CHAPTER IV

Results & Discussion

CHAPTER 4

RESULTS AND DISCUSSION

Pharmacological characterization of a fibrin(ogen)olytic serine protease (Bacethrombolase) from *Bacillus cereus* strain FF01

4.1 Brief introduction

Cardiovascular diseases like ischemic heart disease, acute myocardial infarction, and high blood pressure account for 29% of total mortality rate in the world [6]. One of the significant factors contributing to the etiology of cardiovascular diseases is the imbalance between fibrin formation and fibrinolysis [2]. This patho-physiological condition results in intravascular thrombus formation in the blood vessels (known as thrombosis), posing a clinical challenge in its treatment [139]. Moreover, higher levels of blood fibrinogen (hyperfibrinogemic condition) stimulate thrombotic events and enhance the risk of myocardial infarction through blood coagulation [3]. Lipid proliferation, which is induced by hyperfibrinogemia to the vessel wall, initiates the development of atherosclerosis and contributes to ischemic pathology [140]. Besides, the elevated levels of fibrinogen synthesized by cancer cells also promote proliferated effect by interacting with fibroblast growth factor-II [141].

Under normal conditions, the fibrin deposits within the vessels are dissolved by plasmin. Therefore, the plasmin-like, direct acting fibrin(ogen)olytic enzymes have high therapeutic demand for the treatment of hyperfibrinogemia associated disorders [27, 142, 143]. Notably, administration of commercial thrombolytic agents such as streptokinase, urokinase, and tissue plasminogen activator have been considered as an alternative approach to surgical interventions for removing the blood clot or bypassing the heart blockage [13]. However, these cardiovascular drugs have their own limitations as they demonstrate several side effects like gastrointestinal bleeding and allergic reactions. Further, these drugs are expensive and are unaffordable for common people in the developing countries [4]. Therefore, in order to develop a potent but inexpensive thrombolytic agent of choice, exploration of natural resources for discovering novel or new fibrin(ogen)olytic enzymes possessing superior properties (more thrombolytic potency and/or less toxicity) as compared to the existing commercial drugs has a great demand over the decades.

In the past 10 years or more, several fibrinolytic or fibrin(ogen)olytic proteases have been identified, purified and characterized from the microbes, isolated from various traditional fermented foods [20, 31, 144]. Despite these efforts, except for a few limited examples, fragmentary data is available on bacterial fibrin(ogen)olytic enzymes possessing anticoagulant activity [94, 99]. Thus to bridge the gap, in the present study, biochemical and pharmacological characterization of a fibrin(ogen)olytic serine protease purified from *Bacillus cereus* strain FF01 isolated from a fermented food sample has been reported. Further, in addition to determining its mechanism of anticoagulation, the thrombolytic potency of this enzyme has also been assessed and compared with commercial drugs, such as plasmin and streptokinase, under *in vitro* and *in vivo* conditions. The antiplatelet activity of this enzyme has also been demonstrated in this chapter.

4.2 Results

4.2.1 Screening and isolation of fibrin(ogen)olytic enzyme producing bacteria

In the initial stage of screening procedure, five fermented food samples were collected in test tubes from different places of North-East India at random. Nine bacterial cultures were obtained from the five fermented food samples, Out of which, four bacterial isolates showed zone of hydrolysis at ≥ 20.0 mm (Table 4.1). The pure cultures of these extracellular enzyme producing bacterial colonies were further sub-cultured in M9 medium, and cell-free supernatants were used to determine fibrinolytic to caseinolytic ratio, substrate specificity and *in vitro* thrombolytic activity for selection of potential bacterial strain for further investigation.

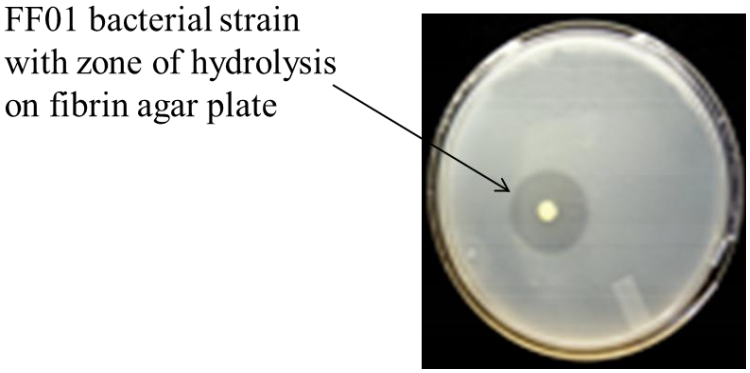

Figure 4.1: Zone of hydrolysis around the FF01 bacterial colonies on fibrin agar medium

The bacteria were isolated by serial dilutions up to 10^{-4} from rice beer alcohol-producing starter culture (dissolved in 1X PBS) plated on a plasminogen-free fibrin agar plate at pH 7.4 containing fluconazole (antifungal agent). The plates were incubated at 37 °C for 48 h to produce zone of hydrolysis by bacterial strain FF01 as stated in experimental procedure described in Materials and Methods section 3.2.2.

Table 4.1: Screening for fibrin(ogen)olytic enzyme producing bacteria from different samples of northeast India

SL No	North-eastern places in India	Sample code	Zone of hydrolysis (mm)
01	Mishing (Arunachal Pradesh)	FF01	29.20
02	Kukurmati (Assam)	FF02A	32.55
03	Kukurmati (Assam)	FF02B	34.54
04	Nopaam (Assam)	FF03	31.75
05	Dibrugarh (Assam)	SF01	23.97
06	Kohima (Nagaland)	SF02	20.16

Note: The medium used was fibrin agar, pH 7.4, incubated at 37 °C for 48 h.

Table 4.2: Identification of fibrin(ogen)olytic enzyme producing potential bacteria

Sl.no.	Name of strains	Fibrinolytic activity (IU/mg)	Caseinolytic activity (IU/mg)	Albuminolytic assay (IU/mg)	Globulinolytic assay (IU/mg)	F/C	Thrombolytic activity (mg of clot lysis/µg of enzyme)
1	FF01	187.3	1.2	80.1	76.2	156	20.2
2	FF02A	139.6	1.6	98	7.3	87.3	10.2
3	FF02B	171.4	0.8	70	7.3	193	22.4
4	FF03	72.2	12.8	60	5	5.6	2.3
5	SF01	105.9	1.9	40.4	131.7	55.1	6.8
6	SF02	117.8	7	18.6	94	16.8	7.2

Note: This was based on fibrinolytic / caseinolytic (F/C) ratio, substrate specificity and thrombolytic activity. All the experiments were performed as described in sections 3.2.2.3, 3.2.2.4, and 3.2.2.5, respectively. Results show average of triplicate experiments.

4.2.2 Taxonomic identification of fibrin(ogen)olytic enzyme producing bacterial strain FF01

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

4.2.2.1 Morphological and biochemical study of fibrin(ogen)olytic enzyme producing bacterial isolates

The results of the biochemical and morphological tests of the selected bacterial isolate are shown in Tables 4.3. Bacterial morphology and the results of the biochemical tests suggested that the FF01 strain belongs to *Bacillus* sp. genus (Bergey's manual of systematic bacteriology, 1999, 2001, 2005).

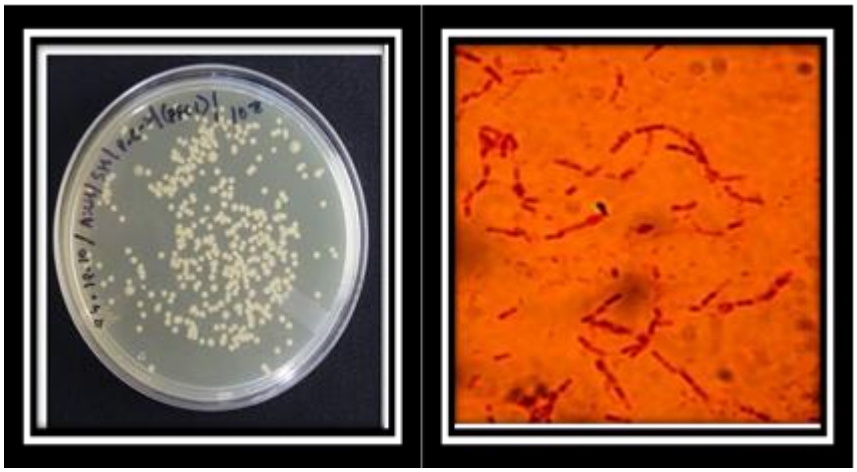

Figure 4.2: Pure culture morphology and gram staining of bacterial strains FF01

Table 4.3: Biochemical and morphological tests of bacterial strain FF01

Characteristics	FF01
Morphology	Rod shaped, motile, Gram negative, border circular, White color, in chain
Spore	No endospore
Growth in Agar	Abundant, white color
Growth in Broth	Good growth, with sediment
pH	4.0-12.0, optimum at pH 8.0
Temperature	Growth range 30-60 °C, optimum at 37 °C
Catalase	Positive
Voges-Proskauer Test	Negative
Methyl Red Test	Negative
Acid from	
D-Glucose	Positive
Sucrose	Negative
Lactose	Negative
D-Mannitol	Negative
Gas from Glucose	Negative
Hydrolysis of	
Casein	Positive
Gelatin	Negative
Starch	Negative
Urease activity	Negative
Utilization of Citrate	Negative
Formation of Indole	Negative
Nitrate reduction	Negative
H_2S production	Positive
Lipid hydrolysis	Negative
Triple sugar Iron agar test	Negative
Litmus milk test	Positive

Note: Experiments were repeated three times to ensure the reproducibility**.**

4.2.2.2 Phylogenetic analysis of bacterial isolates based on 16S rDNA sequencing

The 16S rRNA gene of the strain FF01 was amplified, sequenced and the sequence is shown in Table 4.4.

Table 4.4: Partial DNA sequence of conserved region of 16S rRNA gene of the strain FF01

Bacterial isolate	16S-rRNA gene sequence (amplified region)
Strain FF01	**5′**CAGGCACCTTGTCTACGACTTCCCCCCAATCATCTG GCCCACCTTAGGCGGCTGGCTCCAAAAAGGTTACCC CACCGACTTCGGATGTTACAAACTCTCGTGGTGTGAC GGGCGGTGTGTACAAGGCCCGGGAACGTATTCACCG CGGCATGCTGATCCGCGATTACTAGCGATTCCAGCTT CATGTAGGCGAGTTGCAGCCTACAATCCGAACTGAG AACGGTTTTATGAGATTAGCTCCACCTCGCGGTCTTG CAGCTCTTTGTACCGTCCATTGTAGCACGTGTGTAGC CCAGGTCATAAGGGGCATGATGATTTGACGTCATCC CCACCTTCCTCCGGTTTGTCACCGGCAGTCACCTTAG AGTGCCCAACTTAATGATGGCAACTAAGATCAAGGG TTGCGCTCGTTGCGGGACTTAACCCAACATCTCACGA CACGAGCTGACGACAATCATGCACCACCTGTCACTC TGCTCCCGAAGGAGAAGCCCTATCTCTAGGGTTTTCA GAGGATGTCAAGACCTGGTAAAGGTTCTTCGCGTTG CTTCAAATTAAAACCACATGCT-**3′**

Table 4.5: Homologous search results of 16S rRNA gene partial sequence of strain FF01 using Basic Local Alignment Tool (BLAST) from National Centre Biotechnology Information (NCBI)

Accession	Description	Query coverage	Max identity
FN433030.1	Bacillus sp. partial 16S rRNA gene, isolate CCM11B	99%	100%
GU433107.1	*Bacillus cereus* strain AG1 16S ribosomal RNA gene, partial sequence	99%	98%
FR851254.1	*Bacillus* sp. PR1.7 partial 16S rRNA gene, strain PR1.7	99%	98%
CP001907.1	*Bacillus thuringiensis* serovar chinensis CT-43, complete genome	99%	98%
JF309223.1	*Bacillus* sp. 3541BRRJ 16S ribosomal RNA gene, partial sequence	99%	98%
JF309221.1	*Bacillus* sp. 3374BRRJ 16S ribosomal RNA gene, partial sequence	99%	98%
HQ914780.1	*Bacillus cereus* strain TAUC5 16S ribosomal RNA gene,	99%	98%

Accession	Description	Query coverage	Max identity
	partial sequence		
FJ215792.2	*Bacillus* sp. 3434BRRJ 16S ribosomal RNA gene, partial sequence	99%	98%
HQ727973.1	*Bacillus cereus* strain Aj080319IA-16 16S ribosomal RNA gene, partial sequence	99%	98%
HM771661.1	*Bacillus cereus* strain PPB6 MB6 16S ribosomal RNA gene, partial sequence	99%	98%
HQ234323.1	*Bacillus subtilis* strain B.S.8 16S ribosomal RNA gene, partial sequence	99%	98%
HQ234318.1	*Bacillus subtilis* strain B.S.K 16S ribosomal RNA gene, partial sequence	99%	98%
HQ267763.1	*Bacillus subtilis* strain Bs30 16S ribosomal RNA gene, partial sequence	99%	98%
HM209757.1	*Bacillus cereus* strain GRM808 16S ribosomal RNA gene, partial sequence	99%	98%
HM152752.1	Uncultured *Bacillus* sp. clone Filt.165 16S ribosomal RNA gene, partial sequence	99%	98%
HM152709.1	Uncultured *Bacillus* sp. clone Filt.122 16S ribosomal RNA gene, partial sequence	99%	98%
GQ381280.1	*Bacillus cereus* strain TA2 16S ribosomal RNA gene, partial sequence	99%	98%
HM045841.1	*Bacillus* sp. WJ17 16S ribosomal RNA gene, partial sequence	99%	98%
CP001903.1	*Bacillus thuringiensis* BMB171, complete genome	99%	98%
GU826154.1	*Bacillus cereus* strain Q34 16S ribosomal RNA gene, partial sequence	99%	98%
GU826152.1	*Bacillus thuringiensis* strain P22 16S ribosomal RNA gene, partial sequence	99%	98%

Note: The 16S rDNA sequences from microbes showing up to 98 % identity are given in the table.

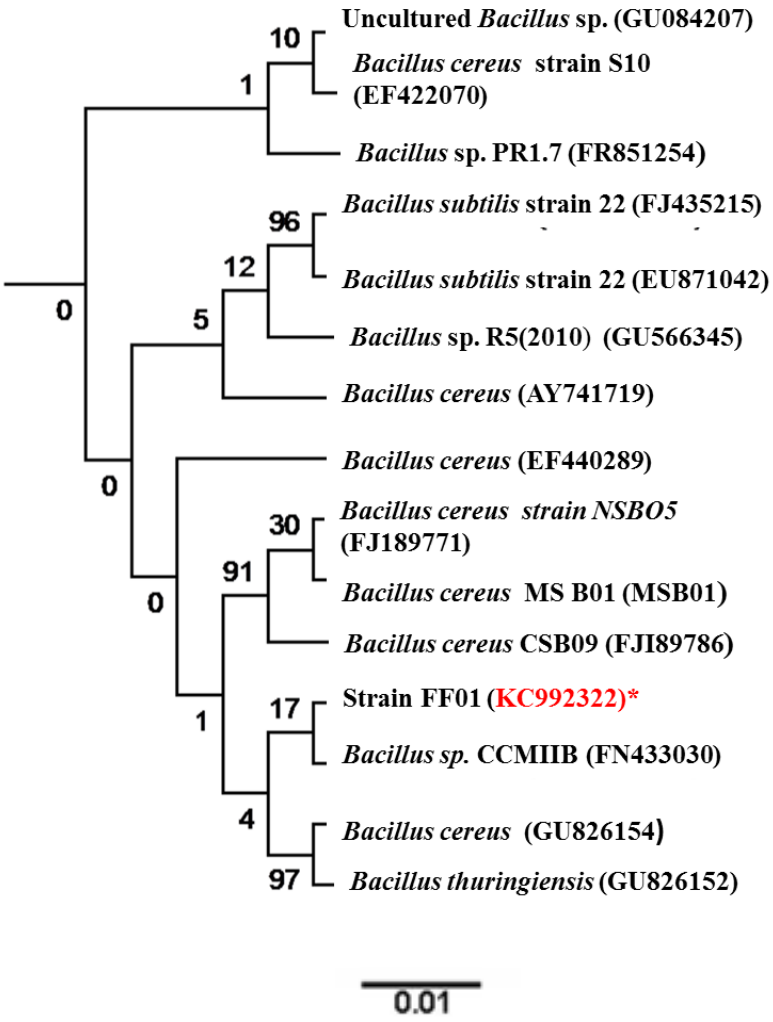

Figure 4.3: Phylogenetic relationships of strain FF01 and other closely related *Bacillus* species based on their 16S rDNA sequences

The tree was generated using the neighbor-joining method and the sequence from *Escherichia coli* strain KesE6 (accession no EU884314) was considered as out-group. The data set was resampled 1000 times by using the bootstrap option and percentage values are given at the nodes. Bar, 0.01 substitutions per site.

4.2.2.3 Phylogeny of strain FF01

A homologous search result of 16S rDNA partial sequence of strain FF01 demonstrated 98% similarity with other species of the genus *Bacillus* (Table 4.5). The phylogenetic tree constructed from the sequence data by the neighbor-joining method (Fig. 4.3) revealed that *Bacillus cereus* (accession no.KC992322) as having 100% 16S rDNA sequence identity representing the closest phylogenetic neighbor of the *Bacillus sp.* strain FN433030 (Fig. 4.3).

4.2.2.4 Phylogenetic analysis of bacterial isolates based on 16S-23S ISR sequences

The 16S-23S ISR of FF01 strain bacterial isolates were amplified and sequenced. The sequences are shown in Table 4.6.

Table 4.6: Homologous search results of 16S-23S lSR partial sequence of strain FF01 using Basic Local Alignment Tool (BLAST) tool from National Centre Biotechnology Information (NCBI).

Accession	Description of bacterium	Query coverage	Max identity
CP001903.1	*Bacillus thuringiensis* BMB171, complete genome	88%	100%
CP001176.1	*Bacillus cereus* B4264, complete genome	88%	99%
EU915688.1	*Bacillus cereus* strain HY-4 16S ribosomal RNA gene, partial sequence	88%	99%
EU871042.1	*Bacillus cereus* strain JL 16S ribosomal RNA gene, partial sequence; 16S-23S ribosomal RNA intergenic spacer, complete sequence; and 23S ribosomal RNA gene, partial sequence	88%	99%
EF117841.1	*Bacillus thuringiensis* serovar kurstaki 16S ribosomal RNA gene, partial sequence; 16S-23S intergenic spacer, complete sequence; and 23S ribosomal RNA gene, partial sequence	88%	99%
EF117840.1	*Bacillus thuringiensis* serovar galleriae 16S ribosomal RNA gene, partial sequence; 16S-23S intergenic spacer, complete sequence; and 23S ribosomal RNA gene, partial sequence	88%	99%
EF117838.1	*Bacillus thuringiensis* serovar colmeri 16S ribosomal RNA gene, partial sequence; 16S-23S intergenic spacer, complete sequence; and 23S ribosomal RNA gene, partial sequence	88%	99%
AY224385.1	*Bacillus cereus* strain BGSC 6A5 rrnI and rrnJ operons, complete sequence	88%	99%
AY224384.1	*Bacillus cereus* strain BGSC 6A5 rrnG and rrnH operons, complete sequence	88%	99%
AE016877.1	*Bacillus cereus* ATCC 14579, complete genome	88%	99%
Z84594.1	*Bacillus thuringiensis* 16S rRNA gene, strain WS 2617	88%	99%
Z84593.1	*Bacillus thuringiensis* 16S rRNA gene, strain WS 2614	88%	99%
CP001907.1	*Bacillus thuringiensis* serovar chinensis CT-43, complete genome	88%	98%
CP002508.1	*Bacillus thuringiensis* serovar finitimus YBT-020, complete genome	88%	98%
CP001746.1	*Bacillus cereus* biovar anthracis str.CI, complete genome	88%	98%

4.2.2.5 Phylogenetic analysis of isolated strain FF01

A homologous search result of strain FF01 demonstrated 100% similarity of 16S-23S ISR sequence with other species of the genus *Bacillus cereus* as shown in Table 4.6. The 16S-23S ISR based phylogenetic analysis showed 99% sequence homology of the strain FF01 with the ISR of *Bacillus cereus* (GenBank accession no X94448) identity, which represented the closest phylogenetic neighbor of the strain FF01 (Fig. 4.4).

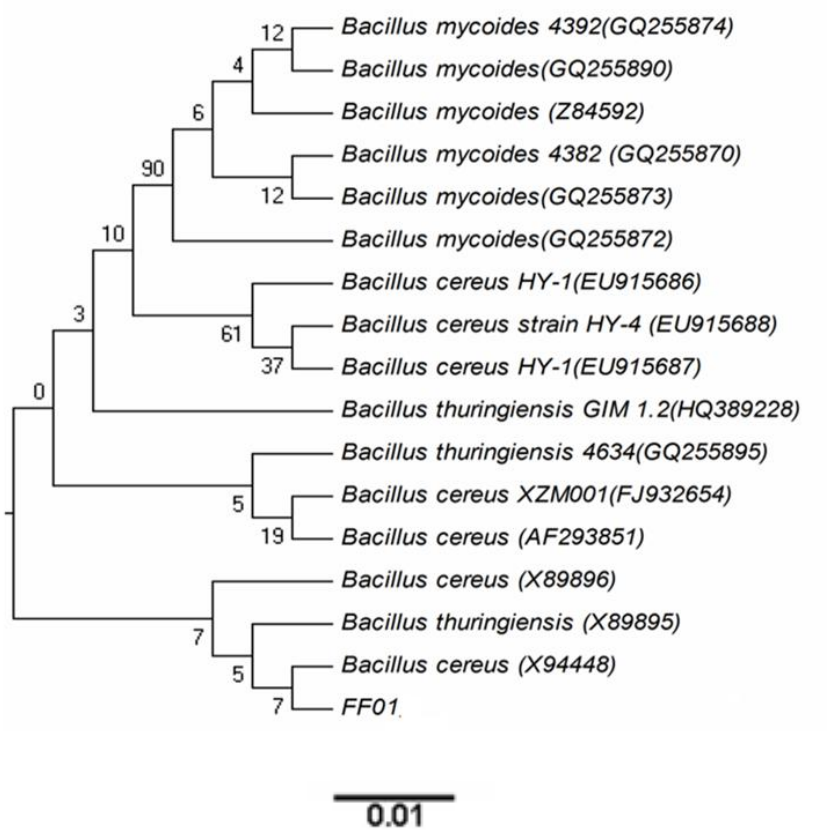

Figure 4.4: Phylogenetic relationships based on 16S-23S ISR sequences of strain FF01 and other closely related *Bacillus* species

The tree was generated using the neighbor-joining method, and the sequence from *Escherichia coli* strain KesE6 (accession no EU884314) was considered as out-group. The data set was resampled 1000 times by using the bootstrap option and % values are given at the nodes. Bar, 0.01 substitutions/site.

Based on phenotypic properties, 16S rDNA and 16S-23S ISR sequences, the bacterial strain FF01 was identified as *Bacillus cereus* strain FF01.

4.2.2.6 Purification of a fibrin(ogen)olytic enzyme from *Bacillus cereus* strain FF01

Unless otherwise stated, all the purification steps were carried out at 4 °C. The fractionation of 40% ammonium sulfate precipitated proteins on a Hi Prep™ Phenyl 16/10 FF column resulted in their separation in one un-bound peak (HIC-I) eluted with 100% buffer B, and one peak (HIC-II) was retained which was consequently eluted from the column with buffer A (Fig. 4.5A). The peak (HIC-II) exhibited fibrinolytic as well as fibrin(ogen)olytic [subsequently referred as fibrin(ogen)olytic enzyme] activities. The re-fractionation of HIC–II through the Hi Load™ Superdex 75 pg 16/60 FPLC column resolved the proteins in three peaks (GF-I to GF-III) (Fig. 4.5B). The peak GF–III exhibited strong fibrin(ogen)olytic activity resulting in an apparent single band of 39.5 kDa in SDS-PAGE under reduced and non-reduced conditions (Fig. 4.6). This protein also showed a single band in fibrin zymography (Fig. 4.6). The molecular weight of GF-III by MALDI-TOF-MS analysis was determined as 38440.12 Da (Fig. 4.7). This purified protease was named Bacethrombase. A summary of purification of Bacethrombase is given in Table 4.7.

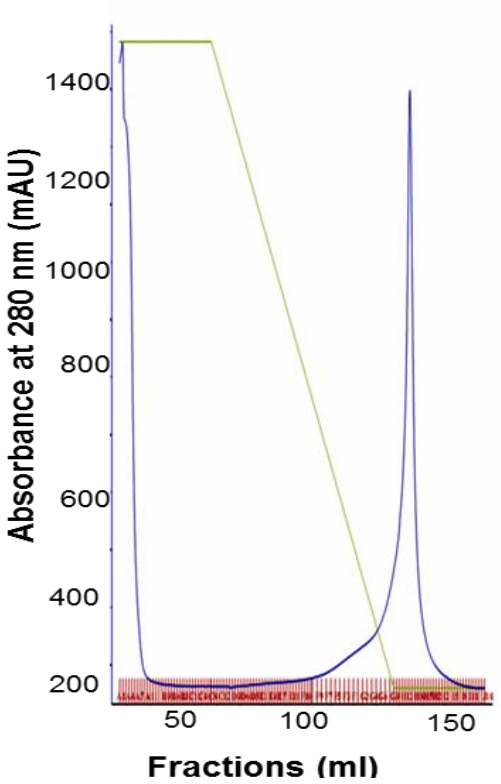

Figure 4.5A: Purification of Bacethrombase from *Bacillus cereus* strain FF01 by hydrophobic interaction chromatography

Ammonium sulfate (40%) precipitated cell free culture supernatant obtained from *Bacillus cereus* strain FF01 was analyzed on a Hi Prep^{TM} Phenyl FF 16/10 column coupled to AKTA purifier 10 FPLC system. Elution of HIC-bound Bacethrombase (HIC-II) was carried out with a linear gradient of 1.0 M ammonium sulfate at a flow rate of 2 ml/min. The elution profile was monitored at 280 nm. The experiment was performed as described in Materials and Methods section 3.2.6.1.3

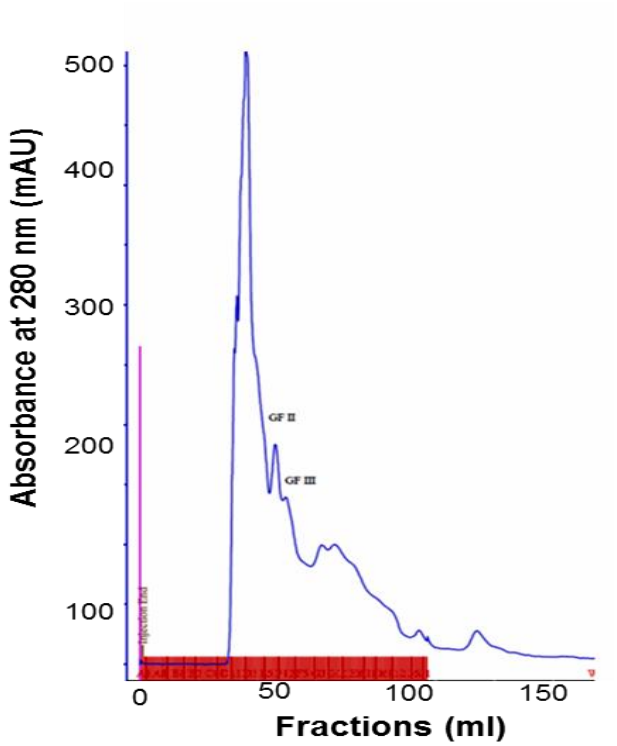

Figure 4.5B: Purification of Bacethrombase from *Bacillus cereus* strain FF01 by gel-filtration chromatography on a Hi Load^{TM} Superdex 75 pg 16/60 FPLC column

Hydrophobic interaction chromatography obtained from partially purified HIC-II was re-fractionated on a Hi Load^{TM} Superdex 75 pg 16/60 FPLC gel-filtration column at a flow rate of 0.5 ml/min at 4 °C. The elution profile was monitored at 280 nm. The experiment was performed as described in section 3.2.7.1.4.

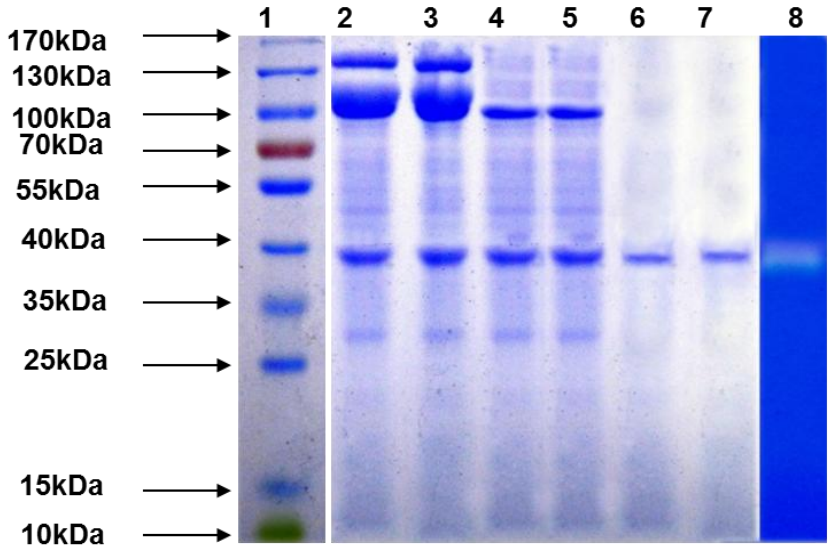

Figure 4.6: Determination of purity of Bacethrombase by SDS-PAGE (12.5%) analysis

Lane 1, protein molecular markers; lane 2, ammonium sulfate precipitated proteins (20 µg) under reduced condition; lane 3, ammonium sulfate precipitated protein (20 µg) under non-reduced condition; lane 4, HIC-II fraction (15 µg) under reduced condition; lane 5, HIC-II fraction (15 µg) under non-reduced condition; lane 6, GFC-III fraction (Bacethrombase, 10 µg) under non-reduced condition; lane 7, GFC-III fraction (Bacethrombase, 10 µg) under reduced condition; and lane 8, fibrin zymography. The procedures were performed as described in Material and Methods sections 3.2.7.1 and 3.2.7.3, respectively.

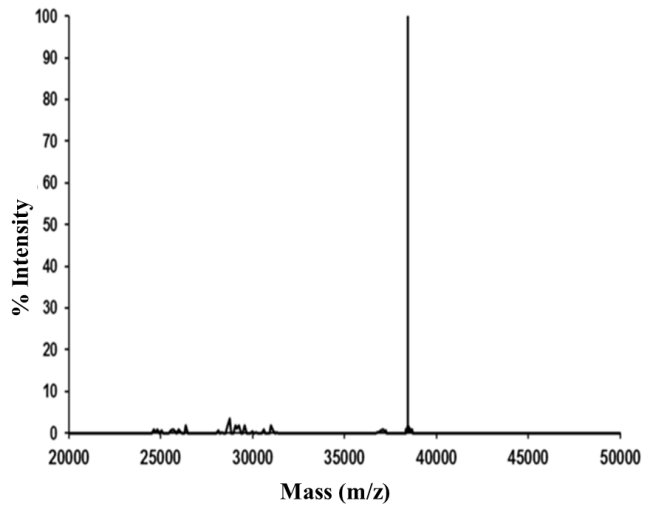

Figure 4.7: MALDI-TOF-MS spectrum of Bacethrombase.

The intact mass of the protein was determined by MALDI-TOF (4800 plus, Applied Biosystems, USA) spectroscopy. Sinapinic acid (Sigma, USA) was used as matrix. The protein samples were desalted using ziptip method (Millipore ZipTip^{TM}). The MALDI-TOF was operated in linear mode with high mass positive acquisition and processing method. The spectrum showed signal at m/z 38440.12 (~38440) corresponding to a single species with molecular weight 39 kDa.

Table 4.7: Summary of purification of Bacethrombase from *Bacillus cereus* strain FF01

Purification steps	Total protein (mg)	Enzyme yield (%)	Total enzyme activity (Units)	Specific activity (Units/mg)	Purification (Fold)
Cell free crude extract (250ml)	194.1	100	15730.1	81.0	1.0
40% Ammonium sulfate precipitation	0.6	2.6	418.0	640.4	7.9
Hydrophobic interaction chromatography	0.3	1.9	305.2	899.2	11.1
Gel-filtration chromatography (Bacethrombase	0.1	1.0	157.4	1349.6	16.6

Note: The enzyme activity was assessed against fibrin at 37 °C at pH 7.4. Unit is defined as µg of L-tyrosine equivalent liberated per ml per min after 30 min incubation at 37 °C. Data represent a typical experiment.

4.2.2.7 Identification of Bacethrombase by N terminal sequencing, peptide mass fingerprinting and amino acid composition analyses.

The blockage of N-terminal amino acid did not allow determining the sequence of Bacethrombase. The BLAST analysis of tryptic peptide sequences of Bacethrombase in NCBI database showed uniqueness of the sequences with no putative conserved domains. However, three of the tryptic peptide sequences of Bacethrombase showed different sequence similarity: (a) AEFALIVR (1018.56 Da) showed 100% sequence similarity with the family of caax amino proteases belonging to *Bacillus cereus* FRI-35 (accession no YP_006599107.1), (b) SFKPQNQVTYAEAVTMIVR (2181.1023 Da) showed 89% sequence similarity with ATP-dependent Clp protease ATP-binding protein isolated from *Bacillus cereus* (accession no WP_000365363), and (c) VQVWIKDDR (1157.6081 Da)

demonstrated 83% sequence similarity with caax prenyl protease 1, putative of *Bacillus cereus* G9241 (accession no EAL14119.1), a well-conserved family of peptidase M48A family.

The amino acid composition analysis of Bacethrombase is shown in Table 4.8. The SWISSPORT databases displayed similarity with lipoprotein signal peptidase (accession number B0S977) belonging to peptidase S8 family (subtilisin family, clan SB) and showing endopeptidase activity. The unique peptide mass fingerprinting data and protein identification based on amino acid composition analysis showed that Bacethrombase is a previously uncharacterized new fibrin(ogen)olytic bacterial serine protease, more particularly it belongs to the MEROPS peptidase family S8 (subtilisin family, clan SB). Table 4.8: Amino acids composition of Bacethrombase (% nmol) The analysis procedure has been described in section 3.2.8.3.

Table 4.8: Amino acids composition of Bacethrombase (% nmol). The analysis procedure has been described in section 3.2.8.3.

Amino acids	Amino acids (% nmol)
GLU	0.6
SER	0.5
GLY	0.7
THR	1.5
CYS	0.2
ARG	1.0
ALA	16.2
TYR	0.3
VAL	0.3
MET	0.6
PHE	47.0
ILE	1.4
LEU	0.3
LYS	19.9
PRO	9.0

4.2.2.8 Secondary structure of Bacethrombase

The native confirmation of Bacethrombase through CD spectrum displayed a negative and a positive peak at 205 nm and 210 nm, respectively (Fig. 4.8). The secondary structure of Bacethrombase when analyzed by CDPRO CLUSTER software suggests that it consists of 14% helix, 6.6% beta-pleated sheet and 79.4% random coil (Fig. 4.8).

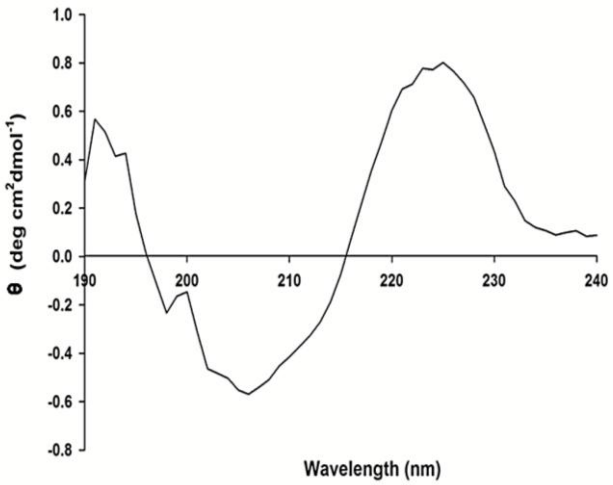

Figure 4.8: Secondary structure of native Bacethrombase by circular dichroism (CD) analysis

Purified protease (1 mg/ml) dissolved in PBS buffer at pH 7.4 was analyzed at 20 °C with a scan rate of 10 nm/min and 0.1 nm resolutions in JASCO J-815 spectrophotometer for secondary structure determination. Average of three scans with no smoothing was reported as CD spectrum of purified protease.

4.2.2.9 Amidolytic activity of Bacethrombase

Bacethrombase demonstrated amidolytic activity towards the chromogenic substrates for plasmin than trypsin; however, it did not show amidolytic activity against other tested chromogenic substrates (Table 4.9).

Table 4.9: Determination of substrate specificity of Bacethrombase

Chromogenic substrate	Activity* (U/mg)
D-Val-Leu-Lys-p-nitroanilidehydrochloride (V0882) (substrate for plasmin)	8.83 ± 0.20
N-(p-Tosyl)-Gly-Pro-Arg-p-nitroanilide acetate (substrate for thrombin)	1.44 ± 0.4

N-Benzoyl-Pro-Phe-Arg-p-nitroanilide hydrochloride (substrate for plasma kallikrein)	0
Nα-Benzoyl-DL-arginine 4-nitroanilide hydrochloride (substrate for trypsin)	0.2 ± 0.1
D-Ile-Pro-Arg-p-nitroanilide dihydrochloride (substrate for t-PA)	0

*Units are defined as µmoles of p-nitroaniline released per min by the enzyme. The experiment was performed as described in section 3.2.7.9

4.2.2.10 Determination of *Km* and *Vmax* of Bacethrombase and plasmin

The *Km* and *Vmax* values of Bacethrombase towards V0882 were calculated at 0.30 µM and 14 ± 0.5 µmol pNA/min, respectively (Fig 4.9); whereas, the *Km* and *Vmax* values of plasmin were calculated at 0.40 µM and 9 ± 0.3 µmol pNA/min, respectively, under the same assay conditions (Fig 4.9).

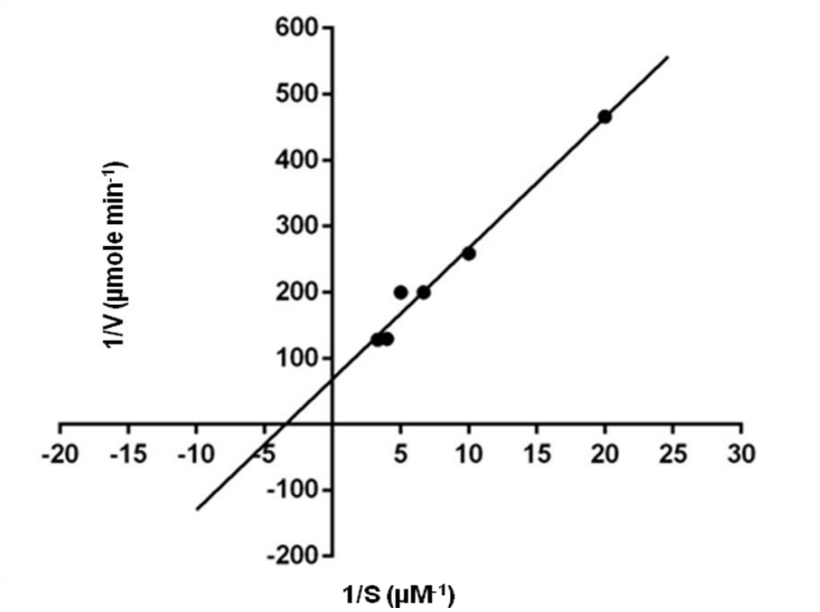

Figure 4.9: Determination of *Km* and *Vmax* values of Bacethrombase against V0882 by Lineweaver-Burk double reciprocal plot

The kinetic properties were determined by incubating 0.2 µM of purified enzyme (Bacethrombase) in different concentrations (0.12 – 1.0 mM) of the above-stated

chromogenic substrate for plasmin (V0882) at 37 °C for 10 min. The enzyme activity was then determined.

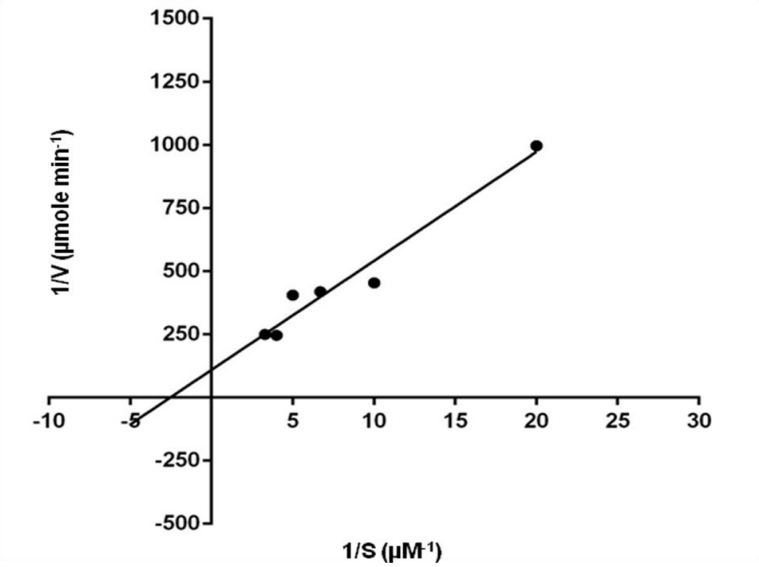

Figure 4.10: Determination of *Km* and *Vmax* values of plasmin against V0882 by Lineweaver-Burk double reciprocal plot

The kinetic properties were determined by incubating 0.2 µM of plasmin in different concentrations (0.12-1.0 mM) of the above-stated chromogenic substrate for plasmin (V0882) at 37 °C for 10 min. The enzyme activity was then determined.

4.2.2.11 Effect of Bacethrombase on extracellular membrane protein hydrolysis

After 24 h incubation at 37 °C, Bacethrombase treated laminin and fibronectin did not show any change in electrophoretic mobility as compared to control (untreated laminin and fibronectin) under the same experimental conditions (Fig. 4.11) suggesting that it does not hydrolyze extracellular membrane proteins. This also indicates non-hemorrhagic nature of Bacethrombase.

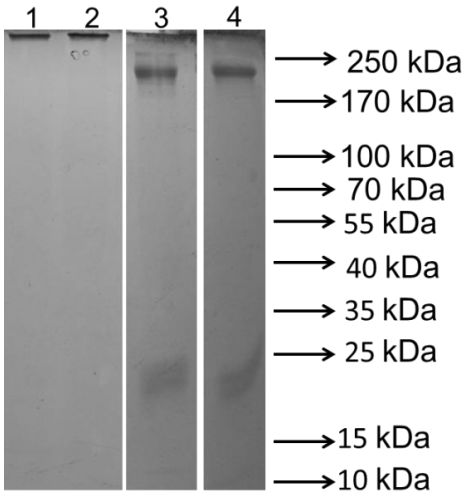

Figure 4.11: Effect of Bacethrombase on extracellular membrane proteins

The extracellular matrix proteins, such as laminin and fibronectin, were prepared in 0.5 M acetic acid to obtain a stock concentration of 1.0 mg/ml. Then 100 µl of stock solution was mixed in 1:15 ratio (w/w) with Bacethrombase dissolved in 50 mM sodium phosphate buffer, pH 7.4. The laminin and fibronectin incubated with buffer served as control. The reaction mixture was allowed to incubate at 37 °C for 24 h. Then, 50 µl aliquots were withdrawn and mixed with an equal volume of denaturing buffer before 10% SDS-PAGE analysis. Lane 1: Laminin (control); lane 2: laminin was incubated with Bacethrombase; lane 3: fibronectin (control); and lane 4: fibronectin was incubated with Bacethrombase.

4.2.2.12 Biochemical properties of Bacethrombase

4.2.2.12.1 Effect of temperature on activity of Bacethrombase

As the temperature increased from 35 to 45 °C, the activity of Bacethrombase also significantly increased; however, further increase in temperature reduced the protease activity (Fig. 4.12). Therefore, 40 °C was considered as the optimum temperature for Bacethrombase activity.

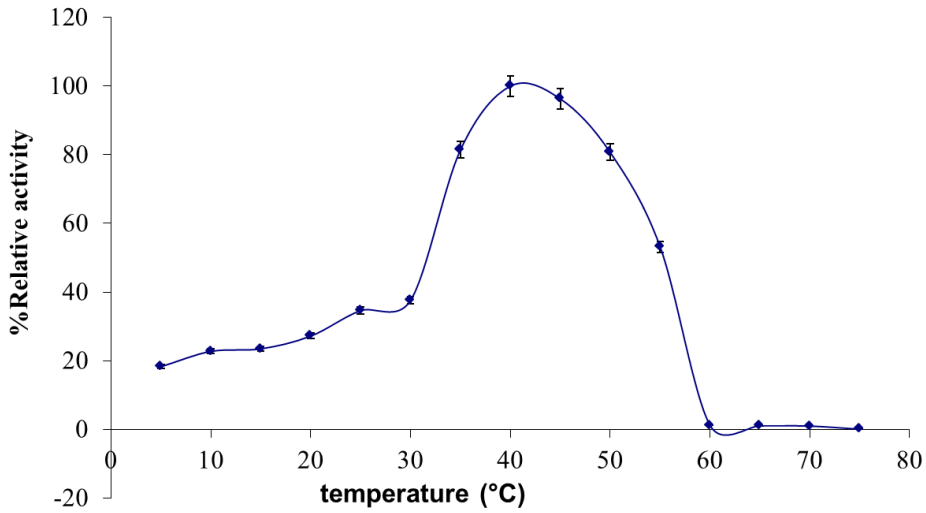

Figure 4.12: Effect of temperature on activity of Bacethrombase

The protease activity was assessed against fibrin at pH 7.4. Values are mean ± SD of triplicate determination. The 100% activity represents at 40 °C.

4.2.2.12.2 Effect of pH on activity of Bacethrombase

Results indicated a significant increase in the protease activity of Bacethrombase when pH of the medium was increased from 8.0 to 9.0 (Fig. 4.13). Therefore, the fibrinolytic activity at pH 8 was considered as optimum pH for enzyme activity.

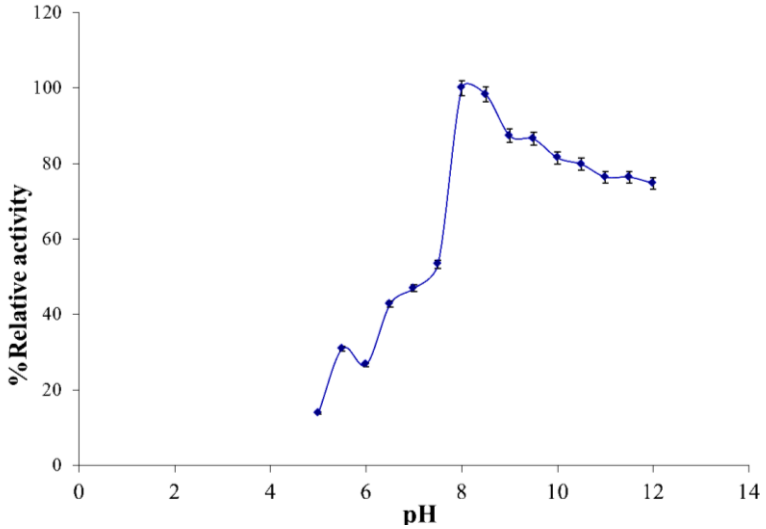

Figure 4.13: Effect of temperature on Bacethrombase activity

The protease activity was determined against fibrin at 37 °C. Values are mean ± SD of triplicate values. The 100% activity represents at pH 8.

The substrate specificity of Bacethrombase is shown in Table 4.10.

Table 4.10: The substrate specificity of Bacethrombase at 37 °C, pH 7.4.

Substrate (1mg/ml)*	**Activity (U/mg)**
Bovine plasma fibrinogen	799.2 ± 1.4
Fibrin	680.1 ± 2.6[a]
Casein	330.3 ± 0.4 [a]
Bovine serum γ-globulin	5.6 ± 0.2 [a]
Bovine serum albumin	291.2 ± 0.2 [a]

Note: Values represents mean ± SD of triplicate determinations. Significance of difference with respect to fibrin(ogen)olytic activity [a]$p < 0.001$.

*Unit is defined as µg of L-tyrosine equivalent liberated per min after 30 min incubation at 37 °C, pH 7.4.

The enzyme demonstrated preferential hydrolysis of bovine plasma fibrinogen followed by fibrin and then serum albumin; however, Bacethrombase displayed poor hydrolytic activity against casein or globulin.

The fibrin(ogen)olytic activity of Bacethrombase at a fibrinogen concentration of 8 mg/ml (~24 µM) increased linearly; thereafter, a steady state was attained (~30 µM). The *Km* and *Vmax* values for fibrin(ogen)olytic activity of Bacethrombase were calculated to be 16.8 ± 2.7 µM and 14.7 ± 0.9 µg/ml/min, respectively (Fig. 4.14).

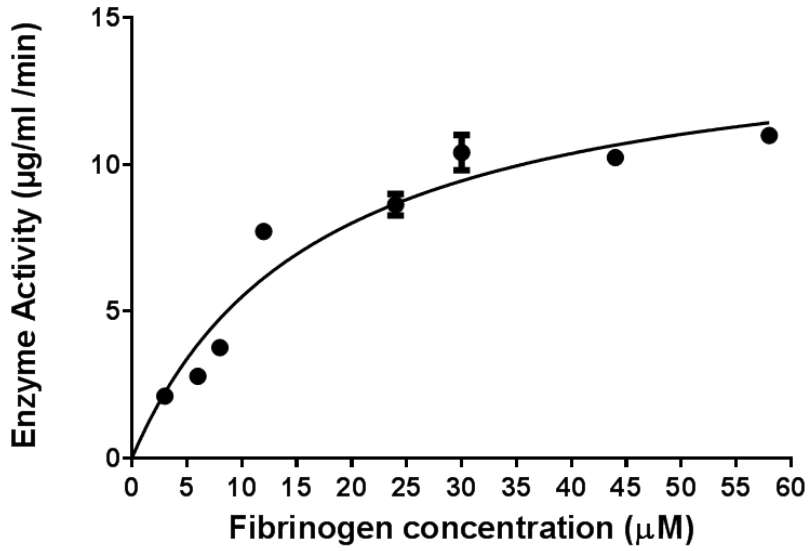

Figure 4.14: Effect of substrate (fibrinogen) concentration on protease activity of Bacethrombase at 2.5 µM to 58.5 µM.

The reaction mixture was incubated at 37 °C for 30 min and the enzyme activity of Bacethrombase was measured at various concentration of fibrinogen (2.5 to 58.5 µM). Values are mean ± SD of triplicate determinations.

4.2.2.12.3 Plasminogen activation property of Bacethrombase

Bacethrombase failed to convert plasminogen to plasmin indicating the lack of plasminogen-activating property (Fig. 4.15).

Streptokinase was added to plasminogen in the presence of chromogenic substrate for plasmin (D-Val-Leu-Lys p-nitroanilide dihydrochloride) as a positive control (curve 1). Bacethrombase was incubated with chromogenic substrate for plasmin (D-Val-Leu-Lys p-nitroanilide dihydrochloride) in the absence of plasminogen at 37 °C for 20 min (curve 2). Bacethrombase was incubated with chromogenic substrate for plasmin (D-Val-Leu-Lys p-nitroanilide dihydrochloride) in the presence of plasminogen at 37 °C, pH 7.4 for 20 min.

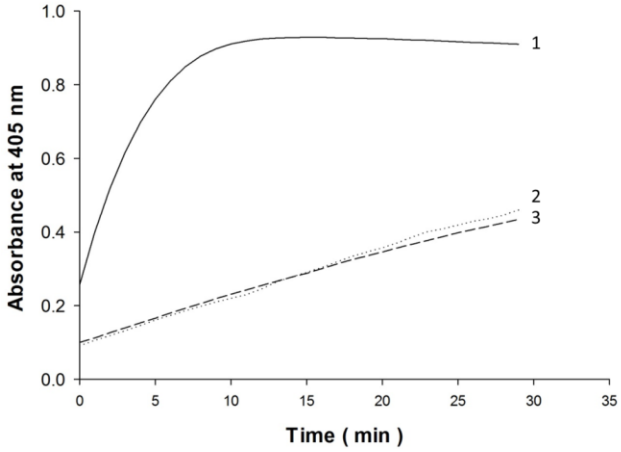

Figure 4.15: Plasminogen activation property of Bacethrombase

4.2.2.12.4 Inhibitory activity of Bacethrombase

The protease activity of Bacethrombase was significantly inhibited by serine protease inhibitors PMSF, TPCK and TLCK, as well as by IAA and pBPP. The enzyme activity remained unaffected by EDTA and DTT (Table 4.11).

Table 4.11: Effect of various inhibitors on fibrin(ogen)olytic activity of Bacethrombase

Inhibitors/Metal ions	Relative activity
Control (without inhibitor/metal ion)	100
Inhibitors (concentration)	
PMSF (2 mM)	18.2 ± 0.3[a]
pBPP (2 mM)	21.3 ± 2.8 [a]
IAA (4 mM)	25.0 ± 0.5 [a]
TPCK(100 µM)	43.1 ± 13.4 [a]
TLCK(100 µM)	22.2 ± 2.8 [a]
DTT (4 mM)	94.2 ± 11.7
EDTA (4 mM)	98.3 ± 2.5
Divalent metal ions (concentration)	
$CaCl_2$ (4 mM)	95.4 ± 0.6
$MgCl_2$ (4 mM)	94.3 ± 3.5
$CoCl_2$ (4 mM)	92.0 ± 4.3
$FeCl_2$ (4 mM)	94.1 ± 5.1
$HgCl_2$ (4 mM)	92.6 ± 2.2

Note: Values represent mean ± SD of triplicate determinations. Difference was significant with respect to control [a]$p < 0.001$.

4.2.2.12.5 Glycosylation on Bacethrombase

4.2.2.12.5.1 Total neutral carbohydrate content Bacethrombase

Bacethrombase was found to contain 66.6 µg neutral carbohydrates per mg of protein, which represent 6.6% of total protein mass.

4.2.2.12.5.2 Sialic acid content of Bacethrombase

Removal of sialic acid from Bacethrombase with neuraminidase under denaturing conditions showed a protein band of 20.2 kDa on SDS-PAGE (Fig. 4.16). Therefore, it may be concluded that the sialic acid content represented 48.86% of total mass of Bacethrombase. However, deglycosyation by PNGase could not be achieved under denaturing conditions. Further, treatment of Bacethrombase with neuraminidase did not result in a change in the SDS-PAGE migration pattern under non-denaturing conditions (Fig. 4.17).

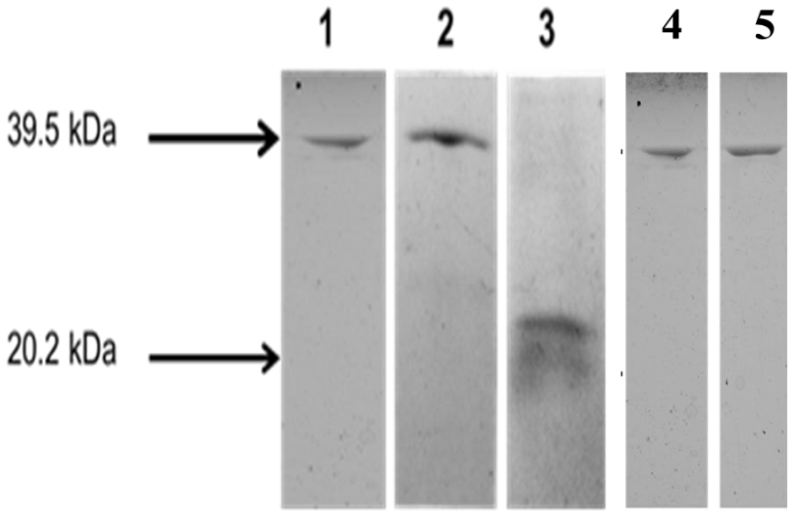

Figure 4.16: Determination of sialic acid content in Bacethrombase by 12.5% SDS-PAGE analysis under denaturation conditions

Bacethrombase (20 µg) was treated with neuraminidase according to manufacturer's protocol (New England Biolabs Inc, Ipswich, MA). After denaturation of enzyme, the reaction mixture was incubated for 4 h at 37 °C and the reaction products were visualized by 12.5% SDS-PAGE under reducing conditions. Lane 1: Bacethrombase (20 µg); lane 2: native (without denaturation) Bacethrombase (20 µg) treated with neuraminidase (500 units) for 1 h at 37 °C; lane 3: denatured Bacethrombase (20 µg) treated with neuraminidase (500 units) for 1 h at 37 °C; lane 4: native (without

denaturation) Bacethrombase (20 µg) treated with PNGase; and lane 5, denatured Bacethrombase (20 µg) treated with PNGase for 4 h at 37 °C.

4.2.2.12.5.3 Kinetics of fibrin degradation by Bacethrombase

The SDS-PAGE analysis of fibrin / fibrinogen degradation by Bacethrombase unambiguously demonstrated that this enzyme preferentially degraded the Aα-chain to Bβ-chain of both fibrinogen (Fig. 4.17 A) and fibrin (Fig. 4.17 B). The γ-chain remained unaffected after incubation for 180 min at 37 °C, pH 7.4. A comparative analysis of fibrin vs. fibrinogen hydrolysis by Bacethrombase showed that during the initial phase of the reaction, Bacethrombase could efficiently degrade the Aα-chain of fibrinogen (Fig. 4.17 A) compared to the hydrolysis of the Aα-chain of fibrin (Fig. 4.17 B) suggesting a higher activity towards fibrinogen.

A comparison of the pattern of fibrin or fibrinogen degradation between Bacethrombase and plasmin by RP-HPLC analysis demonstrated that both these serine proteases have nearly identical cleavage sites in fibrinogen (Fig. 4.18 A) and fibrin molecules (Fig. 4.18 B)

(A)

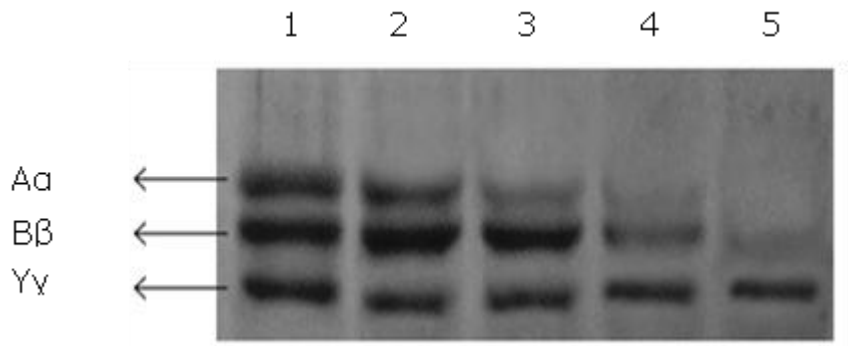

Figure 4.17A: A SDS-PAGE analysis of fibrinogen degradation by Bacethrombase

Fibrinogen was incubated with 1.0 µM Bacethrombase for 60 min at 37 °C and the reaction was stopped by adding 10 µl of TCA in ice cold condition to denature the enzyme. The precipitate was removed by centrifugation at 5000 × g for 10 min. The supernatant (30 µl) containing fibrin degradation products was separated by 12.5% SDS-PAGE under reduced conditions. (A) Lane 1, control fibrinogen (without Bacethrombase); lanes 2 to 6, fibrinogen degradation by Bacethrombase after 30, 60, 90 and, 120 min of incubation, respectively at 37 ºC, pH 7.4.

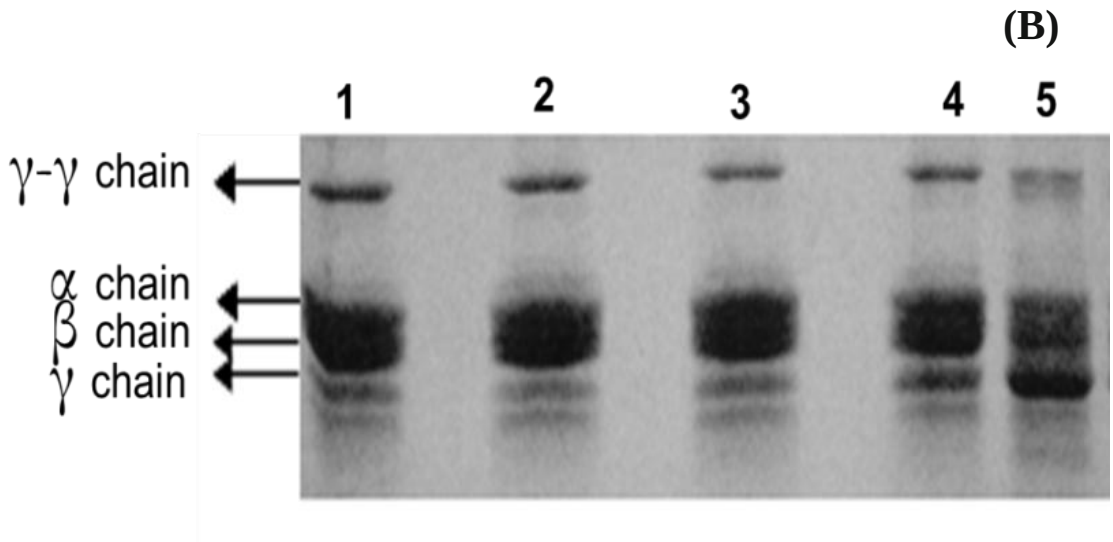

Figure 4.17B: SDS-PAGE analysis of fibrin degradation by Bacethrombase

Fibrin was incubated with 1.0 µM Bacethrombase for 60 min at 37 °C and the reaction was stopped by adding 10 µl of TCA in ice cold condition to denature the enzyme. The clot was removed by centrifugation at 5000 × g for 10 min. The supernatant (30 µl) containing fibrin degradation products were separated by 12.5% SDS-PAGE under reduced conditions. Lane 1, control fibrin (without Bacethrombase treatment); lanes 2 to 6, fibrin degradation by Bacethrombase after 30, 60, 90, 120 and 180 min of incubation, respectively at 37 ºC, pH 7.4.

(A)

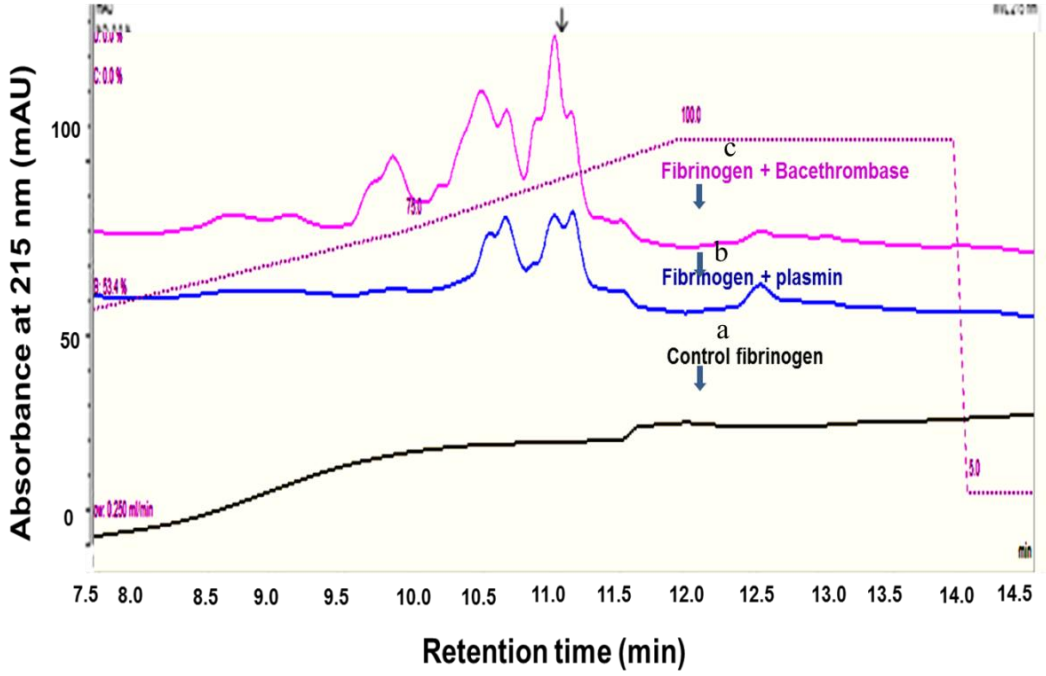

Figure 4.18A: A comparison of fibrinogen degradation between plasmin and Bacethrombase under identical experimental conditions by RP-UHPLC analysis.

Fibrinogen was incubated with 0.6 µM plasmin/ Bacethrombase for 60 min at 37 °C and the reaction was stopped by adding 10 µl of TCA in ice cold condition to denature the enzyme. The clot was removed by centrifugation at 5000 × g for 10 min. The supernatant (100 µl) was loaded on a Acclaim® 300 C_{18} column (2.1 mm x 150 mm, 3 µm, 300 Aº), and peptides were eluted by a linear gradient (0%-100%) of solvent A [100% MilliQ water containing 0.1% (v/v) TFA] and solvent B [90% acetonitrile containing 0.1% (v/v) TFA] at a flow rate of 1 ml/min. Elution of peptide was monitored at 215 nm. (a) Control (fibrinogen + PBS), (b) fibrinogen + plasmin, (c) fibrinogen + Bacethrombase. All the above experiments were conducted in identical conditions and were repeated in triplicate to assure the reproducibility.

(B)

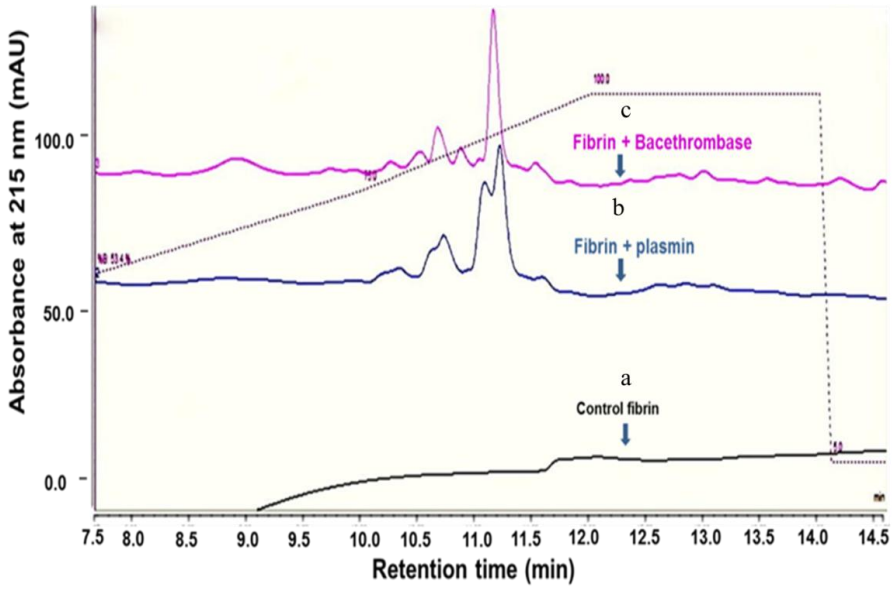

Figure 4.18B: A comparison of fibrin degradation between plasmin and Bacethrombase under identical experimental conditions by RP-UHPLC analysis

Fibrin was incubated with 0.6 µM plasmin/ Bacethrombase for 60 min at 37 °C , pH 7.4 and the reaction was stopped by adding 10 µl of ice cold TCA. The clot was fractionated through a Acclaim® 300 C_{18} column (2.1mm x 150 mm, 3 µm, 300 Aº) as stated previously: (a) Control (fibrin +PBS), (b) fibrin + plasmin, (c) fibrin + Bacethrombase. All the above experiments were conducted in identical conditions and were repeated in triplicate to assure the reproducibility.

4.2.2.13 Pharmacological properties of Bacethrombase

4.2.2.13.1 *In-vitro* thrombolytic activity of Bacethrombase

As compared to streptokinase and plasmin, Bacethrombase showed significantly superior ($p < 0.05$) *in vitro* thrombolytic activity (Fig. 4.19). However, the clot bursting potency of Bacethrombase and plasmin towards dissolving the heat-treated blood clot was reduced to ~70% of their original activity in dissolving the unheated blood clot (Fig. 4.19). Nevertheless, streptokinase exhibited negligible activity in dissolving the heated-treated blood clot (Fig. 4.19).

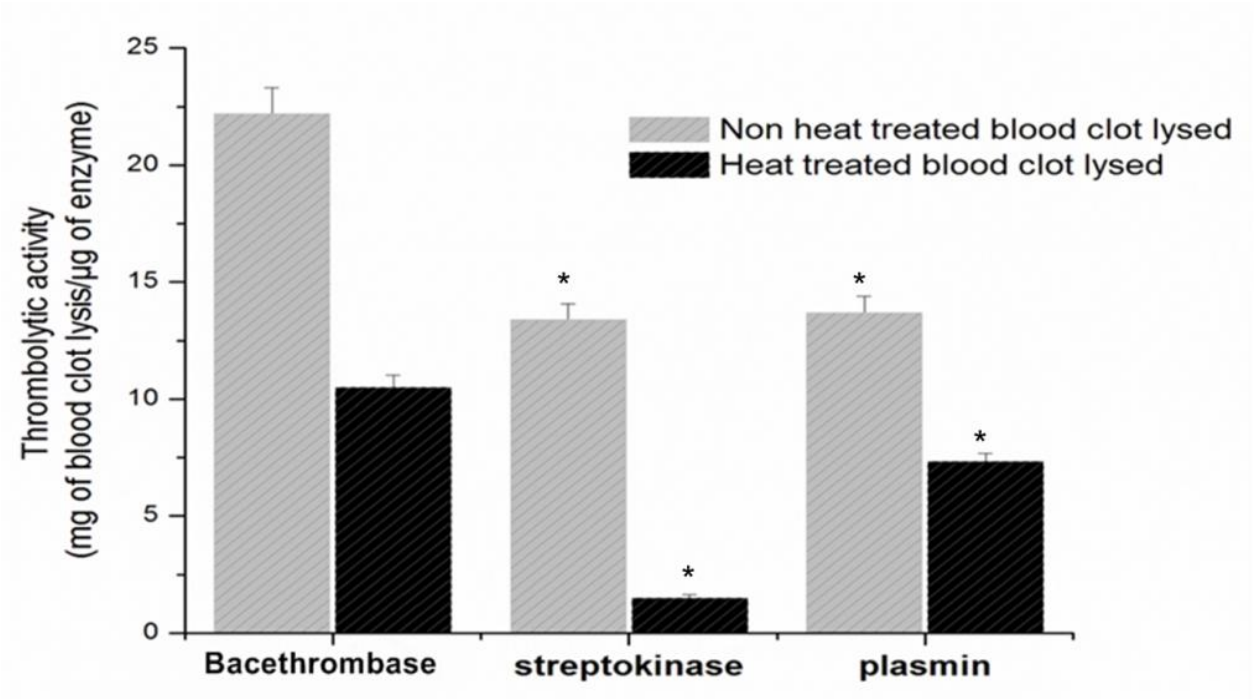

Figure 4.19: A comparison of *in vitro* thrombolytic activity among Bacethrombase, plasmin and Streptokinase at 37 °C, pH 7.4, under identical experimental conditions

Goat blood containing 1.0 ml of 3.8% sodium citrate was allowed to clot at room temperature for an hour after the addition of 100 µl of 250 mM $CaCl_2$. The experiment was done as described in section 3.2.2.4. The *in vitro* thrombolytic activity was measured as mg of blood clot (thrombus) lysed per µg of enzyme. Values are mean ± S.D. of triplicate determinations. Significance of difference with respect to blood clot lysis activity of Bacethrombase $^{*}p < 0.05$.

4.2.2.13.2 Cell cytotoxicity and hemolytic activity of Bacethrombase

At a dose of 15 µg/ml, Bacethrombase did not show *in vitro* cytotoxicity towards HeLa and HT29 cells (Fig. 4.20).

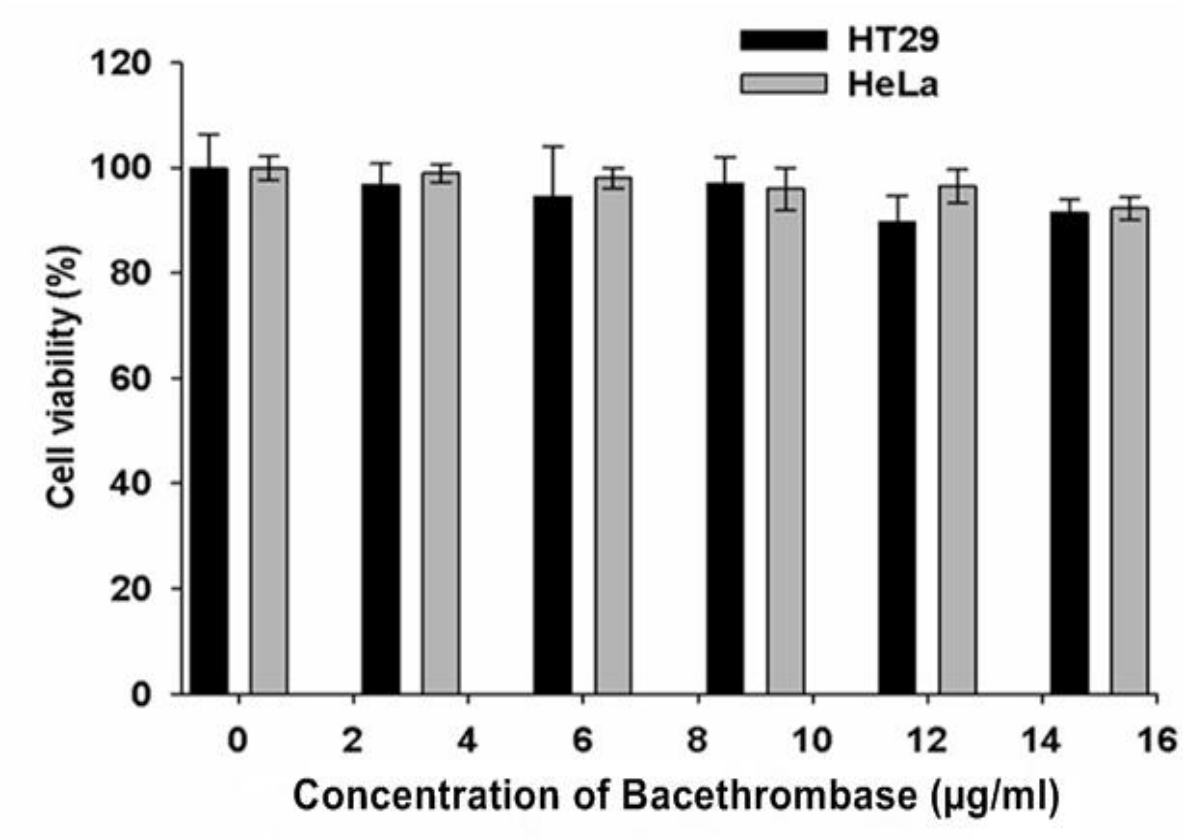

Figure 4.20: Cell cytotoxicity of Bacethrombase on Hela and HT29 cells

Dose-dependent *in vitro* cell cytotoxicity of Bacethrombase (3-15 µg/ml) against Hela and HT29 cell lines was determined. The differences between treated groups were insignificant ($p > 0.05$) with respect to control cells. The experiment was performed as described in section 3.2.11.

4.2.2.13.3 Antiplatelet activity of Bacethrombase

4.2.2.13.3.1 Effect of Bacethrombase on platelet aggregation

Bacethrombase demonstrated dose-dependent antiplatelet effect when tested against PRP (Fig. 4.21A).

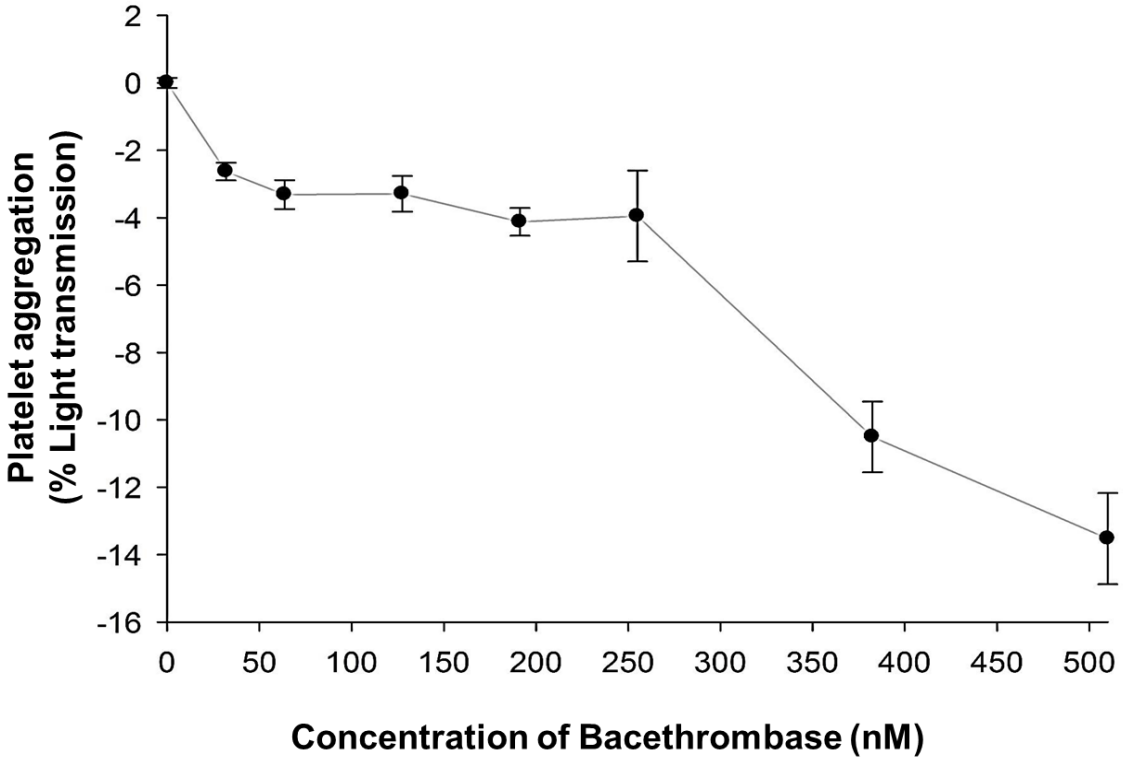

Figure 4.21A: A Dose-dependent platelet deaggregation by Bacethrombase

Different concentrations of Bacethrombase (25-500 nM) were added to 100 µl of PRP or to washed platelets suspension in a 96-well plate, and the experiment was performed as described in section 3.2.13. The data represent mean ± S.D of triplicate experiments.

4.2.2.13.3.2 Effect of Bacethrombase on collagen-induced platelet aggregation

Bacethrombase failed to inhibit the collagen-induced aggregation of PRP (Fig. 4.21B).

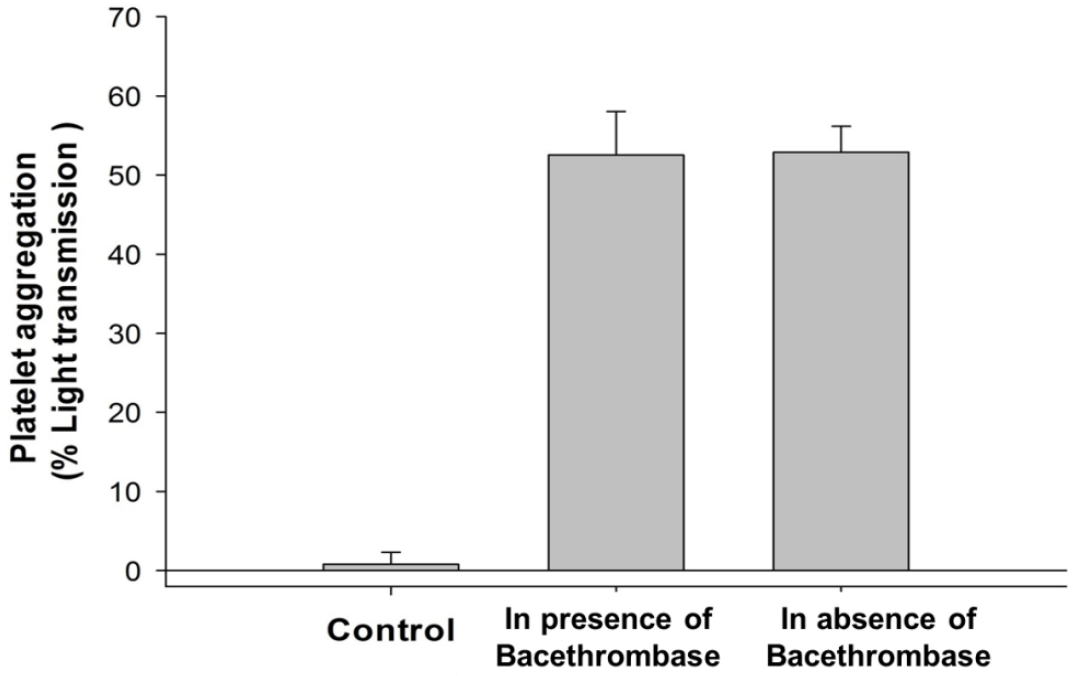

Figure 4.21B: Effect of Bacethrombase on collagen-induced platelet aggregation

Bacethrombase (250 nM) was incubated with PRP at 37 °C for 10 min, and then 1.0 μg/ml of collagen was added to the reaction mixture. The absorbance was recorded at 540 nm. The platelet aggregation induced by collagen was considered as 100% activity. The data represent mean ± S.D of triplicate experiments.

4.2.2.13.3.3 Determination of IC_{50} value of Bacethrombase from the regression analysis of inhibition curve

Bacethrombase showed inhibition of ADP-induced aggregation of PRP in a dose-dependent manner with an IC_{50} value of 10 nM (Fig. 4.21C).

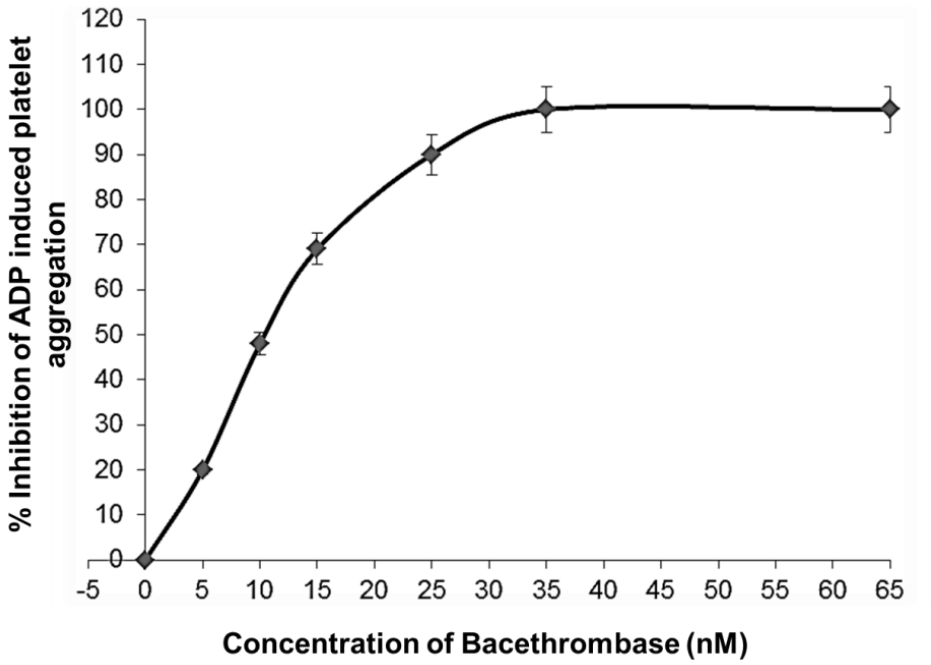

Figure 4.21C: Dose-dependent inhibition of ADP-induced platelet aggregation by Bacethrombase

Different concentrations of Bacethrombase (5.0 to 65.0 nM) were incubated with PRP at 37 °C for 10 min and then 20 µM of ADP was added to the reaction mixture. The percent platelet aggregation by ADP in the absence of Bacethrombase was considered as 100% activity and was used as the base for comparing other values with it. The IC_{50} value of Bacethrombase (that showed 50% inhibition of ADP-induced platelet aggregation) was determined from the regression analysis of inhibition curve.

4.2.2.13.4 Anticoagulant property of Bacethrombase

4.2.2.13.4.1 Effect of Bacethrombase on recalcification time of PPP

Increasing the pre-incubation time of Bacethrombase with PPP before the addition of $CaCl_2$ resulted in a concomitant ($P < 0.05$) increment of Ca^{2+} clotting time of PPP (Fig. 4.22).

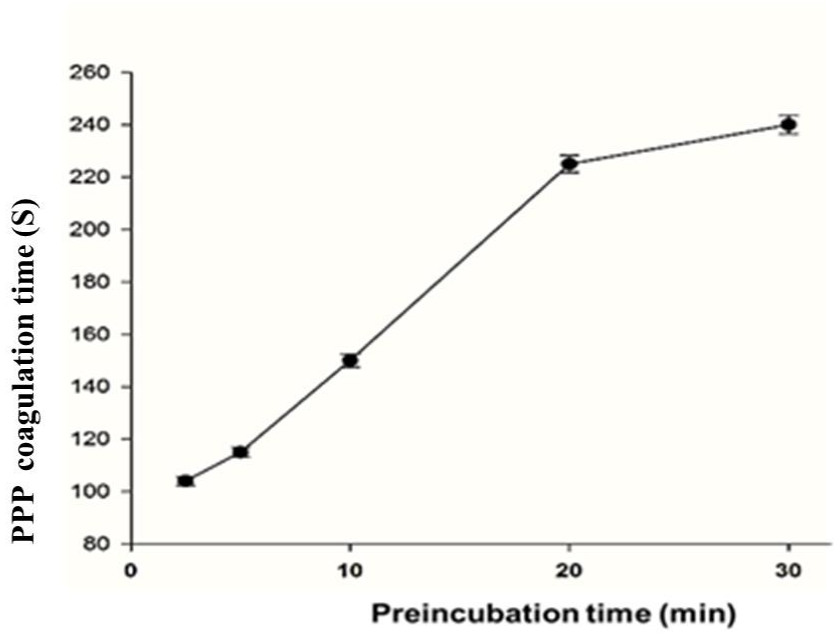

Figure 4.22: Effect of pre-incubation time (0 - 30 min) of Bacethrombase (0.1 µM) / platelet poor plasma (PPP) at 37 °C on Ca^{2+}clotting time of PPP

Platelet poor goat plasma (PPP) was pre-incubated with graded amounts of fibrinolytic enzyme (0.5-4 µg/ml) for 3 min at 37 °C, and then the Ca^{2+} clotting time of PPP was determined by adding 250 mM $CaCl_2$ solution. The clotting time was measured as described in section in 3.2.16.3.5. The data represent mean ± SD of triplicate determinations.

4.2.2.13.4.2 Comparison of anticoagulant activity of Bacethrombase with commercial anticoagulant

A comparison of the dose-dependent anticoagulant activity among Bacethrombase, plasmin, heparin, and warfarin demonstrated their equivalent potency to prolong the Ca^{2+}clotting time of PPP (Fig. 4.23).

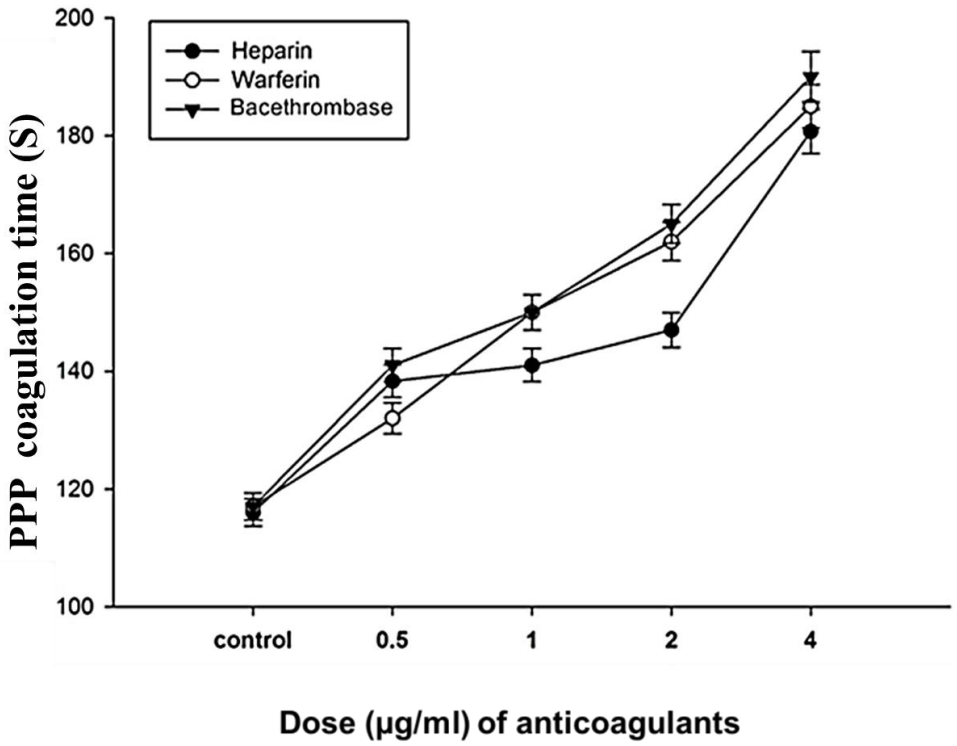

Figure 4.23: A comparison of the dose-dependent anticoagulant activity among Bacethrombase, warfarin, heparin and plasmin under identical experimental conditions

Bacethrombase, warfarin, heparin, and plasmin were incubated individually at different concentrations with 300 µl of platelet poor plasma (PPP) at 37 °C followed by addition of 40 µl of 250 mM $CaCl_2$ solution to initiate coagulation. One unit of anticoagulant activity is defined as a Bacethrombase, plasmin, Warfarin and heparin induced 1s increase in clotting time of PPP compared to clotting time of control plasma. The data represent mean ± SD of triplicate determinations.

4.2.2.13.4.3 Mechanism of anticoagulant action by Bacethrombase

4.2.2.13.4.3.1 Effect on thrombin and FXa inhibition by Bacethrombase

Bacethrombase failed to inhibit the prothrombin activating property of FXa (Fig. 4.24).

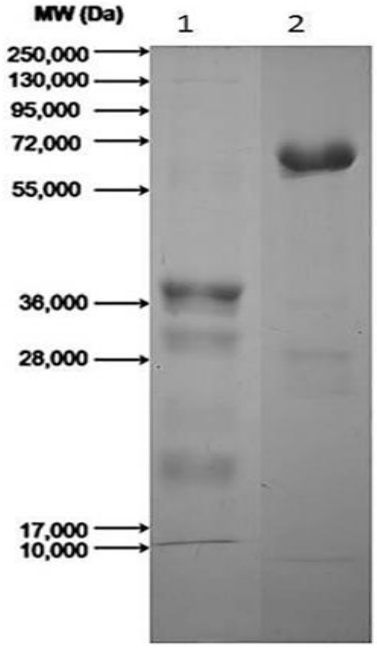

Figure 4.24: Effect of Bacethrombase in inhibition of prothrombin activating property factor Xa

Bacethrombase (0.4 µM) was pre-incubated with FXa (0.13 µM) in 20 mM sodium phosphate buffer (pH 7.4) at 37 °C for 30 min. Thereafter, 1.4 µM of prothrombin and 2.5 mM Ca^{2+} were added to the mixture and was incubated overnight at 37 °C. A control was run parallel in which 1 X PBS was added instead of purified protease. The prothrombin degradation products were analyzed by 12.5% SDS-PAGE under reducing conditions. Lane 1: prothrombin (10 µg) incubated overnight with Bacethrombase (2 µg)-treated factor Xa (0.1 µg) at 37ºC and lane 2: prothrombin (10 µg).

4.2.2.13.4.3.2 Interaction study between Bacethrombase and fibrinogen/thrombin by spectroflurometer

Bacethrombase showed excitation at 280 nM typical of tryptophan residue. The spectrofluorometric study vouched the interaction between Bacethrombase and fibrinogen (Fig. 4.25); however, Bacethrombase did not show interaction with thrombin (Fig. 4.25).

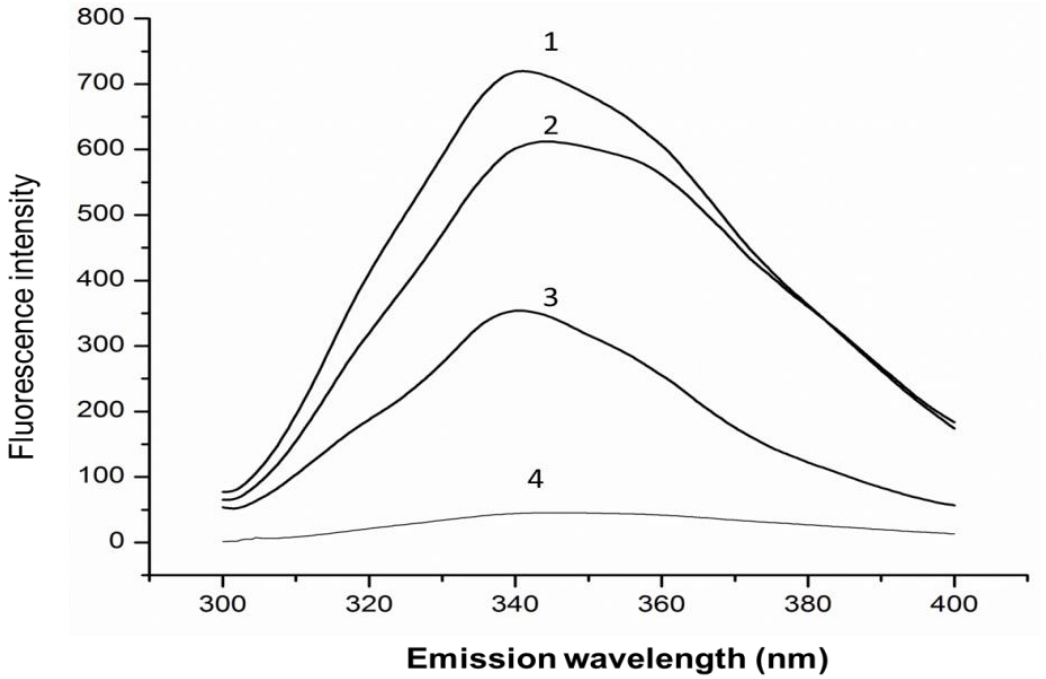

Figure 4.25: Interaction between Bacethrombase and fibrinogen/thrombin by fluorescence spectroscopy

Fluorescence spectra showing (1) interaction of Bacethrombase (0.02 μM) with fibrinogen (0.04 μM), (2) Bacethrombase (0.02 μM), (3) fibrinogen (0.04 μM), and (4) Bacethrombase (0.02 μM) with thrombin (0.04 μM)

4.2.2.13.4.3.3 Inhibition of thrombin

Pre-incubation of Bacethrombase with thrombin did not inhibit the fibrinogen clotting activity of thrombin; albeit, pre-incubation of graded concentrations of Bacethrombase with fibrinogen prior to the addition of thrombin enhanced the fibrinogen clotting time of thrombin in a progressive manner (Fig. 4.26).

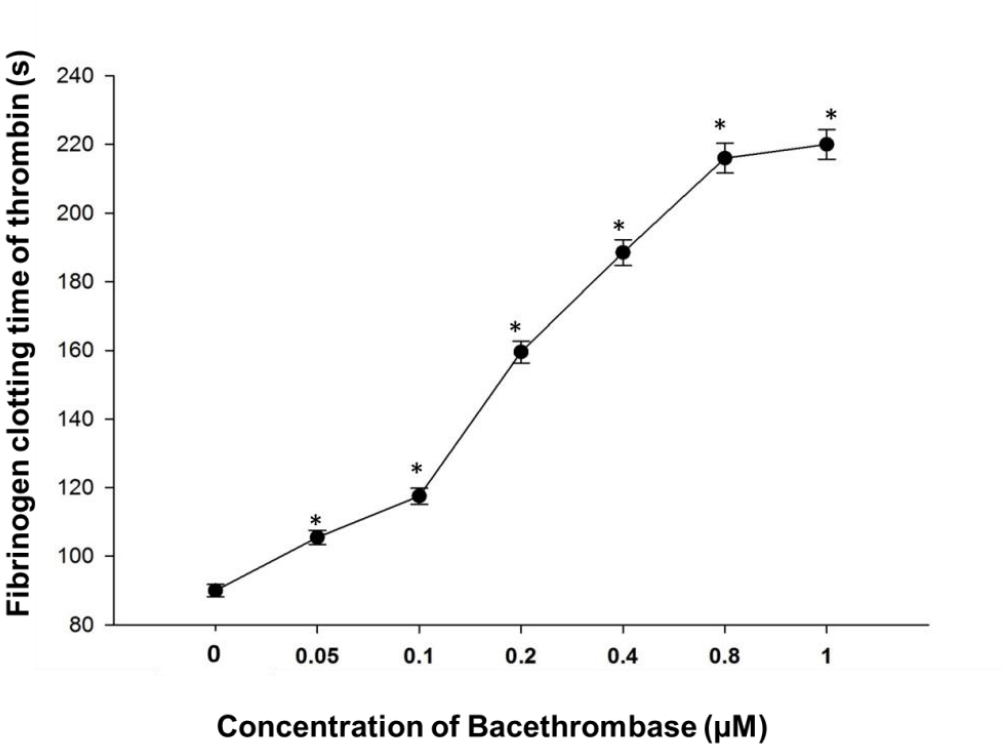

Figure 4.26: Effect of pre-incubation of Bacethrombase with fibrinogen on fibrinogen clotting time of thrombin

Bovine thrombin (3 µl, 10 NIH U/ml) was pre-incubated with increasing concentrations (0.05-1.0 µM) of Bacethrombase in 50 mM sodium phosphate buffer at pH 7.4 or with 1X PBS (control) at 37 °C for 30 min. The fibrinogen clotting activity of thrombin was measured against physiological substrate fibrinogen (0.04 µM). The data represent mean ± SD of triplicate determinations. Significance of difference with respect to control group of rats, *p<0.001, **p<0.05.

4.2.2.13.5 Evaluation of *in vivo* toxicity of Bacethrombase in a rat model

4.2.2.13.5.1 Physiological changes after Bacethrombase administration in rats

An intravenous injection of Bacethrombase (10 mg/kg) did not show any mortality or behavioral changes in treated rats compared to the control group of rats. There were no differences in weight, water and food intake of treated animals compared to control animals (Tables 4.12A-C).

Table 4.12A: Group mean terminal body weight (g) of control and treated rats for 14 days

Group		Terminal body weight (g)		
		0 day	7 days	14 days
Control (n=6)	Mean	213.86	213.97	214.75
	± SD	9.10	8.30	9.80
Treated (n=6)	Mean	218.05	218.44	217.98
	± SD	7.87	9.31	10.22

Table 4.12B: Group mean food consumption (g/kg body weight/day) trends for male Wistar albino rats for 14 days.

Group		Day		
		0	7	14
Control (n = 6)	Mean	5.42	5.46	5.48
	± SD	1.30	2.80	1.60
Treated (n = 6)	Mean	5.19	5.24	5.26
	± SD	0.96	1.2	1.77

Table 4.12C: Group mean water consumption (mL/kg body weight/day) trends Wistar albino rats for 14 days.

Group		Day		
		0	7	14
Control (n = 6)	Mean	190.30	190.11	190.80
	± SD	7.21	7.36	6.45
Treated (n = 6)	Mean	184.06	184.21	184.41
	± SD	9.28	8.80	8.35

Additionally, there was no significant change observed in the ECG (Fig. 4.27) and Penh value of control 0.55 ± 1.56 (mean ± SD) as well as treated rats 0.56 ± 0.45 (mean ± SD).

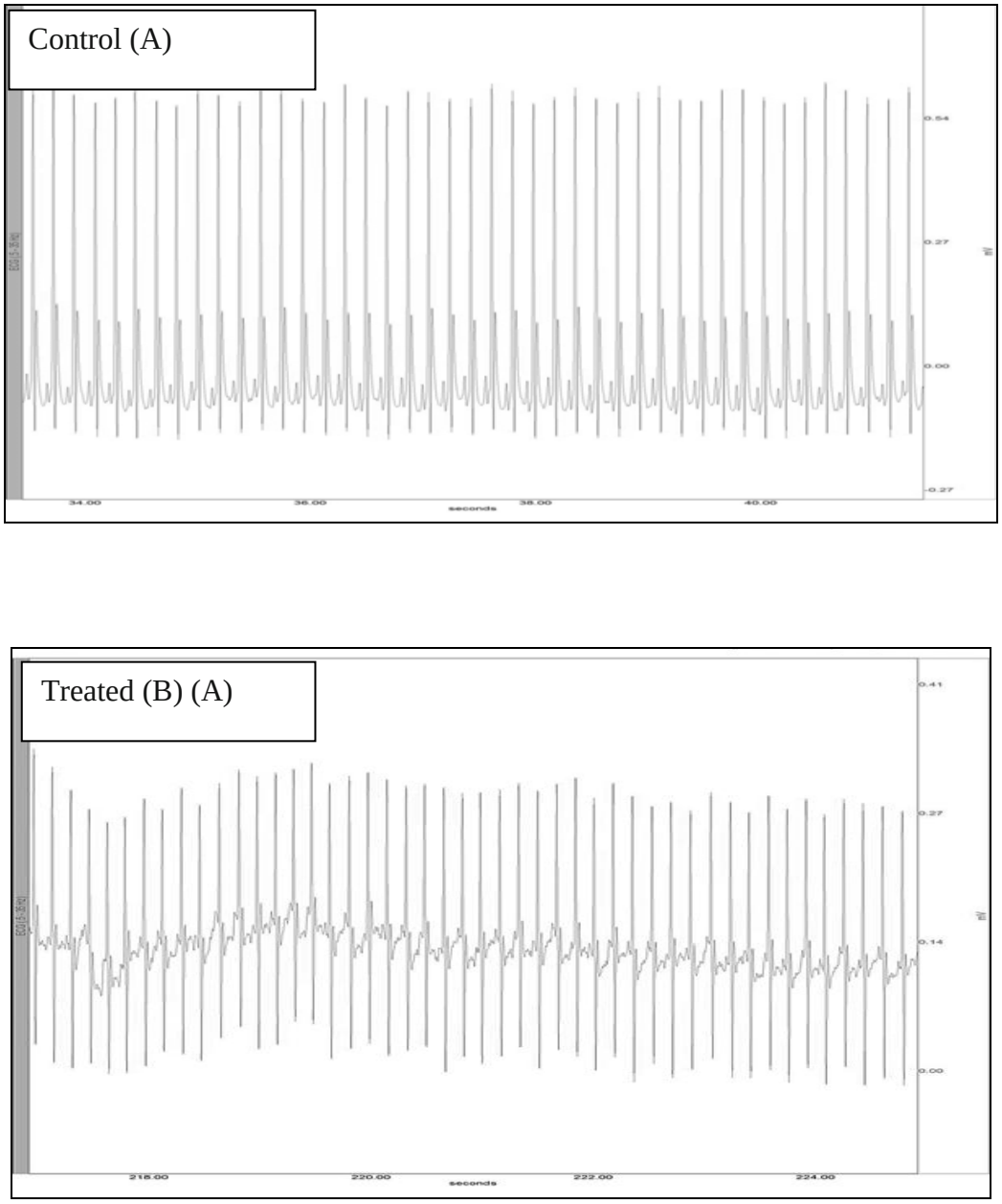

Figure 4.27: Effect of i.v. injection of Bacethrombase on ECG of rats

(A) control and (B) Bacethrombase-treated (10 mg/kg) rats after 14 days of treatment.

4.2.2.13.5.2 Effect of Bacethrombase on hematological parameters in rats

Bacethrombase-treated rats did not show any change in hematological parameters as compared to control group of rats (Table 4.12D).

Table 4.12D: A comparison of hematological parameter in Bacethrombase treated and control rat after 14 days of i.v. injection (i.v.)

Parameters (Unit)		Value (Mean ± SD)	
		Control rat	Treated rat
Total WBC	(m/mm^3)	5.3 ± 0.1	5.2 ± 0.1
Lymphocytes	(%)	49.1 ± 1.5	49.2 ± 0.8
Monocytes	(%)	14.2 ± 1.1	14.7 ± 0.5
Neutrophils	(%)	45.2 ±0.4	44.8 ± 2.9
Eosinophils	(%)	4.8 ± 1.0	5.0 ± 1.2
Basophils	(%)	0.4 ± 0.2	0.4 ± 0.1
Total RBC	(m/mm^3)	6.8 ± 1.1	6.6 ± 0.3
MCV	fl	57.2 ± 0.1	56.8 ± 0.2
HCt	%	44.8 ± 0.9	45.3 ± 1.2
MCH	pg	18.9 ± 1.7	18.5 ± 0.5
MCHC	g/dl	32.3 ± 1.7	29.8 ± 6.2
RDW		10.7 ± 0.31	11.0± 0.3
He	g/dl	11.0 ± 4.4	11.4 ± 0.1
MPV	fl	7.4 ± 0.1	7.3 ± 0.1
PCt	%	3.2 ± 0.1	2.9 ± 0.4
PDW		7.9 ± 0.2	8.0 ± 0.1

Note: Values are mean ± SD of six experiments.

4.2.2.13.5.3 Effect of Bacethrombase on biochemical parameters of serum of treated rat

There was no significant difference ($p > 0.05$) in serum profiles of treated animals compared to control animals after 14 days of Bacethrombase treatment (Table 4.13).

Table 4.13: A comparison of some biochemical properties of serum of control and Bacethrombase-treated rats, after 14 days of i.v. injection at a dose of 10 mg/kg

Parameters (Unit)	Value (Mean ± SD)	
	Control rat	Treated rat
LDL (mg/dL)	12.8 ± 0.3	13.9 ± 0.6
HDL (mg/dL)	18.6 ± 0.2	18.2 ± 0.4
Glucose (mg/dL)	74.5 ± 1.1	74.3 ± 4.4
Urea (mg/ml)	51.8 ±1.6	54.2 ± 2.0
Total protein (g/dL)	6.2 ± 0.1	6.0 ± 0.2
Uric acid(mg/dL)	1.1 ± 0.1	1.1 ± 0.2
Triglyceride	53.2 ± 1.1	53.4 ± 1.7
Cholesterol (mg/dL)	46.8 ± 1.3	47.0 ± 1.4
Creatinine (mg/dL)	0.9 ± 0.1	1.0 ± 0.5
SGPT (U/L)	92.4 ± 2.0	92.3 ± 2.3
SGOT (U/L)	160.2 ± 4.0	160.8 ± 0.2

4.2.2.13.5.4 Evaluation of Histopathology

A representative pictogram of light microscopic histopathological examination of selected organs, such as liver, kidney, spleen, heart in control, of the tested groups revealed normal architecture without detrimental or morphological changes till 14 days after injection of Bacethrombase Figs. 4.28 (A-H).

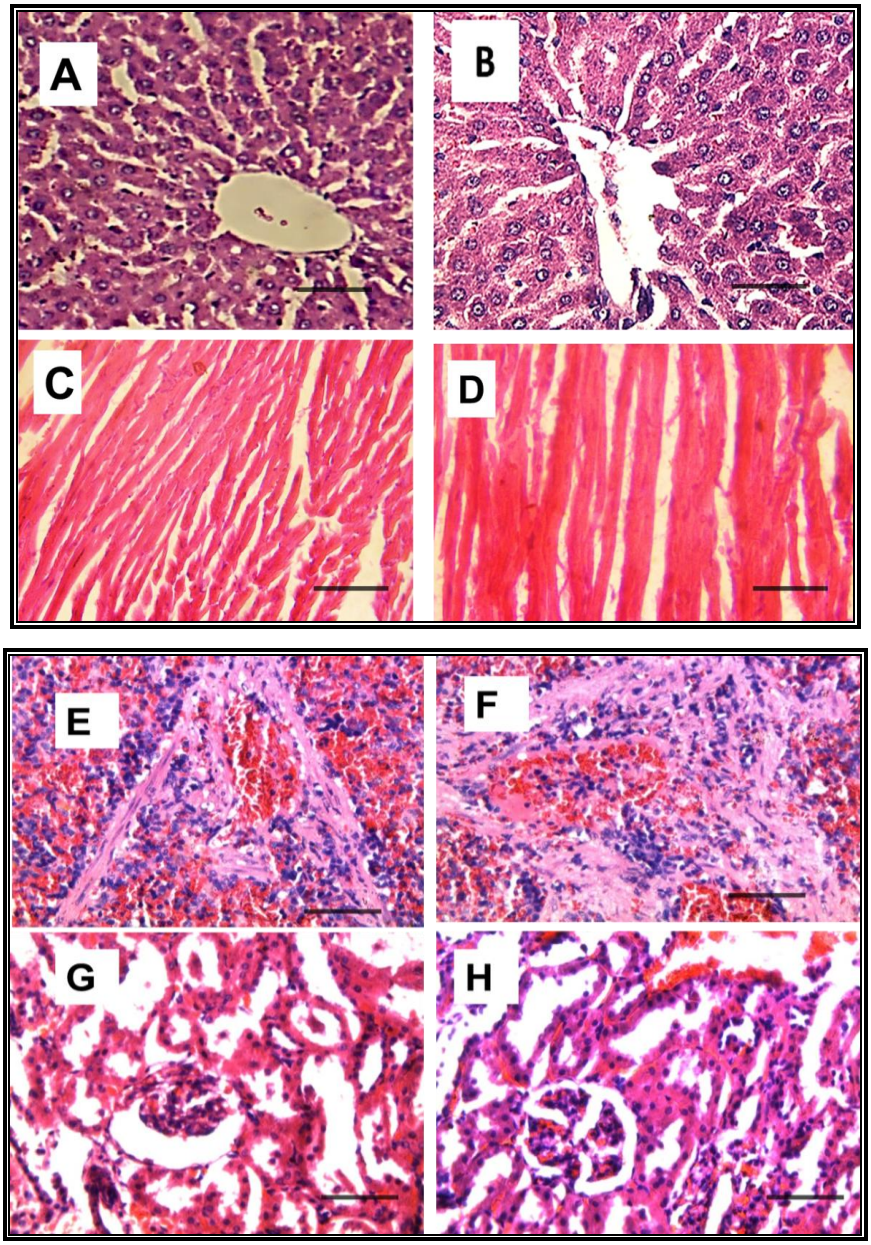

Figure 4.28: (A-H): Light microscopic observation of the effect of Bacethrombase (at a dose of 10 mg/kg) on different organs of treated rats

Both the treated and control groups of rats were sacrificed by euthanasia and spleen, heart, liver, and kidney were dissected out from the euthanized rats. The tissues were washed with 1X PBS to remove the adherent blood and cut into small pieces and placed in 10% formaldehyde. The fixed tissues were dehydrated in graded concentrations of alcohol and embedded in parafilm. The thin sections (5 μm) of the tissues after staining with hematoxylin eosin were observed under a light microscope. Tissues treated with PBS served as control. (A) Control liver tissues, (B) treated liver tissues, (C) control spleen tissues (D) treated spleen tissues, (E)

control heart tissues, (F) treated heart tissues, (G) control kidney tissues, (H) treated kidney tissues with X10 magnification, bar 50 µm.

4.2.2.13.5.5 *In vivo* thrombolytic potency of Bacethrombase

4.2.2.13.5.5.1 Thrombus induction in rat tail with optimized dose of carrageenan

A suitable dose of carrageenan at which thrombus could be formed was determined by i.v. injecting various doses of carrageenan and subsequently correlating with length of thrombus formation. At a carrageenan dose of 0.9 mg/kg or above, the length of thrombus formation remained steady as shown in Fig. 4.29. With increasing doses of carrageenan, the length of thrombus in the rat tail vein also increased up to 0.9 mg/kg. But further increase in the dose of carrageenan did not increase the length of the thrombus. The optimum dose of carrageenan for thrombus formation in the rat tail vein was found to be 0.9 mg/kg.

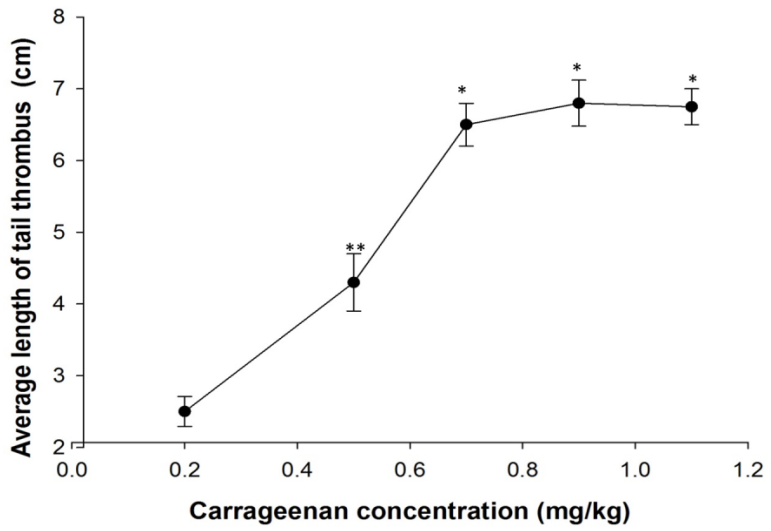

Figure 4.29: Determination of carrageenan-induced thrombus formation in rat tail

Various concentrations (0.2 mg/kg to 1.1 mg/kg) of carrageenan were administered intravenously into the tail vein. The data represent mean ± S.D of triplicate experiments (n=3). Significance of difference with respect to control group of rats, *p<0.001, **p<0.05.

4.2.2.13.5.5.2 *In vivo* thrombolytic potency of Bacethrombase

The mean length of the infarcted region of the tail significantly decreased with dose and time and nearly disappeared after 24 h of Bacethrombase administration as compared to control group of rats. The *in vivo* thrombolytic potencies of Bacethrombase, streptokinase, and plasmin are shown in Table 4.14. Streptokinase, plasmin and Bacethrombase demonstrated identical thrombus dissolving ability at the tested dose of 600 µg/kg (Fig. 4.30 (a-f)).

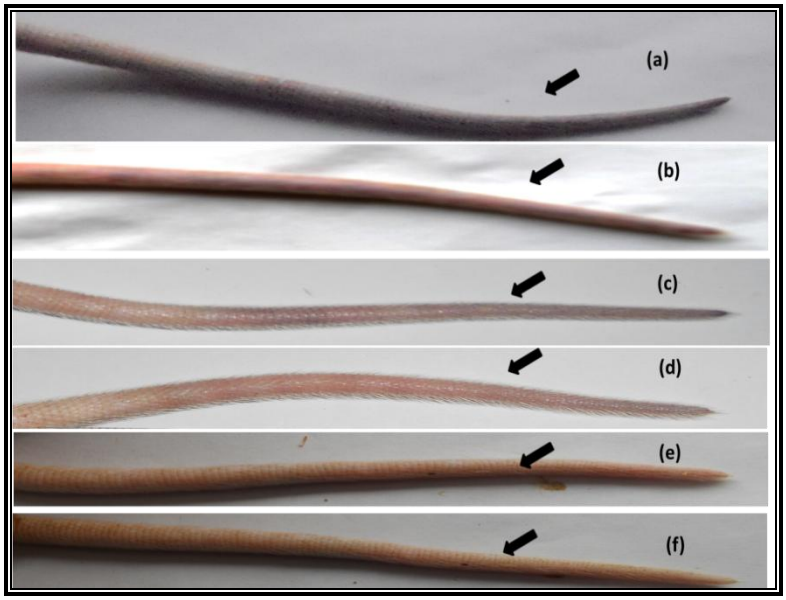

Figure 4.30: (a-f) Dose-dependent thrombolytic effect of Bacethrombase, streptokinase and plasmin

A comparison of thrombolytic potency among Bacethrombase, streptokinase and plasmin after 24 h of intravenous administration in Wistar strain rat (n=6). The arrows indicate thrombus formation area (wine color) in the tail of rats; (a) Group 1, carrageenan (0.9 mg/kg) dissolved in physiological saline; (b) Group 2, carrageenan(0.9 mg/kg) and 200 µg/kg of Bacethrombase; (c) Group 3, carrageenan (0.9 mg/kg) and 400 µg/kg of Bacethrombase; (d) Group 4, carrageenan (0.9 mg/kg) and 600 µg/kg of Bacethrombase; (e) Group 5, carrageenan (0.9 mg/kg) and 600 µg/kg of streptokinase; (f) Group 6, carrageenan (0.9 mg/kg) and 600 µg/kg of human plasmin.

The results of Fig. 4.30 (a-f) have been analyzed in Table 4.14 to compare the *in vivo* thrombolytic potency of Bacethrombase, plasmin and streptokinase under identical experimental conditions.

Table 4.14: A comparison of *in vivo* thrombolytic activity of Bacethrombase, plasmin and streptokinase after 24 h of treatment on Wistar strain rat

Thrombolytic agent	Dose (µg/kg)	Dissolution of thrombus (%)	Length of rat tail thrombus (cm)
Control (1X PBS)	0	0	6.8 ± 0.3
Bacethrombase	200	41.7 ± 0.3[a]	4.5 ± 0.2 [a]
Bacethrombase	400	62.7 ± 5.1[a]	2.1 ± 0.1 [a]
Bacethrombase	600	98.1 ± 0.1	0.1 ± 0.1
Streptokinase	600	98.2 ± 0.1	0.1± 0.1
Plasmin	600	98.5 ± 0.1	0.1 ± 0.1

Note: Thrombus was induced in rat tail by injection of 0.9 mg/kg dose of carrageenan. Values are mean ± S.D. of triplicate determinations. Significance of difference is with respect to blood clot lysis activity of Bacethrombase at a dose of 600 µg/kg, $^{a}p < 0.001$.

4.2.2.14 Anticoagulant potency of Bacethrombase *in vivo* condition

4.2.2.14.1 Dose-dependent activated partial thromboplastin time (APTT) and prothrombin time (PT) of Bacethrombase

In a dose-dependent manner, Bacethrombase-treated rat plasma showed prolongation of activated partial thromboplastin time (Fig. 4.31) and prothrombin time (Fig. 4.31) as compared to control rats' plasma.

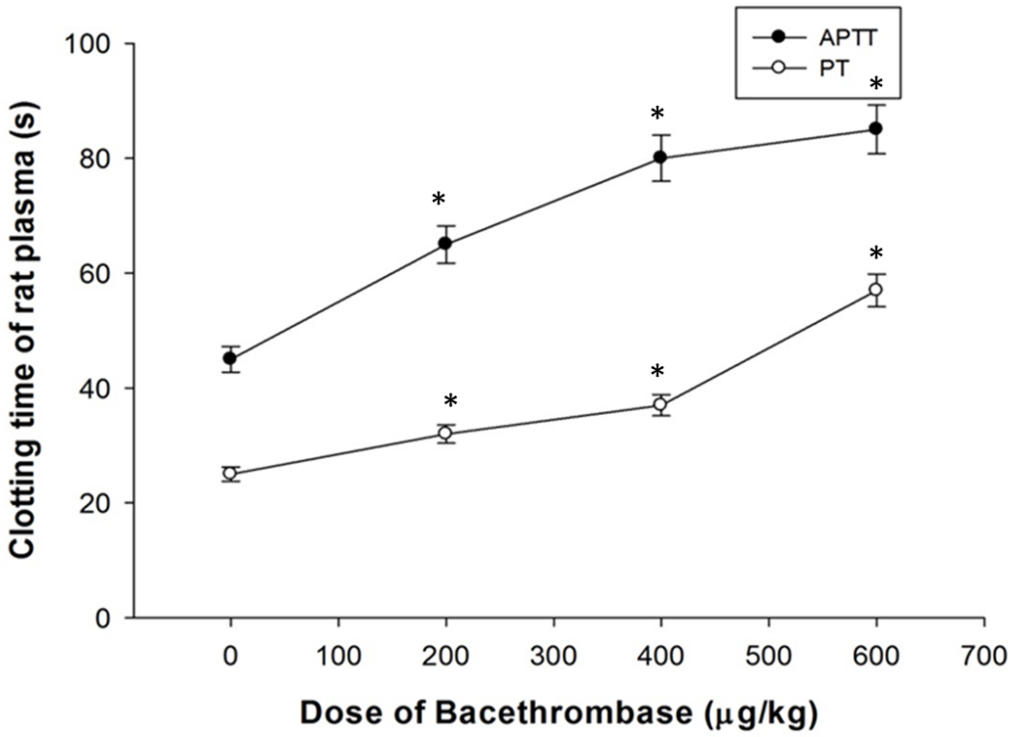

Figure 4.31: Effect of different concentrations of Bacethrombase on activated thromboplastin time (APTT) and prothrombin time (PT) of PPP

The clotting time of rat PPP 2 h was estimated after administration of Bacethrombase. Different doses (200 µg/kg to 600 µg/kg) of Bacethrombase were administered intravenously to measure APTT and PT. The experiments were done as stated in section 3.2.16.3.1 & 3.2.16.3.2, respectively The data represent mean ± S.D of triplicate experiments.

4.2.2.14.2 *In vivo* effect of Bacethrombase on thrombin time (TT) of rat plasma

After 2 h of treatment, it was observed that Bacethrombase prolonged the TT of PPP in a dose-dependent manner in the treated groups of rats, while not in the control groups (Fig. 4.32).

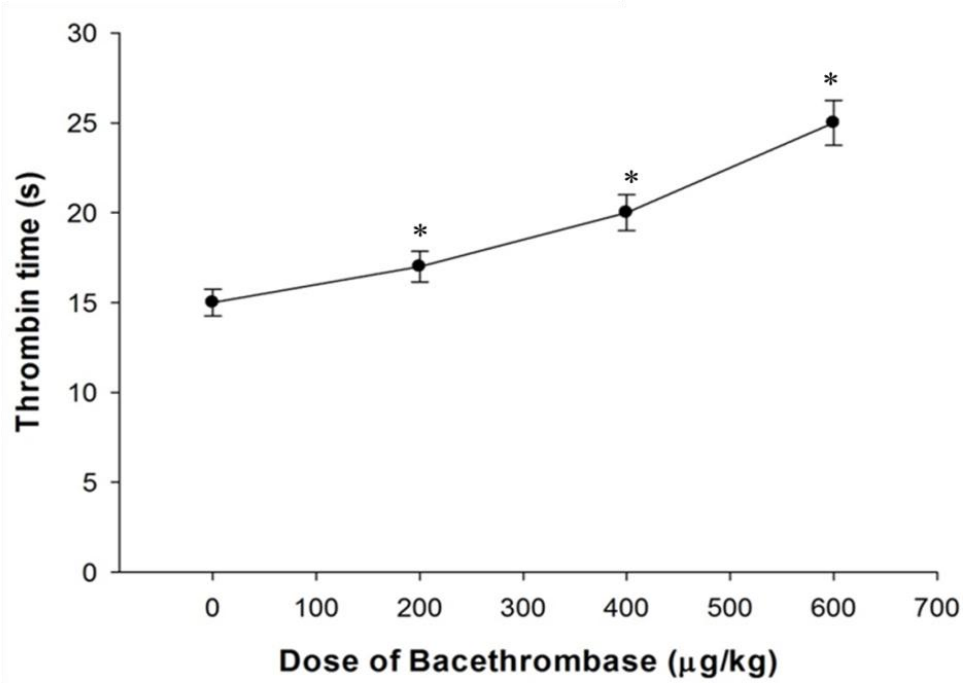

Figure 4.32: Assessment of thrombin time (TT) at various dosages of Bacethrombase

After 2 h i.v. administration of different doses of Bacethrombase (200 µg/kg to 600 µg/kg) in rats, the blood was withdrawn and the thrombin time (TT) was measured against citrated platelet poor plasma. For experimental details in section no 3.2.16.3.3. The data represent mean ± S.D of triplicate experiments.

4.2.2.14.3 *In vivo* dose-dependent fibrin(ogen)olytic activity of Bacethrombase

The significant reduction in fibrinogen level of Bacethrombase-treated rat plasma as compared to control rat plasma suggests that Bacethrombase also degrades fibrinogen in *in vivo* conditions (Fig. 4.33).

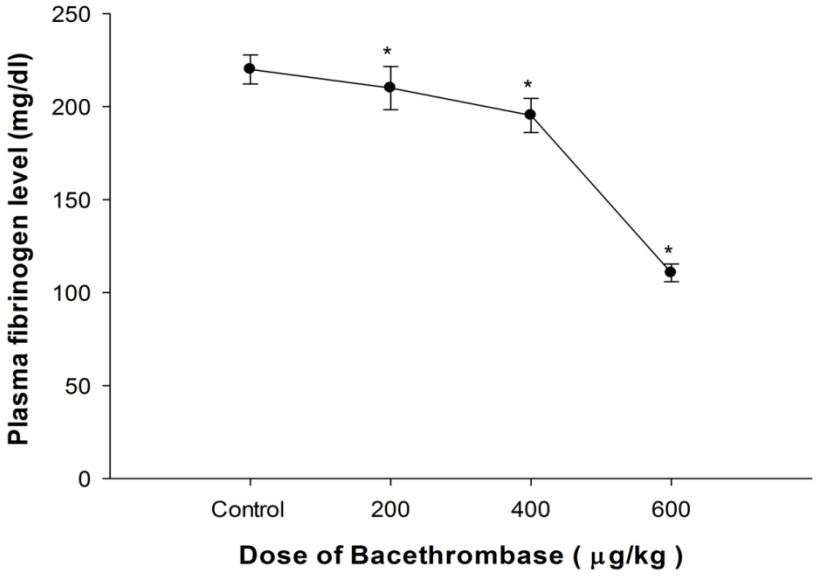

Figure 4.33: The *in vivo* dose-dependent defibrinogenating activity of Bacethrombase

The fibrinogen level in the treated and control group of rat plasma was determined by using commercial diagnostic kits FIBRI-PREST (section 3.2.16.3.4). Each datum represents mean ± S.D of triplicate experiments. Significance of difference is with respect to control group of rats ($p < 0.05$).

4.2.2.14.4 *In vivo* anticoagulant activity of Bacethrombase

Bacethrombase increased Ca^{2+} clotting of PPP in a concentration-dependent manner (Fig. 4.34).

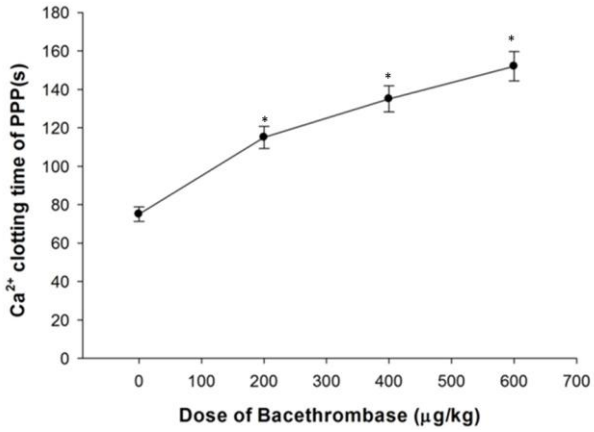

Figure 4.34: Dose dependent *in vivo* anticoagulant action of Bacethrombase

Determination of re-calcification time of PPP from control and Bacethrombase-treated groups of rats. The experiment was done as described in the Materials and Methods section 3.2.16.3.5. Data represent mean ± SD of triplicate determinations. Significance of difference with respect to control group of rats *$p < 0.001$.

4.3 Discussion

Proteolytic enzymes that contain serine in their catalytic site are ubiquitously distributed in nature and perform important biological functions for the producing species. Studies have suggested different evolutionary origins for the serine peptidases; nevertheless, most of them have a common proteolytic mechanism [145]. The present study is the first report on characterization and *in vitro* toxicity assessment of a plasmin-like, fibrin(ogen)olytic enzyme showing strong anticoagulant activity purified from *Bacillus cereus* strain FF01. Notably, many fibrin(ogen)olytic enzymes have been reported from fungus [146] and rhizopus [78]; nevertheless, except for one instance of a bacterial metalloprotease [94], a fibrin(ogen)olytic serine protease of bacterial origin, is yet to be demonstrated. It has already been reported that *Bacillus cereus*, a ubiquitous, spore-forming bacterium is accountable for approximately 2% of food-borne illnesses, and the characteristic symptoms of infection are severe nausea, vomiting and diarrhea [147]. In order to sustain its growth and to obtain nutrients from a particular environment, *B. cereus* may produce a variety of hydrolytic enzymes such as proteases [148]. However, unlike toxins and enzymes from venomous animals [107], some of these bacterial enzymes may not be harmful, instead, may find novel therapeutic and/or diagnostic applications.

The molecular mass of Bacethrombase was found to be higher than few fibrin(ogen)olytic enzymes purified from *Rhizopus chinensis* 12 [78], *Aspergillus fungigatus* [146] as well as from snake venom [149]. Conversely, the molecular weight of Bacethrombase was lower than Brevithrombolase, a fibrin(ogen)olytic enzyme purified from *Brevibacillus brevis* strain FF02B [89]. However, the molecular mass of Katsuwokinase (38 kDa) [55] and Bacethrombase was found to be the same.

The typical secondary structure of Bacethrombase shows similarity with Polyproline–II type model [150] and also closely resembles the secondary structure of a fibrin(ogen)olytic enzyme purified from fermented shrimp paste [23] as well as

Brevithrombolase [89]. The unique peptide mass fingerprinting data and protein identification based on amino acid composition analysis indicates that Bacethrombase is a uncharacterized new fibrin(ogen)olytic bacterial serine protease, belonging to the MEROPS peptidase family S8 (subtilisin family, clan SB). Further, higher fibrinolytic/caseinolytic activity (F/C) ratio of Bacethrombase can be compared to the similar property displayed by purified fibrin(ogen)olytic or fibrinolytic enzymes like subtilsin BPN [90], CK [90] and Bafibrinase [41] indicating the higher fibrin or fibrinogen degradation efficiency of the former enzyme. Furthermore, lower *Km* value of Bacethrombase compared to *Km* value (0.52 mM) of nattokinase towards the chromogenic substrate for plasmin (V0882**)** also suggests its higher potency to degrade the thrombus. Similar to Bafibrinase [41], Bacethrombase did not show any proteolytic effect on extracellular membrane proteins, viz., laminin and fibronectin degradation, which led us to anticipate that it was devoid of hemorrhagic activity, therefore, it may overcome the limitations of many of the currently available thrombolytic drugs [151].

Notably, examples of bacterial fibrinolytic enzymes possessing N-linked and/or O-linked oligosaccharides or sialic acid moieties are very rare. Narasaki et al. [39] have reported the role of N-linked glycosylation on Bacillolysin MA, a bacterial metalloproteinase that produces angiostatin-like fragments from plasminogen. Interestingly, Bacethrombase is the first example of a fibrin(ogen)olytic serine protease that contains sialic acid residues and does not possess N-linked carbohydrates residues. Studies have shown that without denaturation, removal of sialic acid moieties from Bacethrombase was impossible, owing to which the role of sialic acid moieties on functioning of Bacethrombase could not be determined. Noteworthy, fragmentary data have been presented to show the role of sialic acid on biological activity or stability of protein molecules [152].

Inhibition study with group specific reagents or metal chelator has been used to identify the nature of active site in an enzyme and its cofactor requirements during the proteolysis [41, 107, 153]. The significant inhibition of protease activity by serine protease inhibitors indicates the presence of serine, cysteine and histidine amino acid residues in the active site of the Bacethrombase and confirms it as a serine protease. Further, failure to inhibit the protease activity of Bacethrombase by EDTA, strongly indicates that, like Bafibrinase [41], Bacethrombase is not a metalloprotease.

Furthermore, inhibition study with DTT also suggests the absence of intramolecular and intermolecular disulfide linkage(s) in Bacethrombase.

Based on preferred degradation of Aα-chain followed by Bβ-chain of fibrinogen/fibrin molecule, Bacethrombase may be classified as an α,β-fibrinogenase [41]. During the last decade several fibrin(ogen)olytic enzymes have been purified from various natural resources and their biochemical and pharmacological properties have been well characterized [39, 78, 146]. Nevertheless, anticoagulant mechanism of most of the above enzymes has not been investigated in detail. Usually, patients who develop atrial fibrillation may require anticoagulant (known as blood thinner) therapy to prevent the risk of clot formation, which otherwise may initiate cardiovascular diseases [154]. Anticoagulant process is a highly interwoven array of multiple processes. It is either dependent on inhibition of platelets aggregation or intervention of the coagulation cascade. The anticoagulant mechanism of Bacethrombase differs from that shown by Brevithrombolase, a fibrin(ogen)olytic serine protease previously isolated in our laboratory [89], as well as commercial anticoagulant drugs such as warfarin and heparin/AT-III [155, 156]. Our study suggests that the anticoagulant mechanism of Bacethrombase does not depend on non-enzymatic inhibition and/or proteolytic degradation of FXa, prothrombin or thrombin; nevertheless, it is correlated with binding of Bacethrombase to plasma fibrinogen (which is evidenced by spectrofluorometric analysis) to form an enzyme-substrate complex followed by degradation of fibrinogen (substrate) in a dose-dependent as well as time-dependent manner. Moreover, antiplatelet effect of Bacethrombase also contributes in preventing the blood coagulation [33]. These effects lead to progressive incoagulable blood. Further, the fibrin(ogen)olytic activity of Bacethrombase does not decrease at a higher concentration of fibrinogen (>12.0 μM) indicating its therapeutic application to ameliorate hyperfibrinogemia-associated disorders. Besides, Bacethrombase lacks *in vitro* cytotoxicity and hemolytic activity, in addition to having higher *in vitro* thrombolytic potency and fibrinolytic activity than plasmin at physiological conditions (pH 7.4 and 37 °C). This reinforces the therapeutic application of Bacethrombase as a new thrombolytic drug.

In addition to playing a central role in thrombogenesis and in the progression of atherosclerotic lesions, activated platelets are associated with many other diseases

such as diabetes, primary pulmonary hypertension, and cystic fibrosis [157]. A great number of agonists, for example ADP and collagen, bind with purinergic P2Y1 and P2cyc, and GPVI receptors on platelet surface to induce the platelet aggregation [158, 159]. Antiplatelet drugs by virtue of their ability to impede one or more pathways implied in the process of platelet aggregation can modulate the platelet aggregation [157]. However, it has been demonstrated that antiplatelet activity of aspirin, a most popular platelet inhibiting drug, progressively decreases in long-term treated patients [33] demanding the search for new antiplatelet agents. Nattokinase showed antiplatelet property by significant inhibition of collagen and thrombin induced platelet aggregation [117]. To the best of our knowledge, Bacethrombase is the first example of a bacterial fibrin(ogen)olytic enzyme showing potent antiplatelet activity. Our study suggests that Bacethrombase may not bind with collagen GPVI receptor on platelet aggregation. Instead, similar to ADP receptor antagonistic lopidine or clopidogrel, Bacethrombase dose-dependently interferes the binding of ADP to its receptors that leads to inhibition of platelet aggregation [34]. Identification of precise mechanism of inhibition of platelet aggregation by Bacethrombase is our next goal of study.

Intravenous injection of Bacethrombase at a dose of 10 mg/kg, which is almost 5 to 10 times higher than clinical doses of streptokinase and plasmin [89] , did not show any difference in hematological and serum biochemical properties over the control group of rats. Moreover, the histopathological examination of selected organs did not show any sign of toxicity or lethality on Bacethrombase-treated rats. This finding fairly indicates the safety of Bacethrombase for its clinical use in the treatment of cardiovascular diseases [41, 89].

Moreover, the *in vivo* clot-dissolving property of Bacethrombase indicates its thrombolytic potential. Notably, at 600 µg/kg dose, the *in vivo* thrombus dissolving efficacy of Bacethrombase surpassed the same activity displayed by nattokinase [117] and fibrinogenase [160]; thereby, suggesting the superiority of the former thrombolytic enzyme. All these findings clearly indicate that Bacethrombase may offer an attractive potential as a safe thrombolytic agent for clinical uses in hyperfibrinogemia and thrombolytic-associated disorders.