://www.ncbi.nlm.nih.gov>).

Table 3.1: Optimal PCR reaction conditions for amplification of conserved region of 16S-rRNA gene of selected protease secreting bacterial strains

| PCR conditions | Bacterial strains | |
|------------------|--------------------|---------------------|
| | FF01 | FF02B |
| Pre-denaturation | 94°C for 5 min | 90 °C for 2 min |
| Denaturation | 94°C for 1 min | 92 °C for 1 min |
| Annealing | 55°C for 30 sec | 44 °C for 30 sec |
| Synthesis | 72°C for 2 min | 72 °C for 2 min |
| Final extension | 72 °C for 7 min | 72 °C for 6 min |
| Hold | 2 | 2 |
| Cycles | 36 | 36 |

3.2.5.3 PCR amplification of the 16S-23S inter space region (ISR region)

The 16S-23S ISR forward primers 16S/p1 (5'-AGTCTGCAACTCGACTGCGTG-3') and 23S/p2 (5'-CAACCCCAAGAGGCAAGCCTC-3') were used for amplification of 16S-23S ISR region from chromosomal DNA of FF01 bacterial strain ^[122]. In the next step, the 16S-23S ISR amplification the PCR-product was used as a template in a second PCR (Nested PCR) with primers 16S/p3(5'GGAAGGTGCGGCTGGATCACC-3'forward and reverse 23S/p4(5'CCCGAAGCATATCGGTGTTTCG-3')), which anneal to position 11334 to 11355 of the 16S-rRNA gene and position 11770 to 11791 of the 23S-rRNA gene, respectively ^[120, 122]. The nested PCR amplification included the entire ISR and one part of the flanking rDNA (28bp of 16S-rRNA and 83bp of 23S-rRNA).

3.2.5.4 Phylogenetic analysis

The 16S rDNA and ISR sequences of bacteria were aligned with reference sequences showing sequence homology from NCBI database (<http://blast.ncbi.nlm.nih.gov>) using the multiple sequence alignment programme of MEGA4 ^[123]. Phylogenetic trees ^[124] were constructed by distance matrix-based

cluster algorithms viz. unweighted pair group method with averages (UPGMA) and neighbor-joining ^[121] analysis. All the positions containing gaps and missing data were eliminated from the dataset (complete deletion option). An evolutionary tree was established among closely related *Bacillus* sp. to show its distinct phylogenetic position within the genus.

3.2.6 Purification of enzymes

3.2.6.1 Purification of fibrino(geno)lytic enzyme from *Bacillus cereus* strain FF01

3.2.6.1.1 Step-I: Preparation of cell free crude extract

Unless otherwise stated, all the purification steps were carried out at 4 °C. The bacteria were allowed to grow in 250 ml of nutrient broth medium at 37 °C for 24 h and cell-free culture supernatant was obtained by centrifugation (MULIFUGE X 1R, Thermo scientific, USA) at 5000 x g for 15 min.

3.2.6.1.2 Step-II: Ammonium sulfate precipitation

The crude cell free containing proteins were precipitated by gradually adding solid ammonium sulphate under stirring to reach 40 % (w/v) saturation. The mixture was allowed to stand for additional 4 h and then centrifuged at 13700 x g for 30 min. The pellet was re-suspended in 50 ml of 50 mM sodium phosphate buffer containing 1M ammonium sulphate.

3.2.6.1.3 Step-III: Hydrophobic interaction chromatography

The crude enzyme solution was applied on Hi prep TM Phenyl 16/10 FF column (1.6 x 10 cm) pre-equilibrated with 50 mM sodium phosphate buffer, pH 7.4 containing 1M ammonium sulphate. Elution of bound proteins was carried out with a linear gradient of 1-0 M (NH₄)₂SO₄ in the same buffer at a flow rate 2 ml/min. The active fractions were pooled and concentrated by lyophilization.

3.2.6.1.4 Step-III: Gel filtration chromatography

The concentrated sample further eluted on Hi load TM16/60 Superdex75 Pg (1.6 x 60 cm) by 20 mM Sodium phosphate buffer containing 0.15 M NaCl at a flow rate 0.5 ml/min. For all purification steps, the elutes were monitored at 215 nm detector

wavelength in AKTA purifier 10 FPLC equipment. Finally, gel filtration eluted fractions exhibited high fibrino(genolytic) activity were pooled together and used for subsequent studies.

3.2.6.2 Purification of fibrinolytic enzyme from *Brevibacillus brevis* strain FF02B.

3.2.6.2.1 Step-I: Preparation of cell free crude extract

All purification steps were carried out at 4 °C. The cell free extract supernatant was obtained by centrifugation (MULIFUGE X 1R, Thermo scientific, USA) at 5000 x g for 15 min to get the clear supernatant. The supernatant was carefully to a fresh tube.

3.2.6.2.2 Step-II: Hydrophobic interaction chromatography

A measured amount of solid ammonium sulphate was gradually added with constant stirring to 250 ml cell free culture supernatant to get the final salt concentration of 1M. Then 250 ml of the above mixture was loaded on a Hi Prep™ Phenyl FF 16/10 column attached to a Fast Protein Liquid Chromatography (FPLC) system AKTA purifier 10 (Wipro GE Healthcare Biosciences, Uppsala, Sweden). The column was pre-equilibrated with 50mM sodium phosphate buffer, pH 7.4 containing 1M ammonium sulphate. After washing the unbound proteins with two volumes of above equilibration buffer at a flow rate 2 ml/min, the bound proteins were eluted with 50 mM sodium phosphate buffer pH 7.4. Two ml fraction was collected in each tube and elution of proteins was measured at 280 nm. The fractions showing fibrinolytic activity were pooled, desalted and then lyophilized.

3.2.6.2.3 Step-III: Gel filtration chromatography

The dried fraction was re-dissolved in 50 mM sodium phosphate buffer, pH 7.4 and fractionated on a Hi Load™ Superdex 75 pg 16/60 FPLC column previously equilibrated with the same buffer. Elution was carried out with the equilibration buffer containing 0.15 M NaCl at a flow rate of 0.5 ml/min. Protein elution was monitored at 280 nm and 2.0 ml fraction was collected. The gel filtration fractions showing fibrinolytic activity were pooled, desalted, lyophilized and its protein content was determined.

3.2.7 Biochemical characterization

3.2.7.1 Determination molecular weight and purity of purified fibrinolytic /fibrin(geno)lytic enzyme by SDS-PAGE analysis

The SDS-PAGE was carried out with or without reduction of proteins by β -mercaptoethanol as described by Laemmli ^[125]. Briefly, either defined amount of crude protein or purified protease was loaded into 15% or 12.5% separating gel containing 5% (v/v) glycerol (Appendix I-N (i)). Electrophoresis was carried out at a constant current of 15mA until the dye front (bromophenol blue) reached the bottom of the gel. Before staining, proteins were fixed by incubating the gel in 20% TCA for 30 min at room temperature followed by washing the gel thrice in distilled water. Proteins bands were visualized by staining the gel with 1 % (w/v) Coomassie brilliant blue R 250 in methanol: acetic acid:water (4:1:5 v/v/v) and destained with methanol: acetic acid:water (4:1:5 v/v/v), and then photographed. Mobility of the purified protein was compared with the following molecular weight markers: phosphorylase b (97,400), bovine serum albumin (66,000), ovalbumin (43,000), carbonic anhydrase (29,000), soyabean trypsin inhibitor (20,000) and lysozyme (14,300). For determining molecular mass of unknown proteins, the migration profile of protein was measured and the \log_{10} values of the molecular weights of the standard proteins versus their migration distance were plotted. Taking the logarithm R_f allows the data to be plotted as a straight line. The molecular weight of unknown protein was then calculated from the standard curve.

3.2.7.2 Mass spectroscopy

The purity and molecular mass of the enzyme was further affirmed by MALDI-TOF-MS analysis (4800 plus, Applied Biosystems, USA) by using sinapinic acid (Sigma, USA) as matrix. Before application to matrix, the protein samples were desalted using Zip Tip (Millipore ZipTipTM). The MALDI-TOF-MS was operated in a linear mode with high mass positive acquisition and processing method. The matrix solution was prepared by dissolving sinapinic acid (SA) in 60 % acetonitrile (ACN) and 35 % methanol (v/v) at a concentration of 40 mg/mL. The FPLC-purified protein sample dissolve into a MALDI-MS-compatible buffer were desalted using ziptip method (Millipore ZipTipTM) and mixed to 10 ml of SA solution. After a short vortexing, the sample/matrix solution (1.5 μ l) was spotted onto the sample

plate and dried at room temperature. The spotting procedure was repeated twice. MALDI-TOF MS was carried out on a Voyager DE-PRO TOF mass spectrometer (Applied Biosystems, Foster City, CA, USA) equipped with a 337 nm UV nitrogen laser and delayed extraction. The other parameters of the instrument were: grid voltage 92 %, guide wire 0.2 %, bin size 4 ns, and input bandwidth at 20 MHz. The final spectrum was generated from the average of 400 laser shots for each sample. The MALDI-TOF was operated in Linear Mode with High Mass Positive acquisition and processing method. Data generated from MALDI-TOF MS instrument was further processed using Data Explorer 4.0 software (Applied Biosystems, Framingham, MA, USA).

3.2.7.3 Fibrin zymography

Fibrin zymography was performed as described previously Kim *et al* (1998)^[126]. Bovine fibrinogen (0.12 %, w/v, Sigma) dissolved in 20 mM sodium phosphate buffer (pH 7.4) and 100 µl of bovine thrombin (10 NIH unit /ml, Sigma) was copolymerized with 12% (w/v) acrylamide, 0.32 % (w/v) bisacrylamide and 1.5 M Tris/HCl (pH 8.8) in order to make the fibrin running gel. Then 4 % (w/v) acrylamide, 0.11 % (w/v) bisacrylamide and 0.5 M Tris/HCl (pH 6.8) (no fibrinogen) were used for the stacking gel, which was poured into a mini-gel cast (Tarson, India). The samples for analysis were prepared by diluting the culture supernatant 5-fold with zymogram sample buffer (0.5MTris/HCl, pH 6.8, 20% glycerol and 0.5% bromphenol blue). After the prepared samples (10 µl) were loaded into the wells, electrophoresis was carried out in the cold room (4 °C) at a constant 12 mA. After electrophoresis, the gel was incubated for 30 min at room temperature on a rotary shaker in 50 mM Tris/Cl (pH 7.4), which contained 2.5 % Triton X-100. The gel was washed with distilled water to remove Triton X-100, and then incubated in zymogram reaction buffer (30 mM Tris/HCl, pH 7.4) at 37 °C for 16 h. The gel was stained with Coomassie blue for 8 h and then destained. The digested bands were visualized as the non-stained regions of the fibrin gel. For the quantification, the densities of digested bands on the gel were analyzed by video densitometry using Bio 1D Ver. 97.04 (Vilber Lourmat, France).

3.2.7.4 Fibrinolytic assay

For fibrinolytic activity assay, 3.0 ml of thrombin (10 NIH U/ml) was added to 40.0 μ l of 0.6 % (w/v) bovine fibrinogen solution (prepared in 100 mM K-phosphate, pH 7.4) and clot was allowed to form at room temperature. Then the purified protease at a dose of 5.0 mg/ml was added to the clots and the reaction mixtures were incubated at 37 °C for different time intervals. The reaction was stopped by adding 10.0 μ l of ice-cold trichloro acetic acid and supernatant was used to determine the release of free amino acids (tyrosine) at 660 nm using Folin–Ciocalteus reagent. From a standard curve of tyrosine, the fibrinolytic activity was calculated. One unit (U) of fibrinolytic activity is defined as mg of tyrosine liberated per min per ml of enzyme.

3.2.7.5 Fibrin(ogen)olytic assay

The fibrinogenolytic activity was assayed as previously described ^[127]. Briefly, purified protease at a dose of 5 mg/ml was added to 40 μ l of 0.6 % (w/v) bovine fibrinogen solution (prepared in 100 mM K-phosphate buffer, pH 7.4) and the reaction mixture was incubated at 37 °C for 30 min. The reaction was terminated by adding 10 μ l of ice-cold 10 % (v/v) trichloroacetic acid (TCA) and tubes were incubated at 4 °C for 20 min. The reaction mixture was centrifuged at 3000 rpm for 3 min and 20 μ l of supernatant was used to determine the release of free amino acids (tyrosine) at 660 nm using Folin-Ciocalteu's reagent. From a standard curve of tyrosine, the fibrinolytic activity was calculated. One unit (U) of fibrinogenolytic protease activity is defined as μ g of tyrosine liberated per min per ml of enzyme.

The fibrin /fibrinogen degradation pattern was also analyzed by (a) 12.5% SDS-PAGE of fibrin degradation products under denaturing conditions and (b) RP-UHPLC (Dionex Ultimate Mate 3000RSLC, Dreieich ,Germany) analysis of fibrin degradation products on a Acclaim® 300 C₁₈ column (2.1 mm x 150 mm, 3 μ m, 300 A°) ^[107]. Briefly, the fibrin/fibrinogen degradation products (peptides) were eluted from the RP-UHPLC column with a linear gradient of 0–90% acetonitrile (v/v) containing 0.1% (v/v) TFA. The elution of peptides was monitored at 215 nm at a flow rate of 1.0 ml/min.

3.2.7.6 Caseinolytic assay

Caseinolytic activity was evaluated calorimetrically as described by Mukherjee and Maity^[127]. Briefly, 1 % (w/v) of casein in 0.1 M concentration of reaction buffer was incubated with specific amount of crude/ purified protease for 30 min at 37 °C followed by addition of 0.5 ml of 10 % (w/v) ice-cold TCA to stop the reaction. After centrifugation of the mixture, supernatant was transferred to a fresh tube containing 2.0 ml of 2 % (w/v) sodium carbonate in 0.1N sodium hydroxide. The reaction was allowed to continue for 10 min at room temperature followed by addition of 0.5 ml of Folin-Ciocalteus's reagent (1:2 dilutions). After 30 min absorbance was measured at 660 nm. Caseinolytic activity of the crude/ purified protein was calculated from a standard tyrosine curve. One unit (U) of caseinolytic activity is defined as µg of tyrosine liberated per min per ml of enzyme.

3.2.7.7 Protein estimation

The protein concentration was measured by the method of Lowry et al. (1951)^[128] using bovine serum albumin (BSA) as standard. The protein content of the unknown sample was calculated from the standard curve BSA obtained by plotting optical density (660 nm) Vs concentrations of BSA (0.1 to 1.0 mg/ml).

3.2.7.8 Substrate specificity of purified enzyme

The substrate specificity of purified enzyme was determined using 1.0 % (w/v) protein substrate such as casein, bovine serum albumin, globulin, fibrin and plasma fibrinogen at pH 7.4 and 37 °C for 30 min^{[127],[87]}. The reaction was stopped by addition of 10% TCA and one unit of protease activity has been defined as µg of tyrosine liberated from protein substrate per min by enzyme. The specific activity was determined by enzyme activity (units) per mg of protein.

3.2.7.9 Determination of amidolytic activity

The amidolytic activity of purified enzyme (0.2 µM) against various chromogenic substrates (0.2 mM) in a final reaction volume of 0.1 ml (50 mM sodium phosphate buffer, pH 7.4) was determined as described previously^[41]. After incubation for 20 min at 37 °C, the amount of released *p*-nitroaniline was determined by measuring the change in absorbance at 405 nm^[41, 107]. Amidolytic activity (AU) was expressed as µmoles of *p*-nitroaniline released per min by the enzyme. The following

chromogenic substrates (0.2 mM) were used for the amidolytic activity assay: *N*-Benzoyl-Pro-Phe-Arg-*p*-nitroanilide hydrochloride (substrate for plasma kallikrein), *N* α -Benzoyl-DL-arginine 4-nitroanilide hydrochloride (substrate for trypsin), D-Val-Leu-Lys-*p*-nitroanilide hydrochloride (substrate for plasmin), *N*-Benzoyl-Ile-Glu-Gly-Arg-*p*-nitroanilide acetate (substrate for factor Xa), and *N*-(*p*-Tosyl)-Gly-Pro-Arg-*p*-nitroanilide acetate (substrate for thrombin).

3.2.7.10 Effect of pH on fibrin(ogen)olytic / fibrinolytic activity and stability

The purified protease was incubated in different pH buffers (5.0, 6.0, 7.0, 8.0, 9.0, 10.0, 11.0, 12.0 and 13.0) and the protease activity in each buffer was determined. The following buffer (0.1 M) systems were used: citrate- phosphate (pH 4.0), potassium-phosphate (pH 7.0), Tris-HCl (pH 9.0), glycine-NaOH (pH 10.5-11) and glycine-NaOH (pH 13.0). The optimum pH for alkaline protease activity was determined by standard alkaline protease assay using casein/keratin 1.0 % (w/v) as substrate dissolved in the buffer systems mentioned above. To study the protease stability as a function of pH, 2.0 ml of the purified enzymes or 0.1- 4.0 μ g of purified protease was mixed with 2.0 ml of the buffer solutions mentioned above and incubated at 37 °C for desired time period. The protease activity was determined as stated in section 3.2.7.4 and 3.2.7.5. The relative protease activities (%) at various pH interval was determined by considering highest protease activity as 100% at optimum pH.

3.2.7.11 Determination of optimum temperature for protease activity

The optimum temperature for alkaline protease activity was determined by incubating the reaction mixture (containing protease and fibrinogen at optimum pH for 30 min, at different temperatures ranging from 30 to 80 °C. Before the addition of enzyme, the substrate (1.0 % (w/v) or fibrinogen) was pre-incubated at the respective temperature for 10 min. For each assay, a control was also setup with respective temperature. The protease activity was determined as stated in section 3.2.8.4 and 3.2.8.5. The relative protease activities (%) at various temperature interval was determined by considering highest protease activity as 100% at optimum temperature.

3.2.7.12 Effect of protease inhibitors and metal ions

The effect of different divalent cations such as CaCl_2 , MgCl_2 , CoCl_2 , HgCl_2 on enzyme activity was determined by incubating 500 nM of enzyme with different cations (final concentration 4 mM) in 20 mM K-phosphate buffer pH 7.4 for 30 min at 37 °C. The residual enzyme activity was assayed using fibrin/fibrinogen as a substrate. The effects of various inhibitors/ disulfide bonds reducing agent/ metal ion chelator and natural protease inhibitor of plasma were investigated by pre-incubating the enzyme with various inhibitors viz. phenylmethylsulfonyl fluoride (PMSF) (2 mM), *p*-bromophenacyl bromide (pBPP) (2 mM), *N*-*p*-Tosyl-phenylalanylchloromethylketonehydrochloride (TPCK) (100 μM), *N* α -Tosyl-L-lysinechloromethylketone hydrochloride (TLCK)(100 μM), diNa-EDTA(4 mM), dithiothreitol (DTT) (4 mM) and iodoacetamide (IAA) (4 mM) for 30 min at room temperature prior to the fibrinolytic activity assay ^[41]. The remaining enzyme activity after treatment with inhibitor was expressed as percent activity relative to the enzyme activity without inhibitors, which was considered as 100% activity ^[107].

3.2.7.13 Determination of plasminogen activating assay

For the determination of plasminogen activation, 20 ml of plasminogen (10 U/ml) was incubated with purified enzyme in 50 mM phosphate buffer pH 7.4 at 37 °C for 20 min. The formation of plasmin from plasminogen was determined by adding 0.2 mM of V0882 and the absorbance was monitored at 405 nm in a plate reader (MULTISKAN GO, Thermo Scientific, Waltham, USA) continuously for 10 min against the reagent blank. The activity of streptokinase (0.2 nM) under identical experimental conditions was considered as a positive control.

3.2.7.14 Determination of carbohydrate content

The total carbohydrate content was estimated by phenol sulfuric acid method as described in Dubois et al. (1956) ^[129].

Standard curve preparation: Graded amount (0.2 ml to 1 ml) of working standard pipetted into a colorimetric tubes and make up the volume 1ml in each tube with distilled water. Blanks were prepared by substituting distilled water for the sugar solution and 0.05 ml of 80% phenol was added. Then, 5 ml of 96% concentrated sulfuric acid was added rapidly. The stream of acid being directed against the liquid

surface rather than against the side of the test tube in order to obtain good mixing. The tubes were allowed to stand 10 min, and then they were shaken and placed for 10 to 20 min in a water bath at 25 to 30 °C for 20 min before readings were taken. The color was stable for several hours and the absorbance of the characteristic yellow orange color was measured at 490 nm for hexoses.

For sample preparation: 100 µg of the sample into boiling water bath for 3 h with 5 ml of 2.5 N HCl and cool them at room temperature. Neutralized the reaction mixture with solid sodium carbonate. Thereafter, the volume was adjusted to 100 ml and centrifuged it at 1500 rpm. Pipetted 0.2 ml of supernatant in to separate tubes and make up the volume up to 1.0 ml with distilled water. To the reaction mixture, 1.0 ml of phenol was added in each tube. Thereafter, 96 % sulfuric acid solution was added to the above reaction mixture. After 10 min, the tubes were vortexes and incubated at 30 °C for 20 min. The optical density of the solution was measured at 490 nm against reagent blank.

3.2.7.15 Determination of glycosylation on purified enzymes

For determination the extent of N-linked and sialic acid contents, denatured purified enzymes (20 µg) was treated with PNGase (2000 units) and neuraminidase (500 units), respectively for 4 h at 37 °C according to manufacturer's protocol (New England Biolabs Inc, Ipswich, MA). The reaction products were visualized by 15% SDS-PAGE under reducing conditions ^[107]. For partial deglycosylation (glycosylation under native conditions), the denaturation step was eliminated and Bacethrombase (20 µg) was treated with 500 units of neuraminidase for 1h at 37 °C. The reaction products were analyzed by 15% SDS-PAGE under reducing conditions ^[107].

3.2.8 Biophysical characterization

3.2.8.1 Fluorescence spectroscopy

Fluorescence emission spectrum was recorded to determine the interaction between fibrinogen and purified enzyme dissolved in 50 mM sodium phosphate buffer pH 7.4 on a fluorescence spectrometer with a scan speed 10 nm/min, using excitation and emission slits was 10 nm and 6 nm respectively. The excitation wavelength was set at 280 nm and the spectra were recorded from 300 to 420 nm. Wavelength shifts

were estimated by considering the midpoint at two third height of the spectrum. The maximum fluorescence of free protein was also calculated.

3.2.8.2 Peptide mass finger printing analysis

For PMF, Coomassie brilliant Blue-stained protein (purified fibrinolytic/fibrin(ogen)olytic enzyme) band was excised from the gel, reduced and alkylated and then tryptic digested [108]. Tryptic fragments for MALDI-TOF-MS analysis were prepared by using “In gel digestion kit” (PIERCE, USA; Catalog no: 89871). Subsequently, tryptic digest peptides were desalted by Zip Tip^{C18} and spotted the thin film on the target with 0.5 µl isocyanides containing saturated solution of α cyano-hydroxy sinapinic matrix dissolved in 50% (V/V) acetonitrile with 0.1% (V/V) TFA. MALDI mass spectra were recorded in the positive ion mode with delayed extraction on a Reflex II time-of-flight instrument (Bruker-Daltonik GmbH, Bremen, and Germany) equipped with a SCOUT multiprobe inlet and a 337 nm nitrogen laser. The ion acceleration voltage was set at 20.0 kV, the reflector voltage was set at 21.5 kV and the first extraction plate was set at 15.5 kV. Mass spectra were obtained by averaging 50–200 individual laser shots. Further MS/MS spectra were searched against NCBI data base of non-redundant protein sequence (NCBI nr) using the MASCOT search engine (GPS explorer Version 3.6). The tryptic peptide sequences so obtained were subjected to blast search in NCBI nr, Swissrot protein sequences (swiss-prot) and protein data bank proteins against a *Bacillus* database using blastp algorithm (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>).

3.2.8.3 Amino acids composition analysis

For amino acid composition analysis, purified enzyme was hydrolyzed with 6 N HCl at 110 °C for 24 h under vacuum. The hydrolyzate (25 µl) was mixed with 25 µl of ortho-phthalaldehyde (Sigma), 3-merceptopropionic acid and 9-fluorenylmethyl chloroformate (Sigma) reagents in borate buffer. This was followed by addition of 8µl of 1M acetic acid and contents were mixed well. The 3 µl of the resulting mixture was injected in Acclaim RSLC 120, C₁₈ RP-UPLC column (2.1 X 150 mm, 3 µm, 300 Å) coupled to ultimate 3000RSLC (Dionex) UHPLC system. After washing the column by eluent A (10 mmol/L Na₂HPO₄, Na₂B₄O₇ containing 0.5mmol /L NaN₃), the amino acid derivatives were separated by eluent B (CH₃CN /MeOH/H₂O in 45:45:10) at a flow rate of 0.722 ml/min. The elution was monitored

initially at 338 nm for 0-7.2 min and then at 262 nm for the next 7.2 -10.5 min^[130]. The concentration of individual amino acid of the purified enzyme was determined against a calibration curve of standard amino acids derivatives run in the same RP-UPLC column under the identical conditions. The amino acids composition of the purified enzyme was searched in Swiss-prot databases by using the programme AAccompident of Expert Protein Analysis System (ExPASy) onlinesoftware (<http://www.expasy.org/tool/aaccompident>)^[131].

3.2.9 Secondary structure determination

The secondary structure of the purified fibrinolytic enzyme (1.0 mg/ml in PBS buffer, pH 7.4) was determined by circular dichroism (CD) analysis (JASCO J-815, Japan) as described previously^[132]. Each recorded CD spectrum represented an average of 5 scans in the range of 190–240 nm, collected at 0.2-nm intervals, with a spectral band width of 0.1 nm and 4 s integration time. The CD spectra were corrected by subtraction of water blank and expressed in molar ellipticities^[9] (degrees cm² dmol⁻¹), using 113 as mean residue molecular weight^[132]. Measurement was conducted at 20 °C and a scan rate of 10 nm/min. Reported spectra were averages of 3 scans with no smoothing^[132]. Errors in molar ellipticities were estimated to be approximately ± 3% by using yeng reference software. Yang's reference was set for the CD analysis and the secondary structure of purified fibrinolytic enzyme was determined using CDPRO CLUSTER software^[133].

3.2.10 Pharmacological characterization

3.2.10.1 Hemolytic assay

The hemolytic activity of was determined using the method as described by of Mukherjee et al. (2008)^[127]. Briefly, 9 ml of blood was collected from healthy goat in sterile tubes containing 1.0 ml of 3.8 % tri-sodium citrate (anticoagulant), centrifuged at 4300 rpm for 15 min. The platelet poor plasma (PPP) was discarded, and the pellet was re-suspended and washed thrice with PBS (pH 7.4), and diluted to 0.5% in PBS (pH 7.4). The sample (protease) to be tested was placed in different tubes and after that 2 ml erythrocyte suspension was added to each tubes, gently inverted and incubated at 37 °C for 90 min. 1 % Triton X-100 are used as positive control and PBS was used as negative control under identical condition. After

incubation, all the tubes were centrifuged at 10,000 rpm for 10 min to pellet out RBCs. The supernatant was carefully separated out and the absorbance was measured at 540 nm for released hemoglobin using UV-Vis spectrophotometer. The percentage of hemolytic index (%) was calculated by using the following formulae:

$$\% \text{ hemolysis} = \frac{(\text{OD samples} - \text{OD negative control})}{\text{OD positive control} - \text{OD negative control}} \times 100$$

3.2.10.2 Anticoagulant assay

To study the plasma clotting/anticoagulant activity, 3.8 % tri-sodium citrate containing goat blood was centrifuged at 250 g for 10 min to obtain platelet rich plasma (PRP). Then graded concentrations of purified protease were incubated with 300 μ l of platelet poor plasma (PPP) from goat blood for 3 min at 37 °C followed by addition of 40 μ l of CaCl₂ to initiate the coagulation^[87]. In control, plasma was incubated with PBS, pH7.4. One unit of anticoagulant activity, if any, is defined as a purified protease induced 1s increase in clotting time of plasma compared with clotting time of normal plasma under the assay conditions^[127].

3.2.10.3 Thrombolytic assay

The *in vitro* thrombolytic activity of purified fibrinolytic / fibrinogenolytic enzyme (5.0 μ g), plasmin (5.0 μ g) and streptokinase (5.0 μ g) was determined by blood clot lysis method^[41]. As a control, blood clot was incubated with phosphate buffered saline (50 mM sodium phosphate buffer pH 7.4 containing 0.85 % sodium chloride), pH 7.4. The *in vitro* thrombolytic activity was expressed as mg of blood clot (thrombus) lysed per μ g of purified enzymes, or plasmin, or streptokinase as compared to the control (blood clot treated with buffer). In another set of experiments, the blood clot was heated at 80 °C for 30 min to denature the endogenous fibrinolytic factors (plasmin, plasminogen, t-PA, etc.) prior to the thrombolytic activity assay^[41, 89]. The triplicate sets of each experiment ensured the reproducibility of the result.

3.2.11 Cell cytotoxicity assay

Cytotoxic effect of purified protease, if any, was assessed against HeLa (cervical carcinoma) and HT29 (colon adenocarcinoma) cancer cells. Briefly, cells were

grown in 96 well plates (BD Falcon, USA) in Dulbecco's Modified Eagle's Medium (DMEM) (Sigma Aldrich, USA) supplemented with 10% (v/v) fetal bovine serum (PAA, Austria), 100 units/ml penicillin and 100 µg/ml streptomycin (Sigma, USA) at 37 °C, 5 % CO₂ in a humidified incubator. After 24 h of seeding of 1 × 10⁵ cells/ml in a 96-well microtiterplate, the old medium was replaced with respective growth medium containing graded concentrations of purified enzyme (0–15 µg/ml) (an approximate concentration of 0–0.3 µM). The cells were allowed to incubate for 48 h in the above conditions and the cell viability was determined by adding 2,3-bis-(2-methoxy-4-nitro-5-sulphophenyl)-2H-tetrazolium-5-carboxanilide. The percent viability of cells in presence of purified enzyme was determined from a standard curve of untreated cells ^[107].

3.2.12 Assay of thrombin and FXa inhibition

Bovine thrombin (3 µl, 10 NIH U/ ml) was pre-incubated with graded concentrations (0.05-1.0 µM) of purified fibrinolytic / fibrin(ogen)olytic protease enzyme / PBS (control) in 50mM sodium phosphate buffer, pH 7.4 at 37 °C for 30 min. Thereafter, the fibrinogen clotting activity of thrombin (treated as well as control) against its physiological substrate fibrinogen and its amidolytic activity towards chromogenic substrate (0.2mM) N-p-tosyl-Gly-Pro-Arg p-nitroanilide acetate was assayed as described previously ^[107]. In another set of experiments, 15 µg of thrombin was incubated with 2.0 µg of purified fibrinolytic enzyme from 30 – 120 min at 37 °C. The treated and control thrombin (incubated with PBS) were analyzed by 15% SDS-PAGE under reducing conditions ^[125].

For FXa inhibition assay, purified fibrinolytic/ fibrin(ogen)olytic enzyme (0.4 µM) was pre-incubated with FXa (0.13 µM) in 20 mM sodium-phosphate buffer, pH 7.4 of at 37 °C for 30 min. Thereafter, 1.4 µM of prothrombin and 2.5mM Ca²⁺ were added to the above mixture and incubated for overnight at 37°C. A control was run in parallel where instead of purified protease, buffer was added. The prothrombin degradation products were analyzed by 12.5% SDS-PAGE under reducing conditions ^[134].

The fibrinogen clotting activity of purified protease, if any, was assayed as described previously ^[107]. For control, equal volume of 50 mM sodium phosphate buffer, pH 7.4 was added to the human fibrinogen solution (2.5 mg/ml) instead of enzyme and

the reaction was carried out under identical experimental conditions. As a positive control, the fibrinogen clotting activity of thrombin (0.03 NIH U/ml) was also determined ^[107].

3.2.13 Platelet modulating activity of purified fibrinolytic/ fibrin(ogen)olytic enzyme

The effect of purified fibrinolytic / fibrin(ogen)olytic enzyme on platelet aggregation was determined as described by Horn et al. ^[135] with the following modifications. In brief, 3.8% tri-sodium citrate containing goat blood was centrifuged at 250 g for 10 min to obtain platelet rich plasma (PRP). The PRP was re-centrifuged at 650 g for 15 min and the pellet was washed twice in Tyrode buffer (5 mM HEPES, 137 mM NaCl, 2.7 mM KCl, 12 mM NaHCO₃, 0.42 mM Na₂HPO₄, 1 mM MgCl₂, 0.1 % glucose and 0.25 % bovine serum albumin). The pellet was re-suspended in the same buffer, and the volume of the platelet suspension was adjusted with the Tyrode buffer to give an absorbance of 0.15 at 540 nm.

To determine the platelet modulating activity, different concentrations of fibrinolytic / fibrin(ogen)olytic enzyme (25-500 nM) were added to 100 µl of PRP or washed platelets suspension in a 96-well plate and the contents were mixed for 5 s in a microplate reader (Multiskan GO, Thermo Scientific, Waltham, USA). The absorbance was then recorded continuously at 540 nm for 5 min at an interval of 15 s. As a control, the absorbance of PPP as well as PRP was also recorded at 540 nm. The absorbance value of PPP was subtracted from the experimental readings to determine the absorbance only due to platelets in PRP. The extent of the platelet aggregation was measured as an increase in the light transmission, and expressed as a percentage of aggregation, which was calculated by the following formula:

Percent aggregation = $\{(A_{540} \text{ of PRP/washed platelets before the addition of Purified fibrinolytic enzyme / fibrin(ogen)olytic enzyme} - A_{540} \text{ of PRP/washed platelets after the addition of fibrinolytic / fibrin(ogen)olytic enzyme}) \div (A_{540} \text{ of PRP/ washed platelets before the addition of fibrinolytic / fibrin(ogen)olytic enzyme})\} \times 100$, where A_{540} represents absorbance value at 540 nm.

3.2.14 Determination of antagonistic effect of purified fibrinolytic enzyme / fibrin(ogen)olytic enzyme on collagen and ADP-induced aggregation of PRP

To determine whether or not purified fibrinolytic enzyme / fibrin(ogen)olytic enzyme can inhibit the collagen or ADP-induced platelet aggregation, different concentrations of purified enzyme (5- 65 nM) were incubated with PRP for 10 min at room temperature (~23 °C). Then collagen type-IV (1.0 µg/ml) or ADP (20 µM) was added and platelet aggregation was measured as stated above. As a positive control, the platelet aggregation induced by collagen or ADP in absence of purified protease was also determined and was considered as 100% activity. From the regression equation of inhibition curve, the IC₅₀ value of purified protease to inhibit the ADP-induced platelet aggregation was determined.

3.2.15 *In vivo* lethality and toxicity

The lethality and *in vivo* toxicity, if any, of purified protease under study was determined in pathogen free Wister strain albino rats (200 to 250 g) from the laboratory breeding colony of Defense Research Laboratory, Tezpur, Assam. The intravenous toxicity study following a single dose administration of purified fibrinogenolytic enzyme was conducted according to “regulations of the United States Food and Drug Administration proposed ICH guideline (61 FR 43934) and approved by Tezpur University Animal Ethical Committee. Purified fibrinogenolytic enzyme at dose of 10 mg/kg body weight of rats (n=6) in a total volume of 200 µl of PBS, pH 7.4 was administered by intravenous injection (Fig.3.1). The control group of rats (placebo) received the same volume of PBS. Rats were maintained in condition typical North East India (temperature 33-36 °C, relative humidity ≥ 75%). The animals were placed in polypropylene cages, with free access to standard laboratory diet (Pranav Agro Industries Limited, Sangli, and Maharashtra, India) and provided water *ad libitum*. The animals were observed for 14 days after the injection for mortality, clinical sign of toxicity or any behavioral changes. The following parameters were recorded body weight, food and water consumption, grip strength and rectal temperature.



Figure 3.1: Intravenous administration of purified fibrin(ogen)olytic/ fibrinolytic enzyme at a dose of 10 mg/kg in tail vein rats

3.2.15.1 Electrocardiograms (ECG) and whole body plethysmograph

With the help of ECG system (BIOPAC Systems, Inc. USA), P wave, R wave, T wave, ST segment, Q-T intervals, P-R interval, QRS duration and heart rate ECG of both the treated and control groups of animals were recorded before and after the injection and at an interval of 7 days (Fig. 3.2).

The Whole body plethysmograph (Data science international, Made in USA, Model no -600-2400-001) (Fig.3.3) was used to measure the respiratory parameters such as peak expiratory flow (PEF), tidal volume (TV), minute volume (MV) and breaths per min (BPM), peak inspiratory volume (PIF) altogether and expressed in terms of PenH value (Fig.3.3).

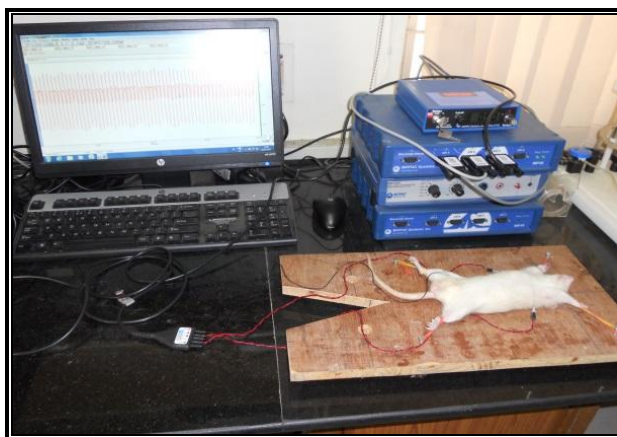


Figure 3.2: Echocardiogram of control as well as treated rats after 14 days of intravenous administration of purified fibrin(ogen)olytic/ fibrinolytic enzyme/ PBS (control) at a dose of 10 mg/kg in tail vein rats

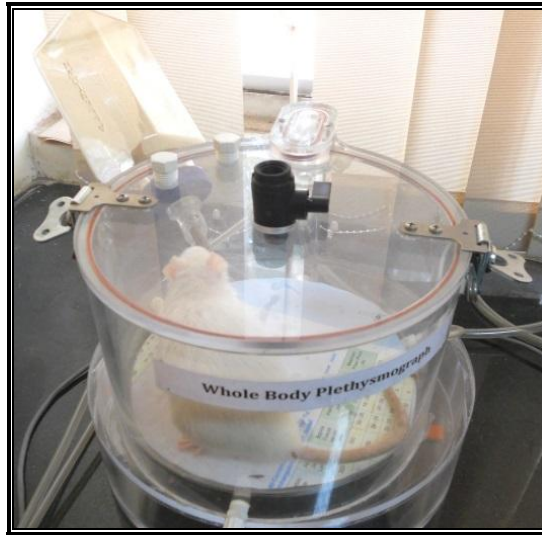


Figure 3.3: Measurement of the PenH value (respiratory system) by whole body plethysmograph after 14 days of intravenous administration of purified fibrin(ogen)olytic/ fibrinolytic enzyme or PBS (control) at a dose of 10 mg/kg in tail vein rat

3.2.15.2 Hematological parameters and serum biochemical profiles

Blood samples were collected from overnight fasted rats after 14 days of injection by venipuncture of retro-orbital sinus with the help of a hematocrit capillary tube (Figure 3.4-3.6).



Figure 3.4: Collection of blood sample from control and treated rats by mechanical disruption of retro orbital sinus.



Figure 3.5: Collection of blood sample from control and treated rats for hematological (blue) and serum profile analysis (red)

The hematological parameters studied were red blood cell (RBC) count, hemoglobin (HB) concentration, hematocrit (HCT), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), platelet (PLT) count, reticulocytes (Rt), white blood count (WBC), and WBC differential counts (lymphocyte, monocyte, and granulocyte). All these parameters were measured using an automatic hematoanalyzer (Milet sesloesing Laboratory, MS-4, Osny, France).

Blood was collected in anticoagulant free tubes and serum was separated by centrifuged at 3000 rpm for 10 min within 1 h after collection. The following biochemical parameters of serum were analyzed-low density lipoprotein (LDL), high density lipoprotein (HDL), glucose (GLU), urea (U), total protein (TP), uric acid (UA), triglycerides (TRIG), cholesterol (CH), creatinine (CR), alkaline phosphatase (ALP) using automatic biochemical analyzer Coralyzer 100.(Tulip Diagostic, Goa, India).

3.2.15.3 Histopathological study

At the end of 14 days, each group of animals was sacrificed by ether euthanasia. The blood sample was collected immediately by puncturing retro orbital sinus from each group of animals for clinical pathology evaluation. The organs such as spleen,

heart, liver, and kidney were dissected out, washed with PBS, pH 7.4 to remove the adherent blood, weighed and then placed in 10% formaldehyde. The fixed tissue were dehydrated in graded alcohol series (50-100%), embedded in parafilm (Paraplast™ resin). The blocks were sliced in 5 µm thick sections and then stained with hematoxylin and eosin and processed routinely for light microscopic observation after hematoxylin–eosin staining and then observed for histopathological changes under a light microscope (LEICA DM 3000) at x100 magnification.

3.2.16 Evaluation of *in vivo* thrombolytic potential of purified fibrinolytic / fibrin(ogen)olytic enzyme in rat model

In order to evaluate the *in vivo* antithrombotic potential of fibrinolytic / fibrin(ogen)olytic enzyme, the carrageenan induced animal model was preferred [136].

3.2.16.1 Carrageenan induced thrombus induction in rat model

The in vivo thrombolytic activity of purified protease was assessed in carrageenan-induced thrombosis in rat model [136] with a slight modification. Before the thrombolytic assessment of protease enzyme, utmost suitable dose of k-carrageenan was optimized for tail thrombosis formation in rats. Briefly, different doses [0.2 mg/kg to 1.1 mg/kg] of carrageenan dissolved in 1X PBS were intravenously administrated in the tail vein of rats. The animals were observed till 24 h for the thrombus formation in the tail of rats and suitable concentration of k-carrageenan was determined based on saturation of wine colour appearance thrombus formation (Fig. 3.6-3.7).



Figure 3.6: BIOPAC system was used to capture rat before intravenous administration of purified enzymes in tail vein.

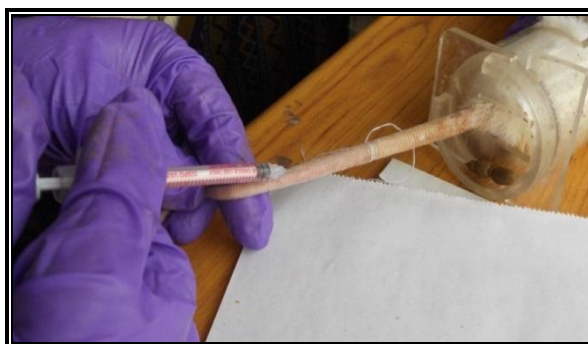


Figure 3.7: Intravenous administration of purified enzyme in carrageenan induced rat tail vein.

3.2.16.2 *In vivo* thrombolytic activity

For the assessment of *in vivo* thrombolytic activity, a total of 36 male rats were randomly subdivided into 6 groups (for each group, $n = 6$). The Group one animals injected with 1 X PBS (50 mM sodium phosphate buffer, 100 mM NaCl, pH 7.4) served as control (placebo). The animals of groups 2, 3, 4 were treated with graded concentrations (200- 600 $\mu\text{g}/\text{kg}$ in 1X PBS) of purified protease in a total volume of 500 μl , and rats of groups 5, 6 were treated with 600 $\mu\text{g}/\text{kg}$ dose of SK (indirect thrombolytic agent via plasminogen activation) and plasmin (direct-thrombolytic agent), respectively. After 30 min of above doses administration, the tails were ligated and 0.9 mg/kg body weight of k-carrageenan was induced through intravenously. The ligature was removed after 15 min of injection. The length of infarcted region was measured and the appearance of wine color thrombus formation in tail was photographed after 24 h of treatment by above thrombolytic agents. The

percentage of *in vivo* thrombus dissolution after 24 h of treatment was calculated by using the following formula ^[137].

$$\% \text{ of thrombus } x = \frac{100}{\text{length of tail thrombus in control rat(cm)}} \times \text{length of tail thrombus in treated rats} \quad (\text{cm}) \text{ ---- (1)}$$

Substituting the %X value of Eq. (1) to Eq. (2)

$$\% \text{ actual thrombus dissolution(Y)} = (100 - \%X) \text{ ---- (2)}$$

3.2.16.3 Coagulation assay

For *in vivo* coagulation assay, the prothrombin time (PT), activated partial thromboplastin time (APTT), thrombin time (TT) and fibrinogen level (Fg) of plasma were determined by using commercial diagnostic kits and following the instructions of the manufacturer's protocol. After 2 h of purified protease infusion, the blood samples were collected from the tail vein of Brevithrombolase treated (200- 600 µg/kg in 1X PBS) as well as from the control group (treated with 1X PBS) of rats in microfuge tubes containing 3.8 % sodium citrate solution (1:9, v/v). The platelet poor plasma (PPP) was obtained by centrifugation of the blood at 2000 g for 10 min at 4 °C. For each experiment PPP was used and the coagulation time (PT, APTT, TT and Ca⁺²-clotting time) of treated as well as control PPP was recorded in a coagulometer (Sysmex CA-1500, Mitalan Keynes, UK).

3.2.16.3.1 Determination of activated partial thromboplastin time (APTT)

The C.K.PREST[®] kit is intended for determination of activated partial thromboplastin time (APTT) according to Hull et al., ^[138]. The APTT of the treated rat plasma being studied and compared with a control reference. In a glass test tube maintained at 37 °C, 0.1 ml of undiluted platelet poor plasma (PPP) of control / treated rat was mixed with reagent vial 1 and incubated at 37 °C for 3 min. To the mixture 0.1 ml of prewarmed (at 37 °C) 0.025 M CaCl₂ was added and clotting time of plasma was measured by coagulometer (Sysmex CA-1500, Mitalan Keynes, UK).

3.2.16.3.2 Determination of prothrombin time (PT)

The LIQUIPLASTIN[®] reagent is used for determination of prothrombin time (PT) ^[138]. The contents of the reagent vials (stored at 2-8 °C) were transferred to a thoroughly clean, dry plastic tube and brought to room temperature before pre-

warming at 37 °C for testing purpose. To a 12 x 75 mm tube, 0.1 ml of (PPP) was added and placed in a water bath for 3 to 5 min at 37 °C. To the reaction mixture 0.2 ml of LIQUIPLASTIN® reagent (prewarmed at 37 °C for at least for 3 min) was mixed and shaken gently to mix the contents. The result was recorded in seconds as soon as the first fibrin strand was visible and the gel/clot formation begins. The test was performed in triplicate and average of triplicate result in seconds represents Mean Normal Prothrombin Time (MNPT).

The result was reported directly in terms of mean of double determination of PT of the test plasma in seconds or International Normalized Ratio (INR).

$$\text{INR} = \frac{\text{Mean of the treated rat plasma PT in seconds}}{\text{MNPT for the reagent *}}$$

3.2.16.3.3 Determination of thrombin time (TT)

For determination of thrombin time (TT), STA®-THROMBIN kit (Stago Diagnostic, France) was used. Two hundred µl of PPP was taken in a prewarmed (at 37°C) glass tube and further incubated it at 37 °C for 2 min. Then, 200 µl of STA®-thrombin was added to measure the clotting time of plasma in seconds by coagulometer (Sysmex CA-1500, Mittan Keynes, UK).

3.2.16.3.4 Determination of fibrinogen (Fg)

For quantitative determination of plasma fibrinogen, FIBRI-PREST ® kit was used. In a prewarmed test tube (at 37 °C) 0.2 ml of test sample was taken and incubated at 37 °C for 2 min. Further, reagent 1 prewarmed at 37 °C was added to the reaction mixture. The plasma fibrinogen concentration in protease treated and control rat plasma was measured with respect to standard calibration curve and expressed in terms of mg/dl.

3.2.16.3.5 Determination of Ca⁺²-clotting time

Effect of pre-incubation of PPP/purified enzymes on plasma clotting time was determined by pre-incubating a fixed concentration (0.1 µM of purified enzymes in 50 mM sodium phosphate buffer, pH 7.4) of purified enzymes with PPP (300 µl) for 0-30 min at 37°C, and then assaying the Ca⁺²-clotting time of PPP [107, 127, 134]. As a control, PPP was incubated with buffer under identical conditions.

3.2.17 Statistical analysis

The statistical analysis of the data was done by Student's t test using the software Sigma Plot 11.0 for windows (version 7.0). The two sets of data showing value of $p \leq 0.05$ was considered significant.