CHAPTER V

Results & Discussion

CHAPTER 5
RESULTS AND DISCUSSION

Characterization of a fibrinolytic enzyme (Brevithrombolase) purified from *Brevibacillus brevis* strain FF02B and assessment of its *in vivo* toxicity as well as *in vivo* thrombolytic activity.

5.1 Brief introduction

An ideal thrombolytic drug should have reasonable cost, no antigenicity, does not affect blood pressure, anticoagulant nature, and low incidence of intracranial and system bleeding [27]. Several microbial fibrinolytic serine proteases have been shown to meet the above criteria. Isolation and characterization of a many fibrinolytic enzymes have already been reported from variety of traditional fermented food samples such as Japanese natto, Korean chungkook Jang soy sauce, edible honey mushroom, Chinese douchi and soyabean shrimp paste [20, 31, 144]. It is to be noted that (a) traditional fermented food samples are excellent sources of fibrinolytic enzyme producing bacteria [16], and (b) such enzyme producing bacteria from fermented food samples of NE India (which is considered as one of the mega biodiversity zones of the world) has never been isolated. Therefore, in this study, an attempt has been made to screen fibrinolytic enzyme producing potential bacteria from various fermented food samples obtained from different regions of north-east India.

It is noteworthy to mention that because of several biological advantages of using microbes isolated from food sources for the production of fibrinolytic enzymes, they may be preferred over the others in the pharmaceutical sector. During the last decade, many direct-acting fibrinolytic enzymes have been purified from microbial resources and their biochemically properties are well characterized [6, 78, 99]. However, there has been little effort to investigate their *in vivo* toxicity, pharmacological properties, and a comparison of their therapeutic (thrombolytic and anticoagulant) potencies with the commercial anticoagulant and thrombolytic drugs. Furthermore, the molecular mechanism of anticoagulant action of most of such enzymes has not been studied in sufficient detail. Therefore, in the present study, an effort has been made to characterize the biochemical and pharmacological

properties, identification of mechanism of anticoagulant action, determination of *in vivo* toxicity, and an assessment of *in vivo* thrombolytic potency of a direct-acting, new fibrinolytic serine protease (Brevithrombolase) purified from *Brevibacillus brevis* strain FF02B, which was isolated from alcohol producing rice beer starter culture (a traditional fermented food) of North-East India. The fibrin-dissolving as well as unique anticoagulant mechanism of Brevithrombolase, as demonstrated in the present study, supports its viability in the development of a superior thrombolytic drug to ameliorate or prevent disseminated intravascular coagulopathy (DIC).

5.2 Results

5.2.1 Screening and isolation of fibrinolytic enzyme producing bacteria:

At preliminary stage of screening, the promising bacterial colonies in which visible zones of hydrolysis were observed on fibrin agar medium were chosen (Table.5.1) and pure culture were obtained. Out of five fermented food samples total nine bacterial strains were isolated, but among them only six isolated bacterial strains showed zone of hydrolysis on fibrin agar medium ≥ 25 mm (Table 5.1). The highest zone of hydrolysis (~34.54 mm) on fibrin agar medium was produced by FF02B strain (Fig. 1) originated from a rice beer producing starter culture.

Figure 5.1: Zone of hydrolysis around the FF02B bacterial colonies on fibrin plate agar medium.

The bacteria was 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 plates at pH 7.4 containing fluconazole. The plates were incubated at 37 °C for

48 h to produce zone of hydrolysis by bacterial strain FF02B as per the experimental procedure described in materials and methods section 3.2.2.

Table 5.1: Screening for fibrinolytic enzyme producing bacteria from different samples of North-East India.

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

SL No	Sample code	Zone of hydrolysis(mm)
01	FF01	29.20
02	FF02A	32.55
03	FF02B	34.54
04	FF03	31.75
05	SF01	13.97
06	SF02	10.16

Among the selected bacteria, the cell-free culture supernatant of the strain FF02B displayed the highest fibrinolytic/caseinolytic ratio and *in vitro* thrombolytic activity, high substrate specificity towards fibrin as shown in (Table 5.2). On the basis of the obtained results, this bacterium was selected for future investigation.

Table 5.2: Identification of fibrinolytic enzyme producing potential bacteria based on fibrinolytic / caseinolytic ratio, substrate specificity and thrombolytic activity.

Name of strains	Fibrinolytic activity*(U/mg)	Caseinolytic activity*(U/mg)	Albuminolytic assay* (U/mg)	Globulinolytic assay* (U/mg)	F/C	Thrombolytic activity(mg of blood clot lysis/µgof enzyme)
FF01	187.3	1.2	80.1	76.2	156	20.2
FF02A	139.6	1.6	98	7.3	87.3	10.2
FF02B	171.4	0.8	70	7.3	193	22.4
FF03	72.2	12.8	60	5	5.6	2.3
SF01	105.9	1.9	40.4	131.7	55.1	6.8
SF02	117.8	7	18.6	94	16.8	7.2

*Unit of protease activity has been defined as µg of tyrosine liberated per min per ml of enzyme at 37 °C, pH 7.4.

All the experiments were performed in triplicates as per described protocol in materials and methods sections 3.2.2.3, 3.2.2.4 and 3.2.2.5, respectively. Average of triplicate results are shown.

5.2.2 Taxonomic identification of fibrinolytic enzyme producing strain FF02B

The results of taxonomic identification by polyphasic approach are shown below.

5.2.2.1 Biochemical and morphological study of fibrinolytic enzyme producing bacterial isolate

The results of the biochemical and morphological tests (Fig 5.2) of the selected bacterial isolate are shown in Table 5.3. Bacterial morphology and the results of the biochemical tests suggested that FF02B belong to the genus *Bacillus* (Bergey's manual of systematic bacteriology, 1999, 2001, 2005).

Table 5.3: Biochemical and morphological tests of bacterial strain FF02B. Experiments were repeated in triplicates to ensure the reproducibility.

Characteristics	FF02B bacterial strain
Morphology	Rod shaped, motile, Gram negative, border circular, White color, in chain
Spore	No endospore
Growth in Agar	Abundant, white colour
Growth in Broth	Good growth, with sediment
pH	4.0-12.0, optimum at pH 11.0
Temperature	Growth range 30-60 °C, optimum at 37 °C
Biochemical fingerprints	
Catalase	Positive
Voges-Proskauer Test	Negative
Methyl Red Test	Negative
Acid from	
D- Glucose	Negative
Sucrose	Positive
Lactose	Negative
D- Mannitol	Negative
Gas from Glucose	Positive
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	Negative
Lipid hydrolysis	Negative
Triple sugar Iron agar test	Positive
Litmus milk test	Positive

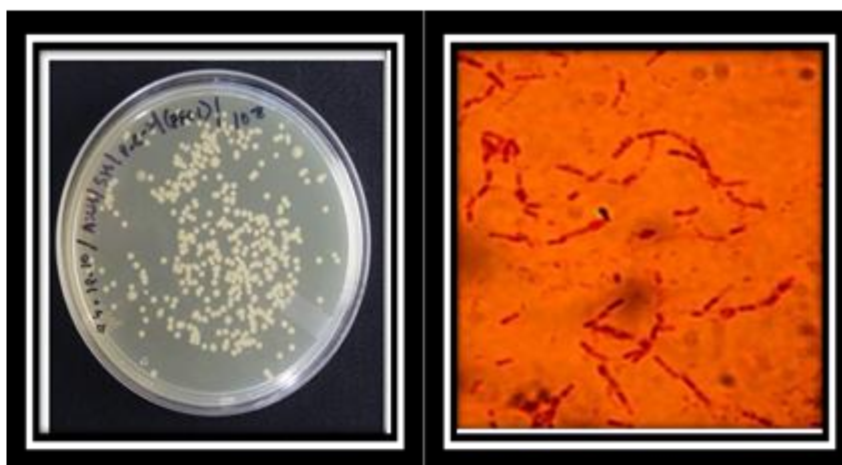


Figure 5.2: Pure culture of colony morphology, Gram staining of bacterial strain FF02B.

5.2.2.2 Phylogenetic analysis of isolated strain FF02B

A homologous search result of strain FF02B demonstrated that 95% to 100% similarity of 16S-rDNA sequence (accession no. KF913005) was observed with other species of the genus *Brevibacillus* as shown in Table 5.4. The phylogenetic tree constructed from the sequence data by the neighbor-joining method showed that *Brevibacillus brevis* (accession no. AB681205.1) showing 95% 16S rDNA sequence identity represented the closest phylogenetic neighbor of the strain FF02B (Fig.5.3).

Table 5.4: Homologous search results of 16S-rDNA partial sequence of strain FF02B using Basic Local Alignment Tool (BLAST) tool from National Centre Biotechnology Information (NCBI).

Sl No.	Accession	Description	Query coverage	Maximum Identity
1	KF913005.1	*Brevibacillus brevis* strain FF02B 16S ribosomal RNA gene, partial sequence	100%	100%
2	AB681205.1	*Brevibacillus brevis* gene for 16S rRNA, partial sequence, strain: NBRC 100599	100%	100%
3	KC873888.1	Uncultured bacterium clone S67_012 16S ribosomal RNA gene, partial sequence	100%	100%
4	JX312638.1	*Brevibacillus agri* strain M73 16S ribosomal RNA gene, partial sequence	100%	100%
5	JX281700.1	*Brevibacillus sp.* NAST12 16S ribosomal RNA gene, partial sequence	100%	100%
6	JF734329.1	*Brevibacillus sp.* DB-3 16S ribosomal RNA gene, partial sequence	100%	100%
7	JF734313.1	*Brevibacillus sp.* HL-2 16S ribosomal RNA gene, partial sequence	100%	100%
8	HQ222834.1	*Brevibacillus agr*i strain FA2 16S ribosomal RNA gene, partial sequence	100%	100%
9	GU320705.1	*Brevibacillus sp.* HKRDNA 16S ribosomal	100%	100%

Sl No.	Accession	Description	Query coverage	Maximum Identity
		RNA gene, partial sequence		
10	KF817656.1	*Brevibacillus limnophilus* strain AG-42 16S ribosomal RNA gene, partial sequence	99%	100%
11	KF817652.1	*Brevibacillus limnophilus* strain AG-38 16S ribosomal RNA gene, partial sequence	99%	100%
12	HM329234.1	Uncultured bacterium clone ncd957e10c1 16S ribosomal RNA ge13ne, partial sequence	99%	100%
13	NR_024822.1	*Brevibacillus limnophilus* strain DSM 6472 16S ribosomal RNA gene, partial sequence	99%	100%
14	HE993879.1	Brevibacillus agri partial 16S rRNA gene, strain Hambi 3346, isolate PMW-17	100%	99%
15	NR_113767.1	*Brevibacillus agri* strain NBRC 15538 16S ribosomal RNA gene, partial sequence	100%	99%
16	AB680888.1	*Brevibacillus agri* gene for 16S rRNA, partial sequence, strain: NBRC 15519	100%	99%
17	KJ735916.1	*Brevibacillus brevis* strain N-421 16S ribosomal RNA gene, partial sequence	100%	99%
18	KJ735912.1	*Brevibacillus brevis* strain N-134 16S ribosomal RNA gene, partial sequence	100%	99%
19	KJ735911.1	*Brevibacillus brevis* strain N-133 16S ribosomal RNA gene, partial sequence	100%	99%
20	JX170207.1	*Brevibacillus agri* strain DH-1 16S ribosomal RNA gene, partial sequence	100%	99%
21	AB971123.1	*Bacillus sp*. NAIST20-7 gene for 16S ribosomal RNA, partial sequence	100%	99%
22	AB971122.1	*Bacillus sp*. NAIST20-2 gene for 16S ribosomal RNA, partial sequence	100%	99%
23	KJ794156.1	*Brevibacillus sp*. BAB-4179 16S ribosomal RNA gene, partial sequence	100%	99%
24	KF101004.1	Uncultured bacterium clone ncd2352g03c1 16S ribosomal RNA gene, partial sequence	100%	99%
25	KF100881.1	Uncultured bacterium clone ncd2351h07c1 16S ribosomal RNA gene, partial sequence	100%	99%
26	AB934971.1	*Brevibacillus brevis* gene for 16S ribosomal RNA, partial sequence	100%	99%
27	HG942103.1	*Brevibacillus sp.* TA_FJ partial 16S rRNA gene, strain TA_FJ	100%	99%
28	KJ482849.1	*Brevibacillus sp.* CV94May 16S ribosomal RNA gene, partial sequence	100%	99%
29	KJ191430.1	*Brevibacillus sp.* ZK3 16S ribosomal RNA gene, partial sequence	100%	99%
30	KJ207212.1	*Brevibacillus sp.* pan5 16S ribosomal RNA gene, complete sequence	100%	99%
31	KF957731.1	*Brevibacillus agri* strain RH01_05 16S ribosomal RNA gene, partial sequence	100%	99%
32	KF830996.1	*Brevibacillus brevis* strain RZ-17 16S ribosomal RNA gene, partial sequence	100%	99%
33	KF913646.1	*Brevibacillus sp.* BAB-99 16S ribosomal RNA gene, partial sequence	100%	99%
34	KF465343.1	Uncultured *Brevibacillus sp.* clone DVPSD3 16S ribosomal RNA gene, partial sequence	100%	99%
35	KF465342.1	Uncultured *Brevibacillus sp.* clone DVPSD2 16S ribosomal RNA gene, partial sequence	100%	99%
36	KF464016.1	Uncultured *Brevibacillus sp.* clone DVASD_J29 16S ribosomal RNA gene, partial sequence	100%	99%
37	KF464015.1	Uncultured *Brevibacillus sp.* clone DVASD_J28 16S ribosomal RNA gene, partial sequence	100%	99%
38	HE985226.1	*Brevibacillus brevis* partial 16S rRNA gene,	100%	99%

Sl No.	Accession	Description	Query coverage	Maximum Identity
		isolate INFU/LS_S1		
39	JX394220.1	*Brevibacillus brevis* strain GY2 16S ribosomal RNA gene, partial sequence	100%	99%
40	KF707493.1	*Brevibacillus agri* 16S ribosomal RNA gene, partial sequence	100%	99%
41	KF641808.1	*Brevibacillus agri* strain Y17 16S ribosomal RNA gene, partial sequence	100%	99%

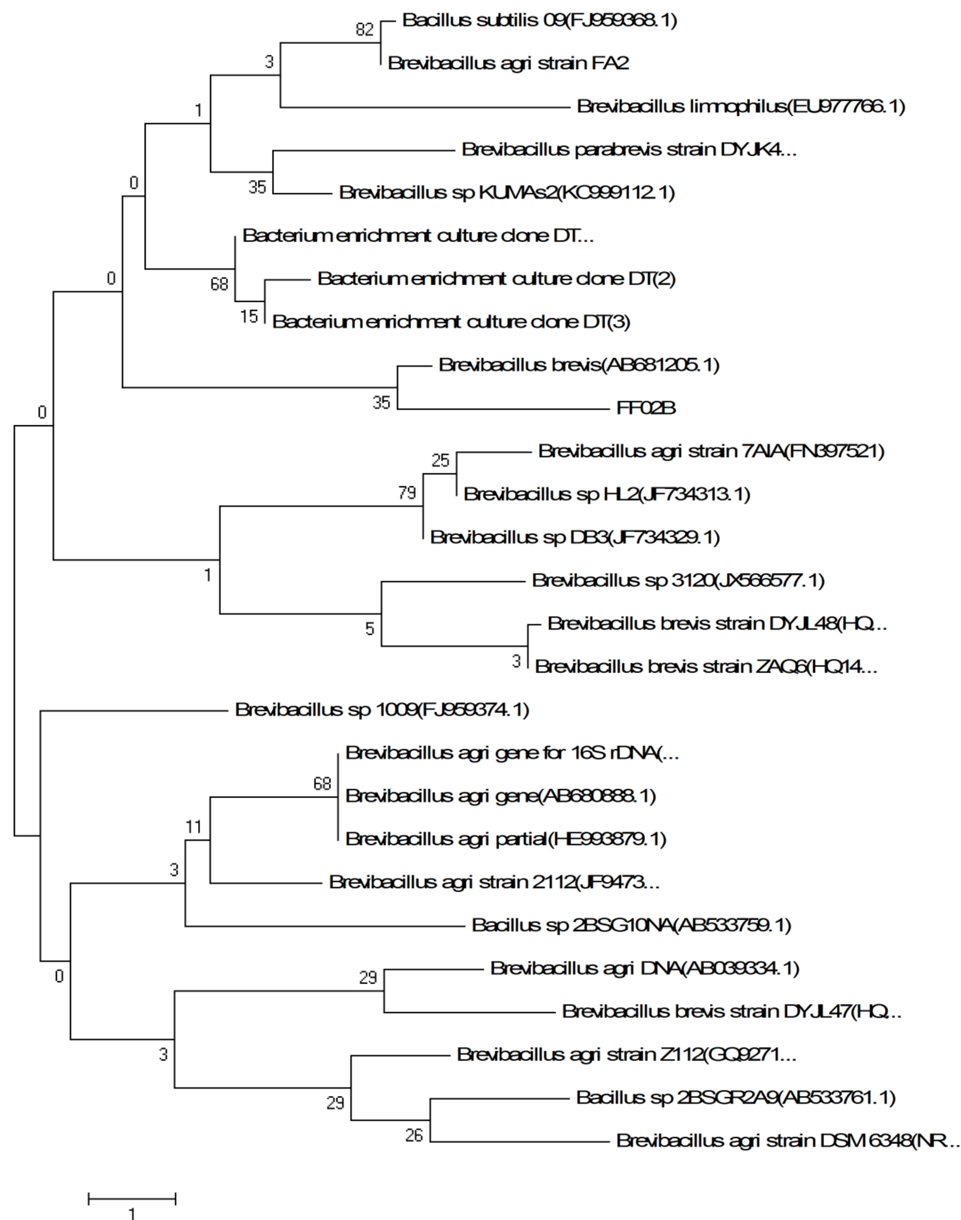


Figure 5.3: Phylogenetic relationships of strain FF02B and other closely related Brevibacillus species based on partial 16S rDNA sequences.

The tree was generated by the neighbor-joining method from the NCBI database (http://blast.ncbi.nlm.nih.gov) using the multiple sequence alignment program of MEGA4 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.

Table 5.5: Homologous search results of 16S-23S lSR partial sequence of strain FF02B using Basic Local Alignment Tool (BLAST) tool from National Centre Biotechnology Information (NCBI).

SL No	Accession	Description	Query coverage	Maximum Identity
1	KF913006.1	*Brevibacillus brevis* strain FF02B 16S ribosomal RNA gene, partial sequence; 16S-23S ribosomal RNA intergenic spacer, complete sequence; and 23S ribosomal RNA gene, partial sequence	100%	100%
2	GQ228684.1	Uncultured *Brevibacillus sp*. clone 3TH4 16S ribosomal RNA gene, partial sequence; 16S-23S ribosomal RNA intergenic spacer, complete sequence; and 23S ribosomal RNA gene, partial sequence	98%	99%
3	AP008955.1	*Brevibacillus brevis* NBRC 100599 DNA, complete genome	98%	90%
4	AF478096.1	*Brevibacillus formosus* clone KI-3F 16S ribosomal RNA gene, partial sequence; 16S-23S internal transcribed spacer, complete sequence; and 23S ribosomal RNA gene, partial sequence	72%	89%
5	NR_103266.1	*Brevibacillus brevis* NBRC 100599 strain NBRC 100599 5S ribosomal RNA, complete sequence	29%	97%
6	X02028.1	*Bacillus brevis* 5S ribosomal RNA	29%	95%
7	M33889.1	*Flexibacter sp.* 5S ribosomal RNA	28%	95%
8	AF478095.1	*Brevibacillus choshinensis* clone Bchosh-1R 16S ribosomal RNA gene, and 16S-23S internal transcribed spacer, partial sequence	24%	91%
9	GU260698.1	Uncultured bacterium 293 genomic sequence	27%	86%

SL No	Accession	Description	Query coverage	Maximum Identity
10	AF478097.1	*Brevibacillus parabrevis* clone KB-1F 16S ribosomal RNA gene, partial sequence; 16S-23S internal transcribed spacer, complete sequence; tRNA-Ile and tRNA-Ala genes, complete sequence; and 23S ribosomal RNA gene, partial sequence	19%	94%
11	AF478094.1	*Brevibacillus brevis* clone JG-1F 16S ribosomal RNA gene, and 16S-23S internal transcribed spacer, partial sequence	16%	97%
12	JQ686656.1	*Bacillus subtilis* strain 8790 16S ribosomal RNA gene, partial sequence; 16S-23S ribosomal RNA intergenic spacer, complete sequence; and 23S ribosomal RNA gene, partial sequence	16%	96%
13	AY763503.1	*Paenibacillus lentimorbus* 16S ribosomal RNA gene, partial sequence; 16S-23S ribosomal RNA intergenic spacer, 5S ribosomal RNA, tRNA-Ile, and tRNA-Ala genes, complete sequence; and 23S ribosomal RNA gene, partial sequence	25%	86%
14	AF478091.1	*Brevibacillus* agri clone Bgala-1R 16S ribosomal RNA gene, and 16S-23S internal transcribed spacer, partial sequence	16%	94%
15	NR_075814.1	*Thermaerobacter marianensis* DSM 12885 strain DSM 12885 5S ribosomal RNA, complete sequence	16%	92%
16	CP002344.1	*Thermaerobacter marianensis* DSM 12885, complete genome	16%	92%
17	EU686608.1	Uncultured bacterium KM3-205-D9 genomic sequence	15%	92%
18	JN644764.1	*Entomoplasma ellychniae* strain ELCN-1 16S ribosomal RNA gene, partial sequence; 16S-23S ribosomal RNA intergenic spacer, complete sequence; and 23S ribosomal RNA gene, partial sequence	9%	100%
19	DQ439660.1	*Mesoplasma corruscae* strain ELCA-2 16S ribosomal RNA gene, partial sequence; 16S-23S ribosomal RNA intergenic spacer, complete sequence; and 23S ribosomal RNA gene, partial sequence	9%	100%
20	DQ439659.1	*Entomoplasma ellychniae* strain ELCN-1 16S ribosomal RNA gene, partial sequence; 16S-23S ribosomal RNA intergenic spacer, complete sequence; and 23S ribosomal RNA gene, partial sequence	9%	100%

SL No	Accession	Description	Query coverage	Maximum Identity
21	AP006627.1	*Bacillus clausii* KSM-K16 DNA, complete genome	23%	80%
22	AF478093.1	*Brevibacillus borstelensis* clone Bborst-1 16S ribosomal RNA gene and 16S-23S internal transcribed spacer, partial sequence; and tRNA-Ile and tRNA-Ala genes, complete sequence	8%	100%
23	AF478092.1	*Brevibacillus borstelensis* clone EE-4_F 16S ribosomal RNA gene and 16S-23S internal transcribed spacer, partial sequence; and tRNA-Ile and tRNA-Ala genes, complete sequence	8%	100%

5.2.2.3 Phylogenetic analysis of isolated strain FF02B

A homologous search result of strain FF02B demonstrated that 100% similarity of 16S-23S lSR sequence (accession no.KF913006) was observed with other species of the genus *Brevibacillus* as shown in Table 5.3. The phylogenetic tree constructed from the sequence data by the neighbour-joining method showed that *Brevibacillus brevis* (accession no AP008955.1) showing 100% 16S-23S lSR sequence identity represented the closest phylogenetic neighbour of the strain FF02B (Fig.5.4).

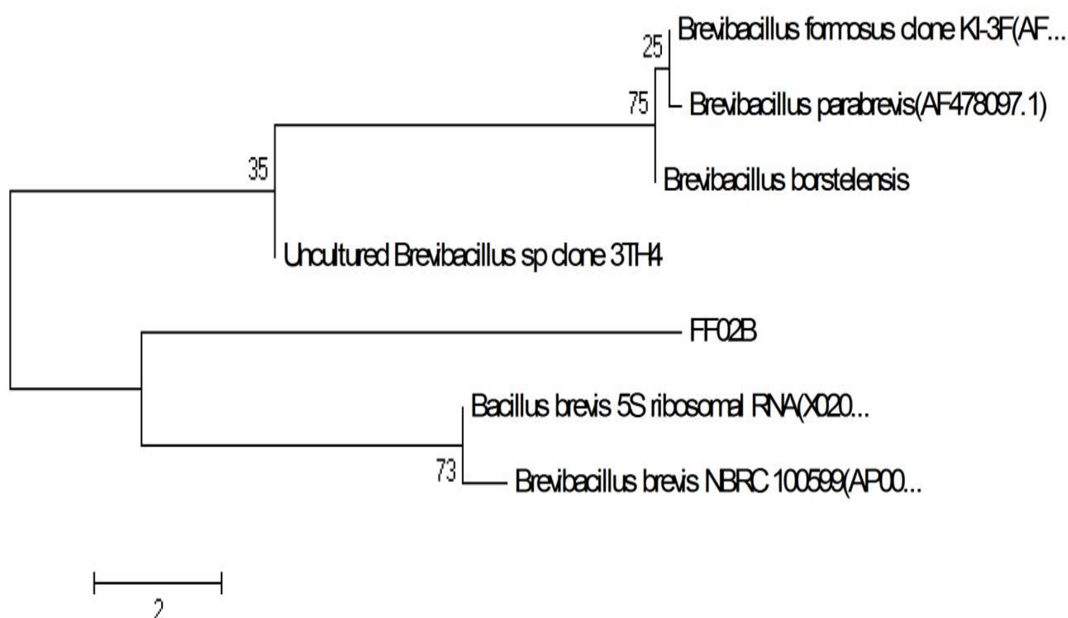


Figure 5.4: Phylogenetic relationships of strain FF02B and other closely related Brevibacillus species based on 16S-23S lSR homologous sequences.

The tree was generated by the neighbor-joining method from NCBI database (http://blast.ncbi.nlm.nih.gov) using the multiple sequence alignment programme of MEGA4 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.

On the basis of phenotypic properties, 16S rDNA and 16S-23S ISR sequences, the bacterial strain FF02B was identified as *Brevibacillus brevis* strain FF02B.

5.3 Purification of a major fibrinolytic enzyme

Fractionation of cell-free culture supernatant on Hi Prep^{TM} Phenyl FF 16/10 FPLC column resulted in the elution of proteins into a single major peak (HIC-I) exhibiting fibrinolytic activity (Fig. 5.5A). Re-fractionation of proteins of HIC-I peak through the gel-filtration column separated them into two peaks, GF-I and GF-II (Fig. 5.5B). The peak GF-II was found to display fibrinolytic activity. The SDS-PAGE analysis of 15 µg protein of the GF-II fraction was found to possess a single distinct band of 55 kDa under both reduced and non-reduced conditions, suggesting it is a monomeric protein (Fig. 5.6). The molecular mass of GF-II fraction by MALDI-TOF-MS analysis was determined as 56043 Da (Fig. 5.7). A summary of purification of this fibrinolytic protease, named Brevithrombolase, is shown in Table 5.6.

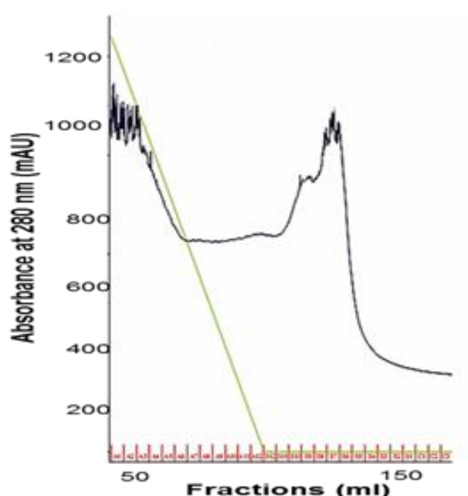


Figure 5.5A: Purification of crude Brevithrombolase from Brevibacillus brevis strain FF02B by Hydrophobic interaction chromatography

The cell-free culture supernatant was adjusted to 1M ammonium sulphate and fractionated on a Hi Prep^{TM} Phenyl FF 16/10 column. Elution of bound fibrinolytic enzyme was carried out with a linear gradient of 1-0 M ammonium sulfate at a flow rate of 2 ml/min. The experimental procedure was carried out as described in materials and methods section 3.2.6.2.2.

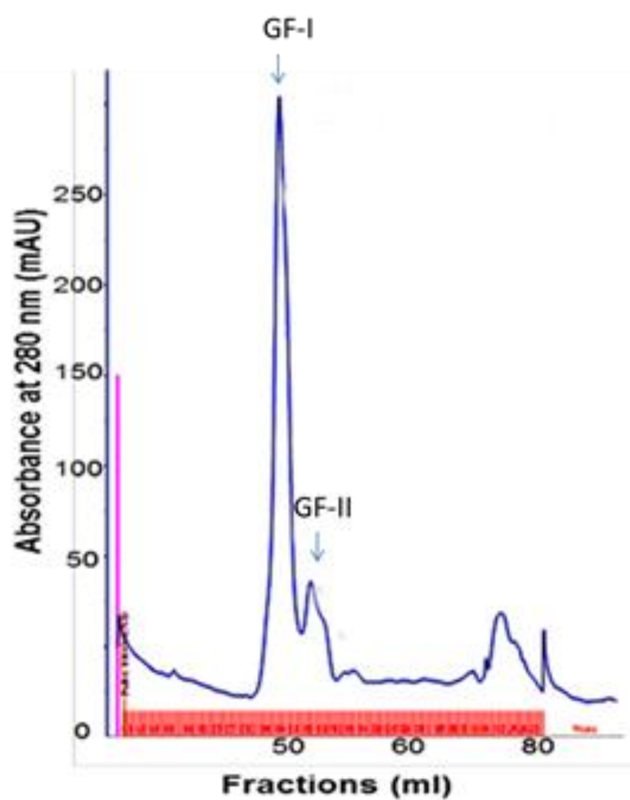


Figure 5.5B: Purification of Brevithrombolase from *Brevibacillus brevis* strain FF02B by Gel-filtration chromatography

Hydrophobic interaction chromatography obtained HIC–I fraction was desalted, lyophilized and re-solubilized in 50 mM sodium phosphate buffer containing 0.15 mM NaCl. Further, it was re-fractionated on a Hi Load^{TM} Superdex 75 pg 16/60 FPLC column, at a flow rate of 0.5 ml/min at 4 °C. The elution of profile was monitored at 280 nm. The fractionation procedure was done as described in material and methods section 3.2.6.2.3.

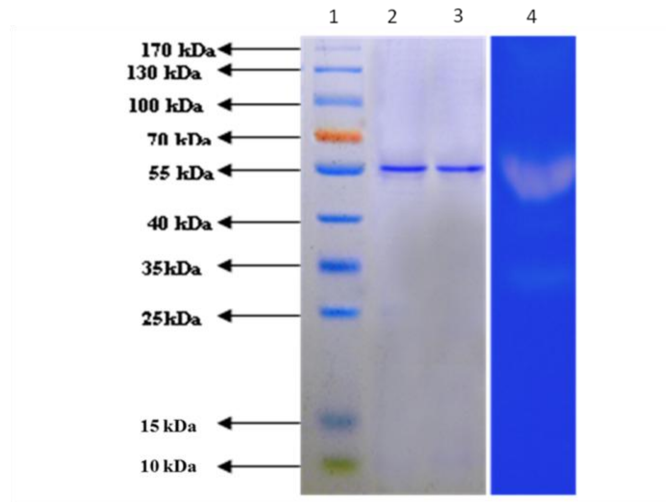


Figure 5.6: Determination of purity and molecular mass of Brevithrombolase by 12.5% SDS-PAGE analysis under reduced and non-reduced conditions.

Lane1, protein molecular markers; lane 2, Brevithrombolase under reduced conditions (15 µg); lane 3, Brevithrombolase under non reduced conditions (15 µg); lane 4, fibrin zymography of Brevithrombolase.

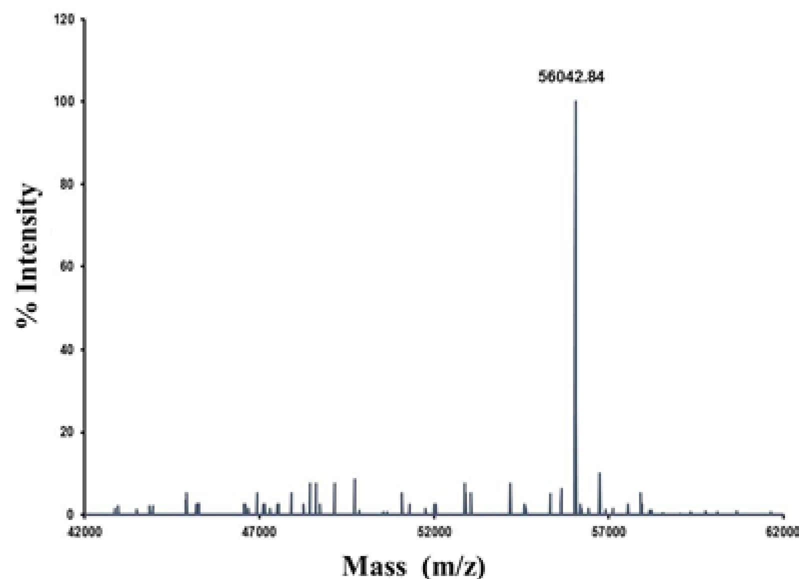


Figure 5.7: MALDI-TOF-MS spectrum of Brevithrombolase.

The intact mass of the purified protein was determined by MALDI-TOF MS (4800 plus, Applied Biosystems, USA) spectroscopy. Sinapinic acid (Sigma, USA) was used as matrix. The protein samples were desalted using ziptip method (Millipore ZipTip™). The MALDI-TOF was operated in Linear Mode with High Mass

Positive acquisition and processing method The spectrum shows signal at m/z 56042.84 (~56043) corresponding to a single species with molecular weight 56 kDa.

Table 5.6: Summary of purification of Brevithrombolase from *Brevibacillus brevis* strain FF02B.The enzyme activity was assessed against fibrin.

Unit is defined as µg of L-tyrosine equivalent liberated per ml per min after 30 min incubation at 37 °C, pH 7.4. Data represent a typical experiment.

Purification steps	Total protein (mg)	Total enzyme activity(Units)	Specific activity (Units/mg)	Enzyme Yield (%)	Purification (fold)
Cell free crude extract (250 ml)	294.0	1538	5.2	100	1
Hydrophobic interaction chromatography	6.3	128.9	20.4	3.9	8.3
Gel-filtration chromatography (Brevithrombolase)	0.4	26.8	64.9	1.7	12.4

5.4 Identification of Brevithrombolase by N-terminal sequencing, peptide mass fingerprinting and amino acid composition analysis

The N-terminal amino acid of Brevithrombolase was found to be blocked and therefore, its sequence could not be determined. By PMF analysis, Brevithrombolase did not show significant match with any protein deposited in the NCBI databases. The BLAST analysis of the tryptic peptide sequences of Brevithrombolase in the NCBI databases against *Brevibacillus brevis* (taxid1393) protease showed uniqueness of the sequences; however, no putative conserved domains could be detected. Nevertheless, one of the tryptic peptides of Brevithrombolase viz INHNIAALNTLNR (1463.79 Da) showed 84% query coverage with a serine protease (accession no WP_016738823), 84% query coverage with peptidase S41 (accession no. WP_016742648), and 61% query coverage with another serine protease sequence (accession no. WP_017247196) reported from *B. brevis*. Another tryptic peptide sequence, viz. NNILSQASQAMLAQANQQPQNVLQLLR (2991.53 Da), of Brevithrombolase demonstrated 62% query coverage, 86% identity with ATP-dependent Clp protease (accession no WP_017252045), 92% query coverage, with 57% identity with extracellular serine protease precursor (accession no. YP_002771256), and 81% query coverage with 57% identity with peptidase C56

family protein (accession no. YP_002775300) reported from *Brevibacillus brevis*. It is to be noted that all the above mentioned matching sequences of proteases reported in the NCBI databases are not purified and characterized in the laboratory, but they represent non-redundant protein sequence, which may be annotated on many Ref Seq genomes from the same, or different species of *Brevibacillus*. When analyzed through the swiss-prot databases, the amino acid composition of Brevithrombolase displayed a similarity with ATP-dependent Clp protease proteolytic subunit 3 (accession number Q9X7R9), which belongs to peptidase S14 family exhibiting serine-type endopeptidase activity (Table 5.7). The PMF as well as amino acid composition analyses suggest that Brevithrombolase is a previously uncharacterized, new fibrinolytic serine protease purified from *Brevibacillus brevis* strain FF02B.

Table 5.7: Amino acids composition of Brevithrombolase (% nmol)

Amino acids	Percent composition (% nmol)
ASP	0.7
GLU	5.6
SER	0.5
GLY	8.2
THR	31.8
CYS	1.7
ARG	19.6
ALA	5.1
TYR	0.9
MET	2.5
PHE	13.5
ILE	0.6
LEU	0.7
PRO	8.7

5.5 Secondary structure of Brevithrombolase

The CD spectrum of native Brevithrombolase showed a negative peak and a positive peak at 205 and 210 nm, respectively. The analysis of secondary structure through CDPRO CLUSTER software suggested that Brevithrombolase consists of 30.6% alpha helix and 69.4% random coil (Fig. 5.8).

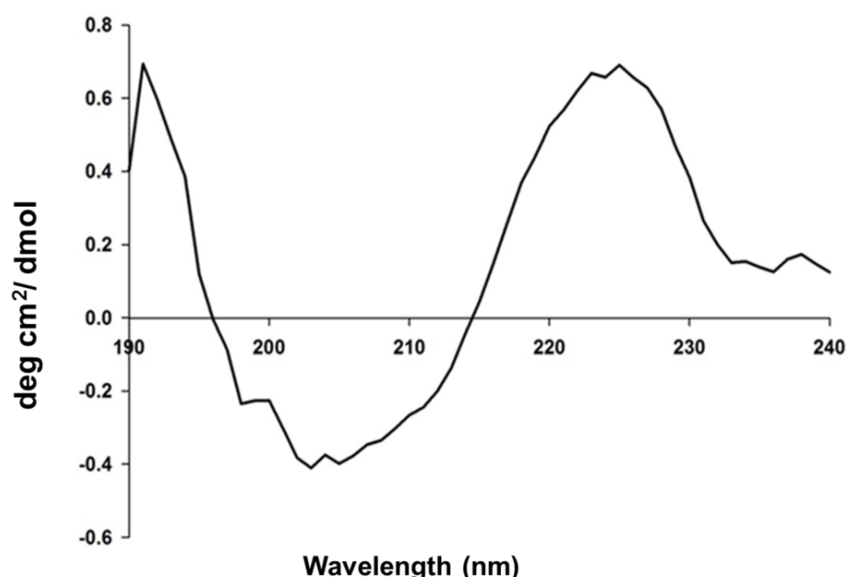


Figure 5.8: Determination of secondary structure of native Brevithrombolase by circular dichroism (CD) analysis

1 mg/ml of purified protease dissolved in PBS buffer, 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. Averages of three scans with no smoothing were reported as CD spectrum of purified protease.

5.6 Amidolytic activity of Brevithrombolase

Brevithrombolase showed highest amidolytic activity against chromogenic substrate for plasmin (V0882); it did not show appreciable activity against other tested chromogenic substrates (Table. 5.8).

Table 5.8: Determination of substrate specificity of Brevithrombolase Values are mean ± S.D of triplicate determinations.

*Units is defined as µmoles of p-nitroaniline released per min by the enzyme.

Substrate	Activity* (U/mg)
Chromogenic substrate*	
D-Val-Leu-Lys-p-nitroanilidehydrochloride (V0882)	1247.7± 6.2
N-Benzoyl-Pro-Phe-Arg-p-nitroanilide hydrochloride	0
*N*α-Benzoyl-DL-arginine 4-nitroanilide hydrochloride	0.5 ± 0.02
N-Benzoyl-Ile-Glu-Gly-Arg-p-nitroanilide acetate	0
N-(p-Tosyl)-Gly-Pro-Arg-p-nitroanilide acetate	0

The K_m and V_{max} values of Brevithrombolase towards its most preferred chromogenic substrate V0882 were determined at 0.39 mM and 14.3 µmol pNA/min, respectively (Fig. 5.9).

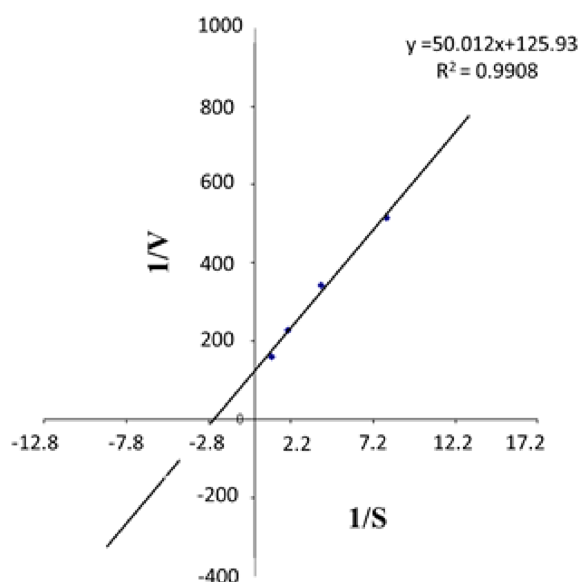


Figure 5.9: Determination of Km and Vmax of Brevithrombolase by Lineweaver-Burk double reciprocal plot

The kinetic properties were determined by incubating 0.2 µM of purified enzyme (Brevithrombolase) with different concentrations (0.12–1.0 mM) of the chromogenic substrate for plasmin (V0882) at 37 °C for 10 min and then determining the enzyme activity.

5.7 Biochemical properties of Brevithrombolase

5.7.1 Effect of temperature on activity of Brevithrombolase

The effect of temperature on Brevithrombolase showed that the activity of Brevithrombolase was significantly increased from 25 to 45 °C and beyond this temperature protease activity declined sharply (Fig. 5.10). Therefore, 37 °C was considered as the optimum temperature for Brevithrombolase activity.

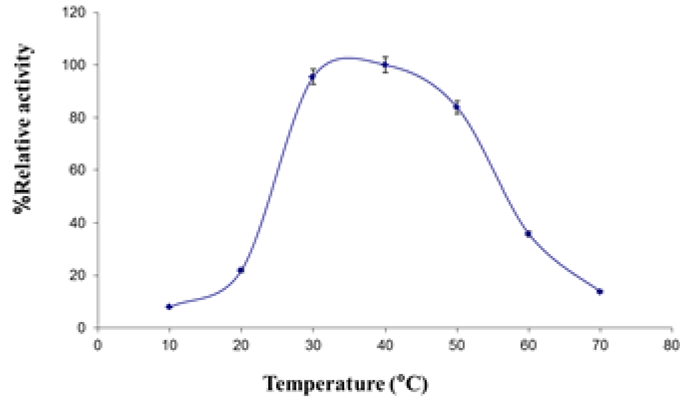


Figure 5.10: Effect of temperature on Brevithrombolase activity. Values are mean of ± SD of three replicates.

5.7.2 Effect of pH on activity of Brevithrombolase

The effect of pH on Brevithrombolase activity was determined at various pH values and assaying the enzyme activity at a particular pH. Results showed that with an increase in the pH of the medium from 6.0 to 8.0, the protease activity was enhanced significantly (Fig. 5.11). Therefore, the fibrinolytic activity at pH 7.4 was considered as optimum pH for enzyme activity.

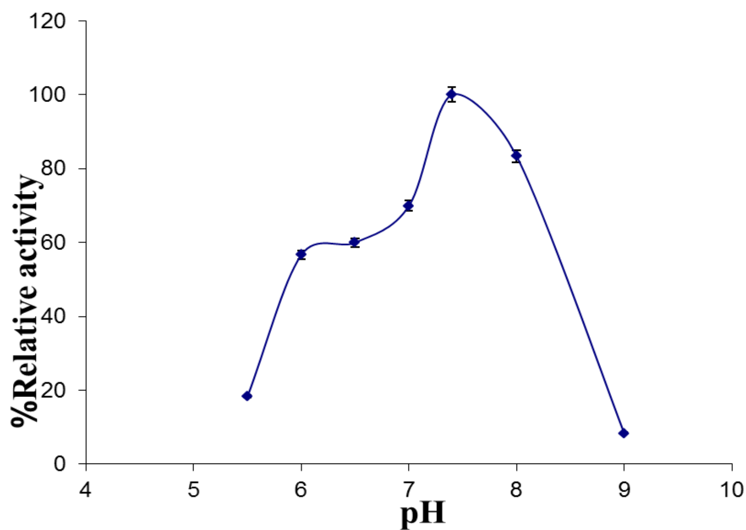


Figure 5.11: Effect of pH on Brevithrombolase activity. Values are mean ± SD of three replicates.

5.7.3 Substrate specificity

Among the various protein substrates tested, Brevithrombolase showed highest substrate specificity towards fibrin, followed by plasma globulin and then fibrinogen; however, it showed poor caseinolytic activity (Table 5.9).The fibrinolytic activity of Brevithrombolase was found to be higher ($p < 0.05$) compared with the fibrinolytic activity of plasmin at pH 7.4 and 37 °C.

Table 5.9: Determination of substrate specificity of Brevithrombolase

*Unit is defined as μg of L-tyrosine equivalent liberated per min per ml after 30 min incubation at 37 °C, pH 7.4. Significance difference with respect to fibrin. [a]p < 0.001.

Protein Specific activity (U/mg)*	substrate
Fibrin	452.0 ± 22.5
Bovine serum γ-globulin	105.4 ± 4.5[a]
Bovine plasma fibrinogen	30.2 ± 1.5[a]
Casein	0.2 ± 0.04[a]
Bovine serum albumin	0[a]

5.7.4 Plasminogen activation property

Brevithrombolase did not show plasminogen activation through conversion of plasminogen to plasmin, suggesting it was devoid of plasminogen-activating property (Fig. 5.12).

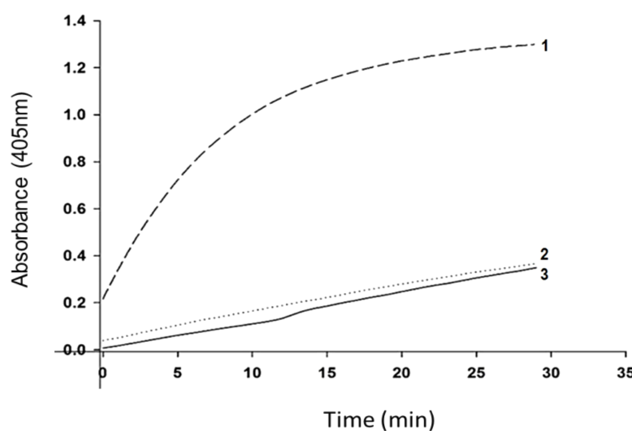


Figure 5.12: Plasminogen activation property of Brevithrombolase

Streptokinase was added with plasminogen in the presence of chromogenic substrate for plasmin (D-Val-Leu-Lys p-nitroanilide dihydrochloride) as a positive control (curve 1); Brevithrombolase was incubated with chromogenic substrate for plasmin (D-Val-Leu-Lys p-nitroanilide dihydrochloride) in absence of plasminogen at 37 °C for 20 min (curve 2); Brevithrombolase was incubated with chromogenic substrate for plasmin (D-Val-Leu-Lys p-nitroanilide dihydrochloride) in presence of plasminogen at 37 °C for 20 min (curve 3).

5.7.5 Inhibition studies of Brevithrombolase

The fibrinolytic activity of Brevithrombolase was significantly inhibited by serine protease inhibitors PMSF, TPCK, and TLCK and by IAA, pBPP and DTT (Table 5.8).

Table 5.10: The effect of inhibitors on fibrinolytic activity of Brevithrombolase

Values represent mean ± SD of triplicate determinations. Significance difference with respect to control: $^{a}p < 0.05$, $^{b}p < 0.001$

Inhibitors(concentration)	Enzyme activity
Control (without inhibitor)	100
PMSF (2mM)	52.9 ± 0.8^{a}
TPCK(100μM)	16.5 ± 1.3^{a}
TLCK (100μM)	51.5 ± 2.3^{a}
DTT (4mM)	92.8 ± 1.9
IAA (4mM)	49.9 ± 0.4^{a}
EDTA(4mM)	21.1 ± 2.1^{a}
pBPP(2mM)	78.5 ± 0.4^{b}

5.7.6 Glycosylation on Brevithrombolase

Brevithrombolase was also found to possess 53.9 μg carbohydrates per mg of protein which represents 5.3% of total protein mass of the protease.

5.7.6.1 Sialic acids residues in Brevithrombolase

Treatment with PNGase did not result in deglycosylation of Brevithrombolase albeit neuraminidase-treated Brevithrombolase showed a protein band of 20.2 kDa on SDS-PAGE (Fig.5.13) indicating sialic acid content represents 45.7% of total mass of Bacethrombase. Conversely, treatment of Brevithrombolase with neuraminidase

without denaturing the enzyme did not result in a change in the SDS-PAGE migration of Brevithrombolase.

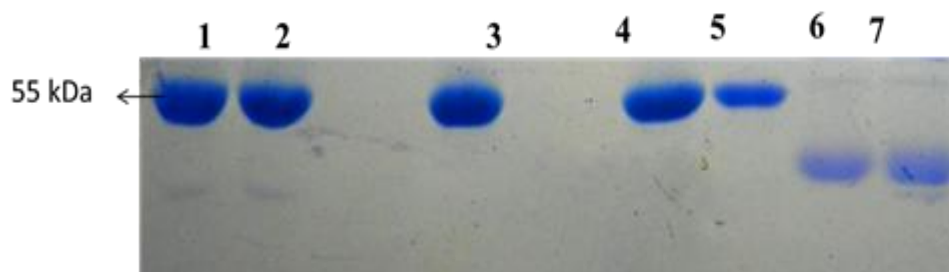


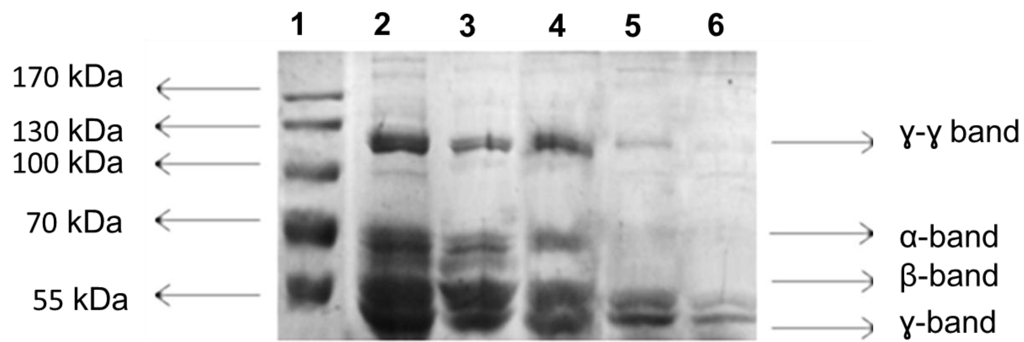
Figure 5.13: 15% SDS-PAGE (reducing conditions) analysis of native and deglycosylated Brevithrombolase

Lane 1, Brevithrombolase (20 µg); lanes 2 & 3, native (without denaturation) Bacethrombase (20 µg) treated with PNGase; lanes 4 & 5, denatured Bacethrombase (20 µg) treated with PNGase; lane 6, native (without denaturation) Bacethrombase (20 µg) treated with neuraminidase (500 units) for 1 h at 37 °C; lane 7, denatured Bacethrombase (20 µg) treated with neuraminidase (500 units) for 1 h at 37 °C.

5.7.7 Kinetics of fibrin degradation by Brevithrombolase

The kinetics of fibrin degradation indicated that Brevithrombolase or plasmin preferentially degraded the Aα chain of fibrin (Figs. 5.14A-B). With an increase in incubation time to 180 min, the Bβ chain also underwent degradation, but the γ-chain of fibrin remained intact after 3 h of incubation at 37 ºC (Figs. 5.14A). Nevertheless, Brevithrombolase could not degrade fibrinogen under identical experimental conditions. The analysis of fibrin degradation by Brevithrombolase or plasmin through the RP-HPLC resulted in an identical elution profile of fibrin-degraded peptides; but, between Brevithrombolase and plasmin, the fibrinolytic activity of the former enzyme was found to be higher at equimolar concentration (Fig. 5.15). Further, RP-HPLC analysis of fibrin degradation indicates that Brevithrombolase and plasmin probably have the identical cleavage sites in the fibrin chain (Fig. 5.16).

(A)



(B)

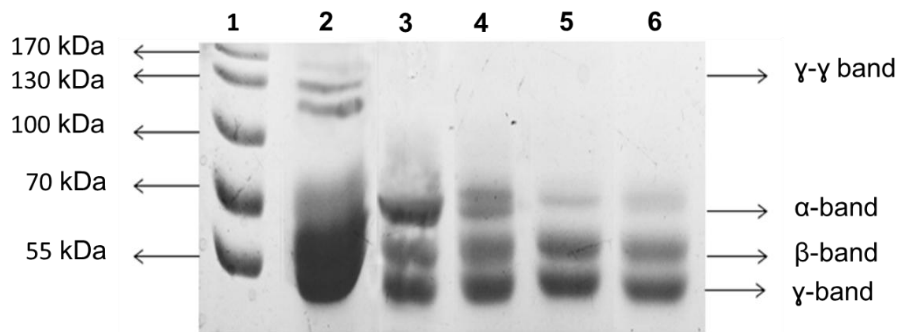


Figure 5.14: A comparison of fibrin degradation between 1.0 μM of (A) Brevithrombolase, and (B) plasmin:

Fibrin was incubated with 1.0 μM plasmin/ Brevithrombolase for 60 min at 37 °C and reaction was stopped by adding 10 μl of 10% 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. (A) Lane 1, control (fibrin without Brevithrombolase/plasmin); lanes 2 to 6, fibrin degradation by Brevithrombolase after 30, 60, 90, 120 and 180 min of incubation, respectively at 37 ºC. (B) Lane 1, control (fibrin without plasmin); lanes 2 to 6, fibrin degradation by plasmin after 30,60, 90, 120 and 180 min of incubation, respectively at 37 ºC.

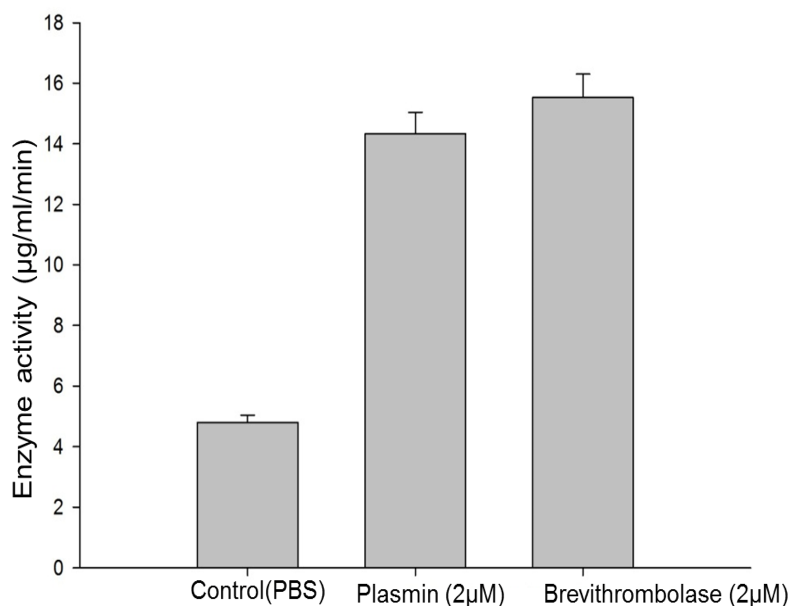


Figure 5.15: A comparison of fibrinolytic activity between plasmin and Brevithrombolase under identical experimental conditions (37 ºC, pH 7.4).

Fibrin was incubated with 2.0 µM plasmin/ Brevithrombolase for 60 min at 37 °C and fibrinolytic activity was determined as per the described in section 3.2.7.4. Phosphate buffered saline (1X PBS) was served as a control. Values are mean ± SD of three replicates.

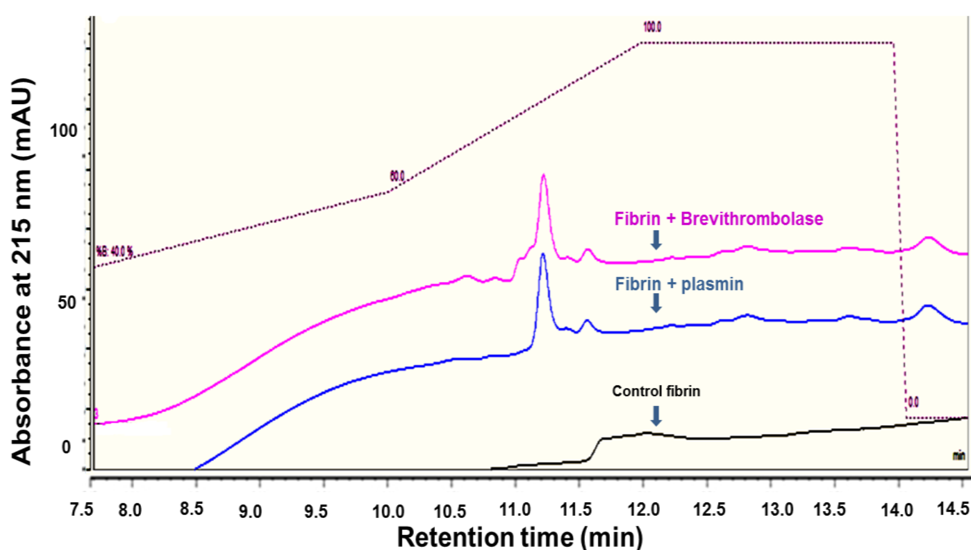


Figure 5.16: Comparison of RP-UPLC elution profile of fibrin degradation products, generated by plasmin/Brevithrombolase, under identical experimental conditions.

Fibrin was incubated with 10 µg plasmin/ Brevithrombolase for 60 min at 37 °C and 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 peptide were eluted by using 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.

5.8 Pharmacological properties of Brevithrombolase

5.8.1 Thrombolytic activity of Brevithrombolase

A comparison showed that *in vitro* clot bursting strength of Brevithrombolase surpassed that of streptokinase or plasmin under identical experimental conditions (Table 5.11). However, the thrombolytic potency of plasmin as well as Brevithrombolase towards the heat-treated blood clot was reduced to ~60% of their potency that they displayed towards dissolving unheated blood clot (Table 5.11). Nonetheless, streptokinase demonstrated negligible activity in dissolving the heated blood clot (Table 5.11).

Table 5.11 A comparison of in vitro thrombolytic activity among Brevithrombolase, plasmin and Streptokinase under identical experimental conditions

Values are mean ± S.D. of triplicate determinations. Significance of difference with respect to blood clot lysis activity of Brevithrombolase, $^{a}p < 0.05$, $^{b}p < 0.001$

Enzyme	Value (mg of blood clot lysed/µg of protein)
A. Non-heated blood clot	
Brevithrombolase	17.7 ± 0.1
Plasmin	13.7 ± 1.2[a]
Streptokinase	13.4 ± 0.3[b]
B. Heat-treated blood clot	
Brevithrombolase	9.5 ± 0.4
Plasmin	8.0 ± 0.3[a]
Streptokinase	0.1 ± 0.1[b]

5.8.2 Cell cytotoxicity and hemolytic activity of Brevithrombolase

Brevithrombolase was found to be non-cytotoxic towards HeLa and HT29 cells (Fig. 5.17) and did not show hemolytic activity against mammalian erythrocytes.

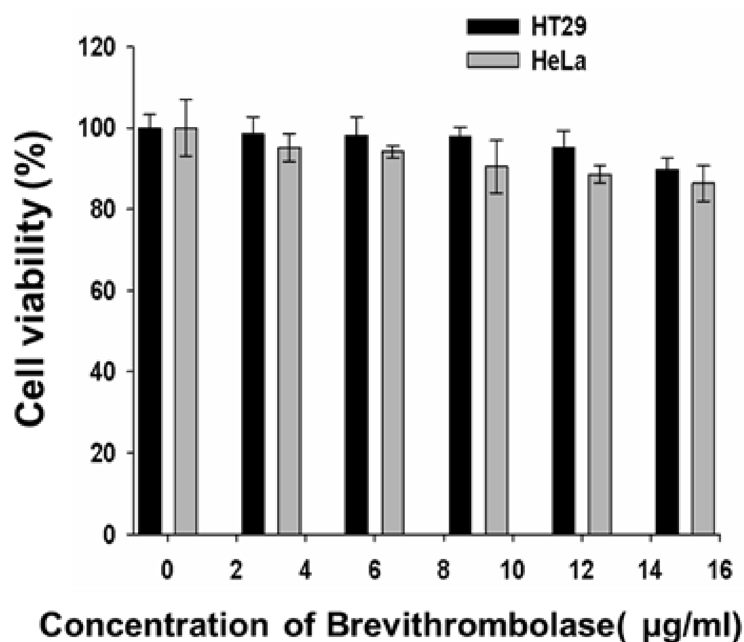


Figure 5.17: Cell cytotoxicity of Brevithrombolase on Hela and HT29 cells. Dose-dependent *in vitro* cell cytotoxicity of Brevithrombolase (2-15 µg/ml) against Hela and HT29 cell lines was determined. The differences in treated values are statistically insignificant ($p > 0.05$) with respect to control cells. The experiment was carried out as mentioned in materials and methods section 3.2.11.

5.8.3 Anticoagulant property of Brevithrombolase

5.8.3.1 Comparison of anticoagulant activity of Brevithrombolase with commercial anticoagulant

A comparison of the dose-dependent anticoagulant activity of Brevithrombolase, plasmin, heparin, and warfarin on PPP is shown in Fig. 5.18. All these four samples showed almost the same anticoagulant power ($p > 0.05$) to prolong the Ca^{2+}-clotting time of PPP (Fig. 5.15).

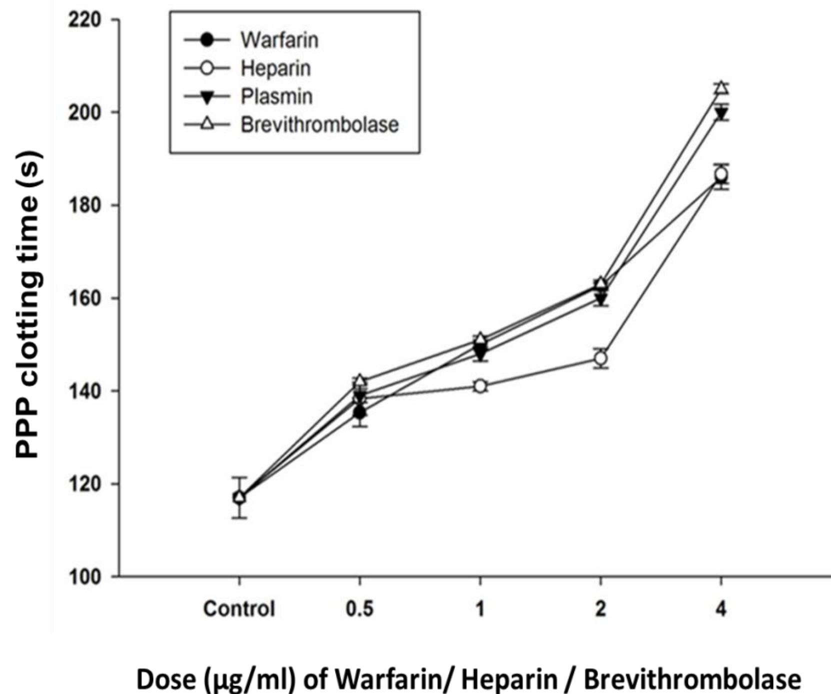


Figure 5.18: A comparison of the dose-dependent anticoagulant activity among Brevithrombolase, warfarin, heparin and plasmin under identical experimental conditions.

Different concentrations (µg/ml) of warfarin/ heparin/plasmin in dose dependent manner was incubated at 37 °C with 300 µl of platelet poor plasma (PPP) obtained from goat blood followed by addition of 40 µl of 250 mM calcium chloride solution to initiate coagulation. One unit of anticoagulant activity has been defined as anticoagulant induced 1s increase in clotting time of PPP compared to clotting time of control plasma. Data represent mean ± SD of triplicate determinations.

5.8.3.2 Effect of Brevithrombolase on recalcification time of PPP.

All these four samples showed almost the same anticoagulant power ($p > 0.05$) to prolong the Ca^{2+}-clotting time of PPP. Furthermore, increasing the pre-incubation of Brevithrombolase with PPP before addition of $CaCl_2$ resulted in a significant ($p < 0.05$) prolongation of Ca^{2+} clotting time of PPP compared with Ca^{2+} clotting time of control PPP (Fig. 5.19).

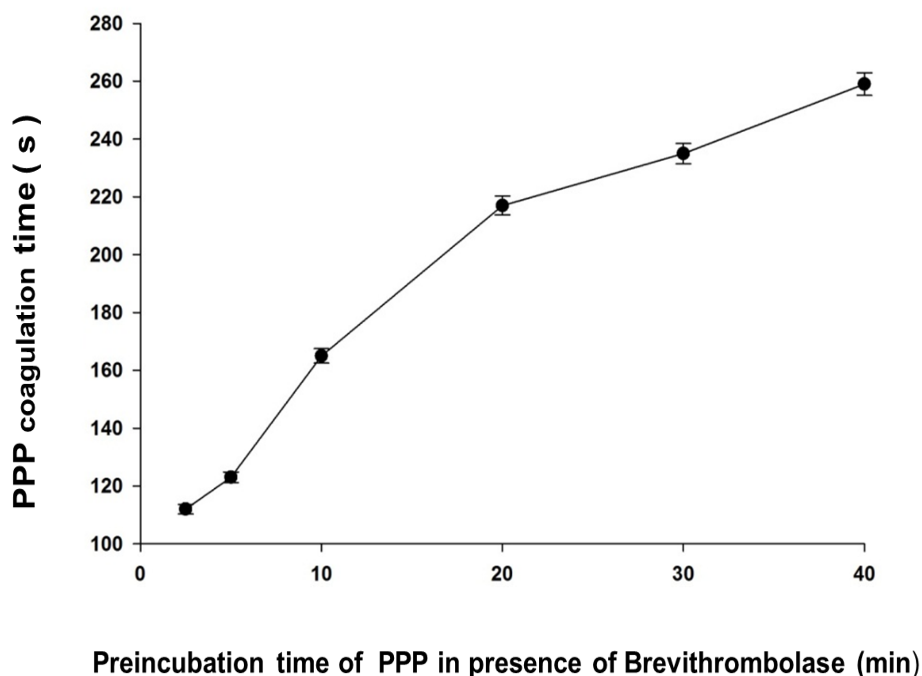


Figure 5.19: Effect of pre-incubation (0–40 min) of Brevithrombolase/platelet poor plasma (PPP) on Ca^{2+}cloting time of PPP.

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 calcium chloride solution. The clotting time was measured as described in 3.2.10.2. Data represent mean ± SD of triplicate determinations.

5.8.4 Anticoagulant mechanism of Brevithrombolase

5.8.4.1 Thrombin and FXa inhibitory activity

Brevithrombolase failed to affect the prothrombin activating property of FXa (Fig. 5.20) suggesting it did not interact with FXa under normal physiological conditions.

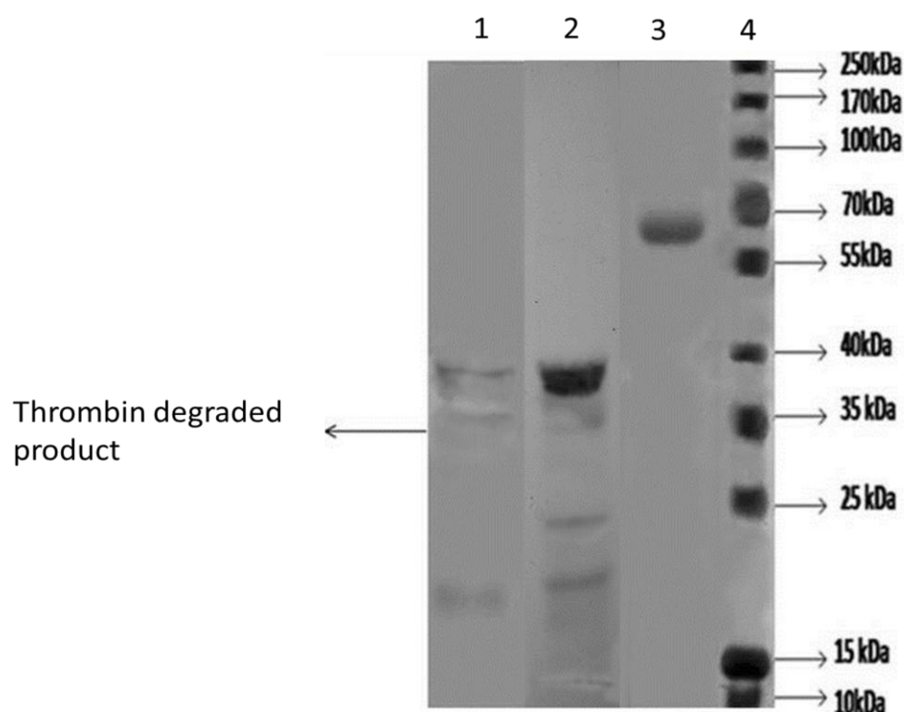


Figure 5.20: Effect of Brevithrombolase in FXa inhibition as analyzed by 12.5% SDS-PAGE.

Purified enzyme (0.4 µM) was pre-incubated with FXa (0.13 µM) in 20 mM sodium phosphate buffer at pH 7.4, 37 °C for 30 min. Thereafter, 1.4 µM of prothrombin and 2.5 mM Ca^{2+} were added to the above mixture and it was incubated overnight at 37°C. A control was run in parallel where instead of Brevithrombolase, 1X PBS was added. The prothrombin degradation products were analyzed by 12.5% SDS-PAGE under reducing conditions. Lane 1) prothrombin (10µg) incubated with Brevithrombolase (2 µg) treated Factor Xa (0.1 µg) at 37 ºC for overnight; lane 2) prothrombin (10 µg) incubated with Factor Xa (0.1 µg) at 37 ºC for overnight; lane 3) prothrombin (10 µg); lane 4) protein molecular weight marker.

However, Brevithrombolase dose-dependently inhibited the amidolytic (Fig. 5.21) as well as fibrinogen clotting activity of thrombin (Fig. 5.22).

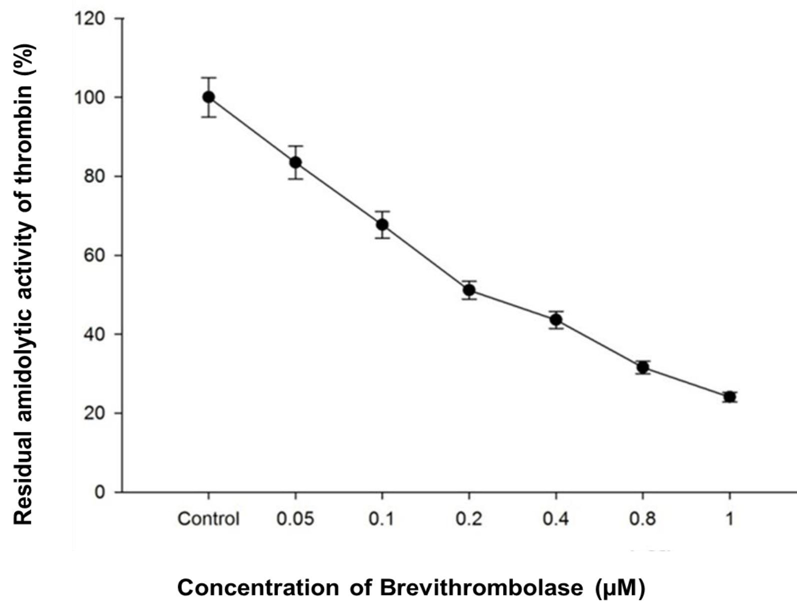


Figure 5.21: Dose-dependent inhibition of amidolytic activity of thrombin by Brevithrombolase.

Bovine thrombin (3 µl, 10 NIH U/ ml) was pre-incubated with increasing concentrations (0.05–1.0 µM) of Brevithrombolase in 50 mM sodium phosphate buffer at pH 7.4 or with 1 X PBS (control) at 37 °C for 30 min. The amidolytic activity of thrombin was measured against N-p-tosyl-Gly-Pro-Arg-pNA acetate (0.2 mM). Dose-dependent residual amidolytic activity of Brevithrombolase was calculated with respect to control reference considered as 100%. Values are mean ± SD of triplicate determinations.

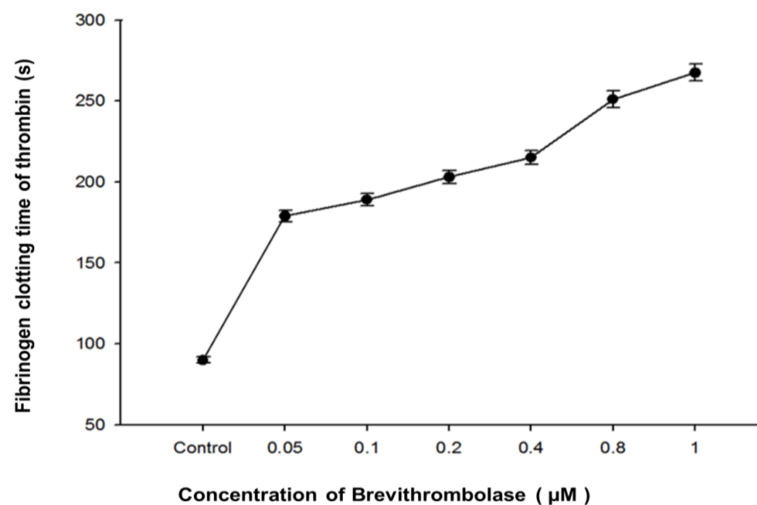


Figure 5.22: Effect of Brevithrombolase (0.05 -1 µM) on fibrinogen clotting time of thrombin.

Bovine thrombin (3 µl, 10 NIH U/ml) was pre-incubated with increasing concentrations (0.05–1.0 µM) of Brevithrombolase 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 (treated as control) was measured against its physiological substrate fibrinogen. Data represent mean ± SD of triplicate determinations.

5.8.4.2 Analysis of interaction between thrombin and Brevithrombolase by spectrofluorometric study

The spectrofluorometric study also indicated an interaction between Brevithrombolase and thrombin, which resulted in an increase in fluorescence signal of Brevithrombolase: thrombin (1:1) complex compared with the fluorescence signal shown by Brevithrombolase or thrombin alone at 340 nm (Fig. 5.23).

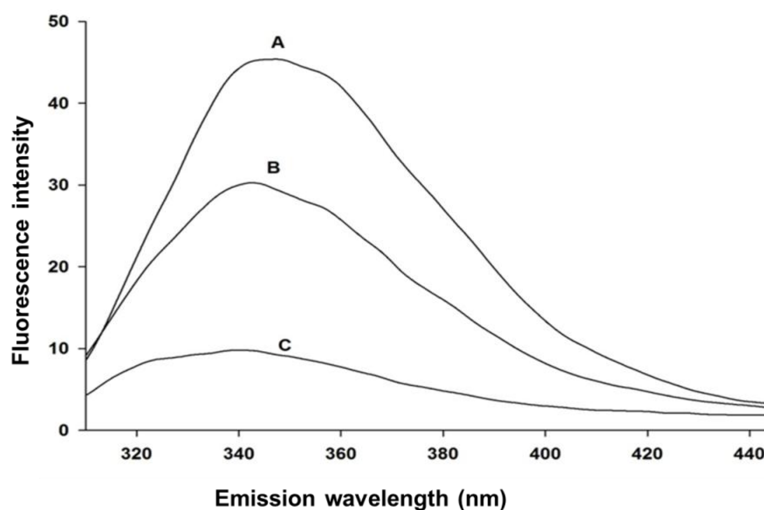


Figure 5.23 Interaction between Brevithrombolase and thrombin by fluroscence spectroscopy.

Fluroscence spectra showing (A) interaction of Brevithombilase (0.02 µM) with thrombin (0.04 µM), (B) Brevithrombolase (0.02 µM), and (C) thrombin (0.04 µM).

5.8.4.3 Thrombin degradation property of Brevithrombolase

The SDS-PAGE analysis suggested degradation of thrombin by Brevithrombolase in a time-dependent manner (Fig. 5.24). After incubation at 37 °C with Brevithrombolase, thrombin was dissociated in two subunits and larger subunit was disappeared while the incubation period was extended till 120 min.

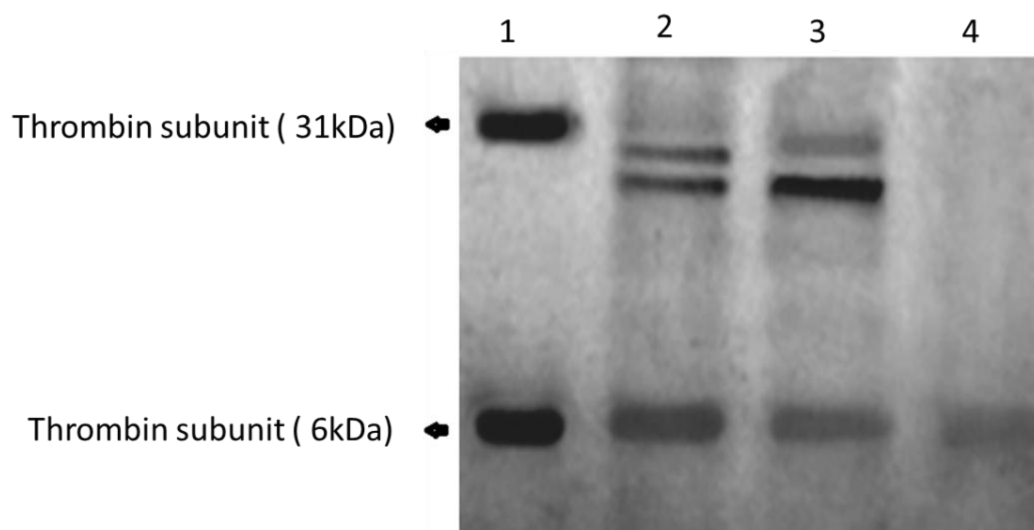


Figure 5.24: Kinetics of thrombin degradation by Brevithrombolase at 37 °C.

135 µM of thrombin (10NIH U/ml) was incubated with 7.0 µM of Brevithrombolase in 50 mM sodium phosphate buffer at pH 7.4 or with 1X PBS (control) at 37 °C for 30 to 120 min. The thrombin degraded products were analyzed by 15% SDS-PAGE under reduced conditions. Lane 1, control thrombin; lanes 2-4, thrombin treated with Brevithrombolase (7.0 µM) for 30, 60 and 120 min, respectively.

5.9 *In vivo* toxicity in rats

5.9.1 Physiological changes after Brevithrombolase administration in rats

An intravenous injection of Brevithrombolase (10 mg/kg) did not result in mortality or behavioral changes in treated rats compared to the control group of rats. There were no differences in weight, water and food intake, [Table 5.12 (A-C)] and ECG of treated animals compared to control animals (Fig. 5.25).

Table 5.12A: 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
	Mean	221.86	223.37	222.75
Control(n=6)	± SD	9.50	8.30	9.80
Treated	Mean	214.05	214.44	214.68
(n=6)	± SD	7.87	9.31	10.22

Table 5.12B: Group mean food consumption (g/kg body weight/day) trends for male Wistar albino rats for 14 days.

Group		Day		
		0	7	14
	Mean	5.22	5.34	5.78
Control(n=6)	± SD	1.31	2.87	1.64
	Mean	4.89	5.24	5.34
Treated (n=6)	± SD	0.96	1.2	1.77

Table 5.12C: Group mean water consumption (mL/kg body weight/day) trends Wistar albino rats for 14 days.

Group		Day		
		0	7	14
	Mean	191.31	193.12	193.80
Control(n=6)	± SD	7.22	7.16	6.85
	Mean	187.06	188.21	188.41
Treated (n=6)	± SD	9.38	8.70	8.65

5.9.2 Effect of Brevithrombolase on hematological parameters of rats

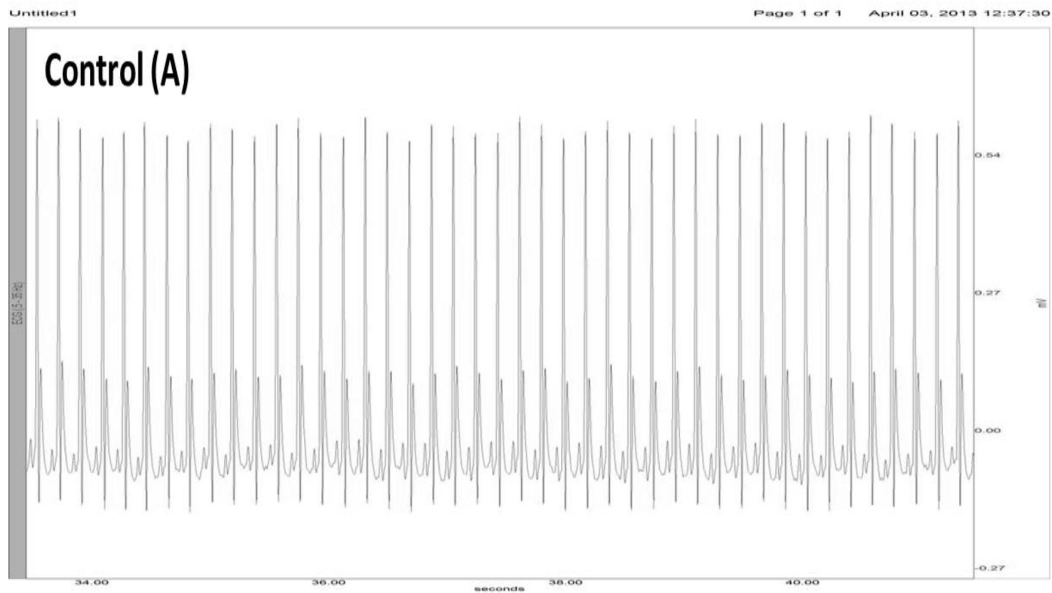
There was no change in hematological parameters of treated animals compared to control rats (Table 5.13).

Table 5.13: A comparison of hematological parameter in Brevithrombolase (10 mg/ml) treated and control rat after 14 days of injection (i.v).

Values are mean ± SD of six experiments. Significance difference with respect to control

Parameters (Unit)		Value (mean ±SD)	
		Control rat	Treated rat
Total WBC	(m/mm^3)	5.0 ± 0.1	5.2 ± 0.1
Lymphocytes	(%)	51.3 ± 3.5	54.5 ± 1.0
Monocytes	(%)	13.3 ± 1.3	14.5 ± 1.0
Neutrophils	(%)	44.3 ± 4.1	45.0 ± 0.4
Eosinophils	(%)	5.8 ± 1.2	5.3 ± 1.7

Parameters (Unit)		Value (mean ±SD)	
		Control rat	Treated rat
Basophils	(%)	0.3 ± 0.3	0.5 ± 0.1
Total RBC	(m/mm^3)	7.1 ± 1.2	8.6 ± 0.4
MCV	fl	57.5 ± 0.2	57.7 ± 0.1
HCt	%	40.8 ± 6.9	46.3 ± 2.2
MCH	pg	19.2 ± 2.7	19.0 ± 0.70
MCHC	g/dl	33.5 ± 4.7	28.0 ± 7.29
RDW		10.7 ± 0.31	11.0± 0.3
He	g/dl	14.0 ± 4.4	11.2 ± 0.1
MPV	fl	7.0 ± 0.1	7.1 ± 0.1
PCt	%	3.0 ± 0.1	3.0 ± 0.45
PDW		7.7 ± 0.2	7.7 ± 0.1



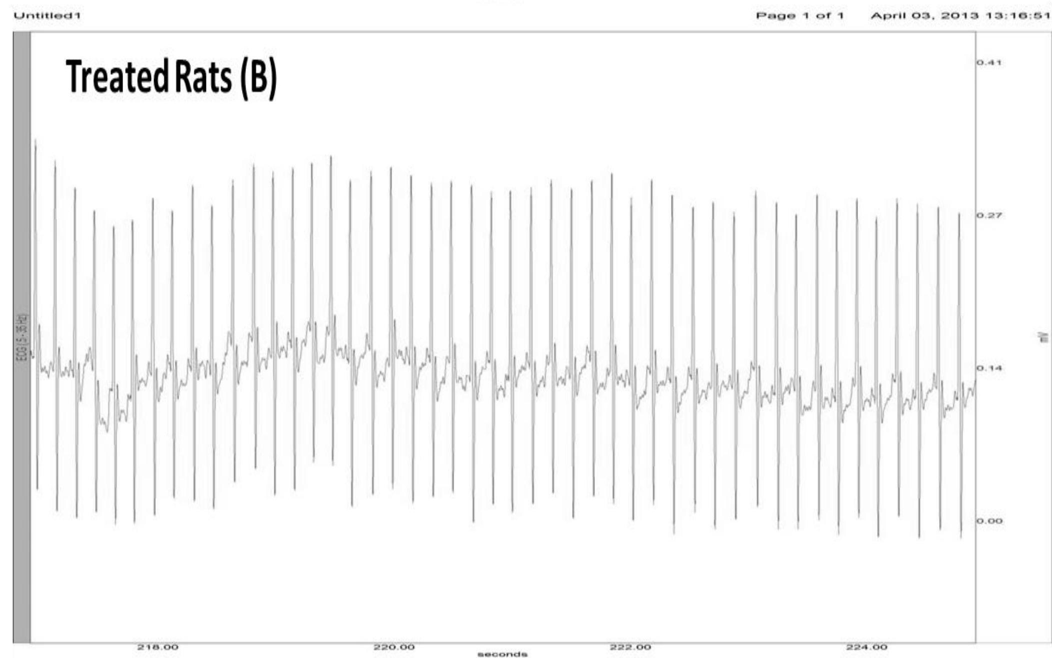


Figure 5.25: Effect of *i.v* injection of Brevithrombolase (10mg/kg) on ECG of treated rat.

(A) Control and (B) treated rats after 14 days of Brevithrombolase injection.

5.9.3 Biochemical changes in serum profile after Brevithrombolase administration in rats

There was no difference in the tested parameters of serum from Brevithrombolase-treated animals compared to control animals after 14 days of treatment (Table 5.14). Although, the level of glucose was slightly increased in Brevithrombolase treated rats; however, its range did not exceed the normal limits.

Table 5.14: A comparison of some biochemical properties of serum of control and Brevithrombolase-treated rats

Brevithrombolase was injected i.v. at a dose of 10 mg/kg and after 14 days of treatment, blood was withdrawn. Significance of difference with respect to control : [a]$p < 0.05$

	Value (mean±SD)	
Parameters (Unit)	**Control rat**	**Treated rat**
LDL(mg/dL)	13.3 ± 0.4	14.3 ± 0.8
HDL(mg/dL)	18.8 ± 0.5	18.1 ± 0.7
Glucose (mg/dL)	70 ± 2.1	78.1 ± 4.4[a]
Urea (mg/ml)	53.8 ± 1.6	54.1± 2.9
Total protein (g/dL)	6.3 ± 0.2	6.2 ± 0.3
Uric acid(mg/dL)	1.3 ± 0.1	1.3 ± 0.1
Triglyceride	52.1 ± 1.3	53.8 ± 2.7
Cholesterol (mg/dL)	46.8 ± 1.3	47.5 ± 2.4
Creatinine (mg/dL)	1.0 ± 0.1	1.0 ± 0.1
SGPT(U/L)	91.3 ± 2.8	92.9 ± 3.5
SGOT(U/L)	162.3 ± 4.9	162.4 ± 6.2

5.9.4 Histopathological examination of tissues from Brevithrombolase-treated rat

The light microscopic examination of tissues obtained from the rats after 14 days of Brevithrombolase treatment did not show any gross morphological change, pathological alteration, inflammation or intravascular coagulation in various organs (Figs. 5.26A-H).

Both the treated and control groups of rats were sacrificed by euthanasia and spleen, heart, liver, and kidney were dissected from the euthanized rats. The tissues were washed with 1X PBS to remove the adherent blood. They were then cut into small sizes 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 were served as control. (A) control liver tissues, (B) treated liver tissues, (C) control spleen tissues.(C) treated spleen tissues. (E) control heart tissues, (F) treated heart tissues, (G) control kidney tissues (H) treated kidney tissues. X10 magnification.

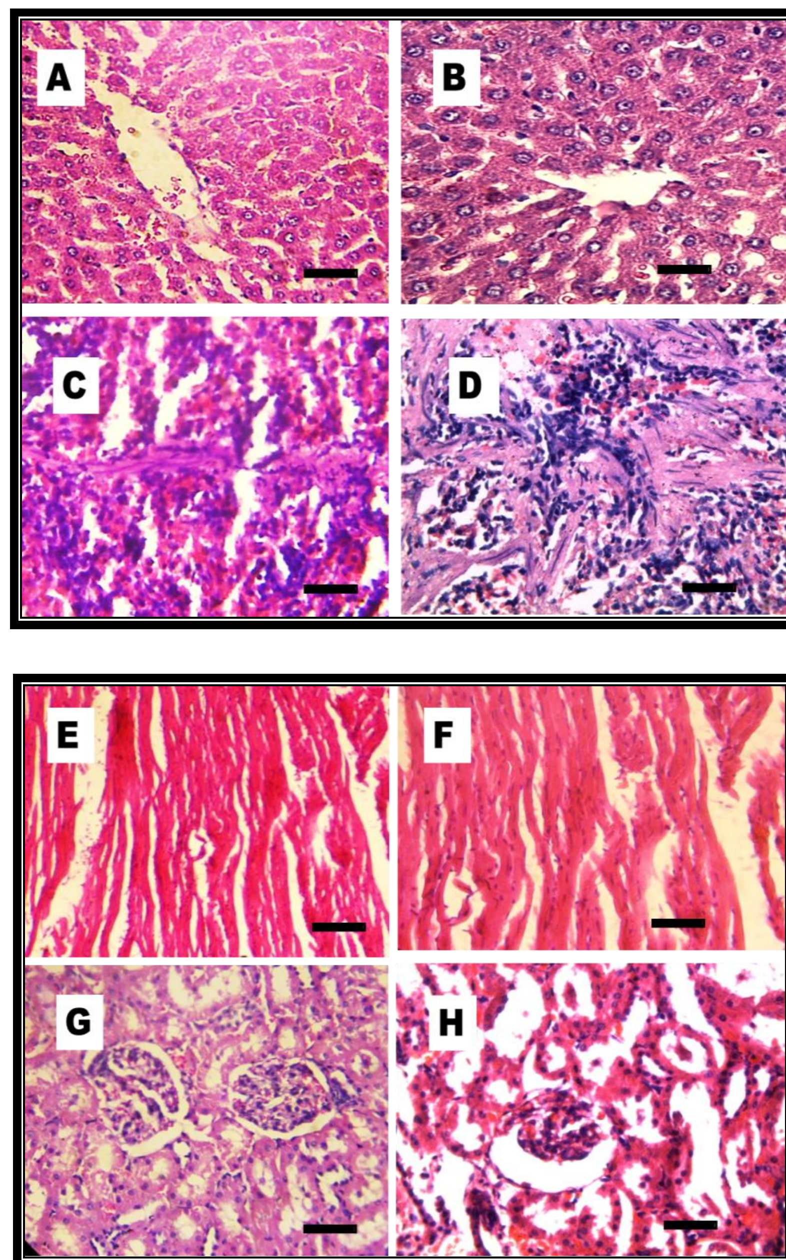


Figure 5.26(A-H): Light microscopic observation of the effect of Brevithrombolase (at a dose of 10 mg/kg) on different organs of treated rats.

5.10 Effect on tail bleeding and clotting time of rat after Brevithrombolase administration

The tail bleeding time and *in vitro* clotting time of blood from the Brevithrombolase-treated group of rats were found to be significantly higher ($P < 0.01$) compared with control group of rats (Fig. 5.27).

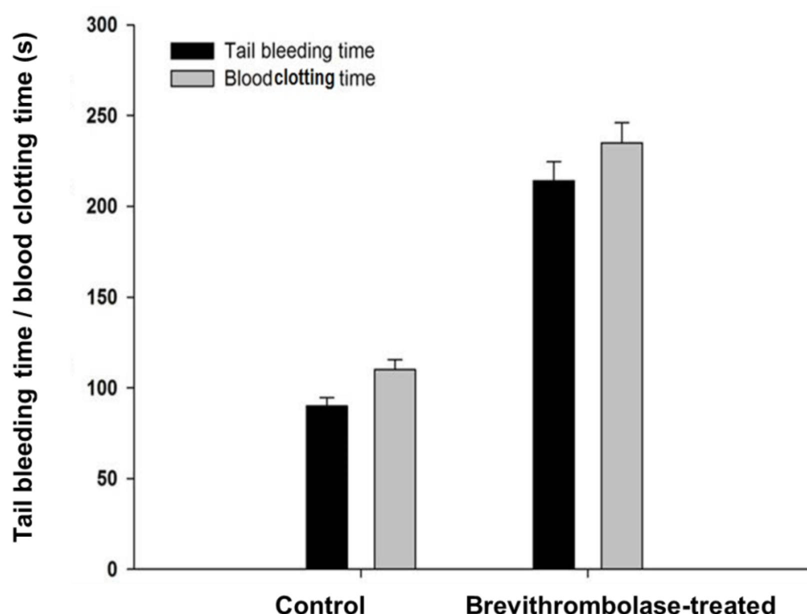


Figure 5.27: The *in vivo* effect of Brevithrombolase on tail bleeding and blood clotting time in Wistar strain albino rats

Bleeding and clotting times were recorded 2 h after the i.v .administration of Brevithrombolase at a dose of 10 mg/kg. Data represent mean ± S.D of triplicate experiments. Significance of difference with respect to control (placebo) group of rats, $p < 0.001$

5.11 *In vivo* thrombolytic activity of Brevithrombolase

The mean length of the infarcted region of the tail significantly decreased with time and nearly disappeared after 24 h of Brevithrombolase infusion, in a dose-dependent manner (Fig.5.28). Conversely, the thrombus that formed in the tail vein of the control group of rats did not decrease with time (Fig. 5.28). The *in vivo* thrombolytic

potencies of Brevithrombolase, SK, and plasmin are shown in Table 5.14. At a dose of 600 μg/kg, the thrombus dissolution property of Brevithrombolase, SK, and plasmin was found to be identical (Table.5.14).

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

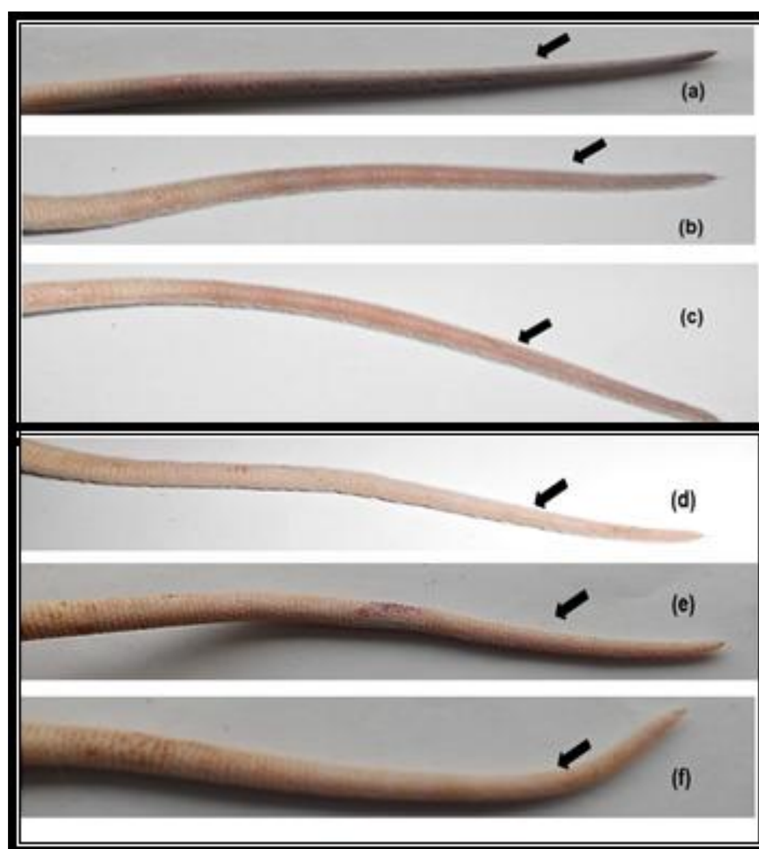


Figure 5.28: Dose-dependent thrombolytic effect of Brevithrombolase

Table 5.15: A comparison of *in vivo* thrombolytic activity of Brevithrombolase, plasmin and Streptokinase after 24 h of treatment on Wistar strain rat

		***In vivo* thrombolytic activity**	
Thrombolytic agent	**Dose (μg/kg)**	**Dissolution of thrombus (%)**	**Length of rat tail thrombus (cm)**
Control (1X PBS)	0	0	7.1 ± 0.3
Brevithrombolase	200	40.85 ± 0.3[b]	4.2 ± 0.2 [b]
Brevithrombolase	400	64.79 ± 5.1[b]	2.5 ± 0.1 [b]
Brevithrombolase	600	98.6 ± 0.1	0.1 ± 0.1
Streptokinase	600	98.6 ± 0.1	0.1± 0.1
Plasmin	600	98.6 ± 0.1	0.1 ± 0.1

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 with respect to in vivo thrombolytic activity of Brevithrombolase at a dose of 600 μg/kg,[b] $p<0.001$.

5.12 Anticoagulant activity of Brevithrombolase in *in vivo* condition

5.12.1 Dose-dependent activated partial thromboplastin time (APTT) and prothrombin time (PT) of Brevithrombolase

The dose-dependent treatment of Brevithrombolase showed a prolongation of the APTT, PT in ex vivo (Fig.5.30), compared to the placebo-control group of rats.

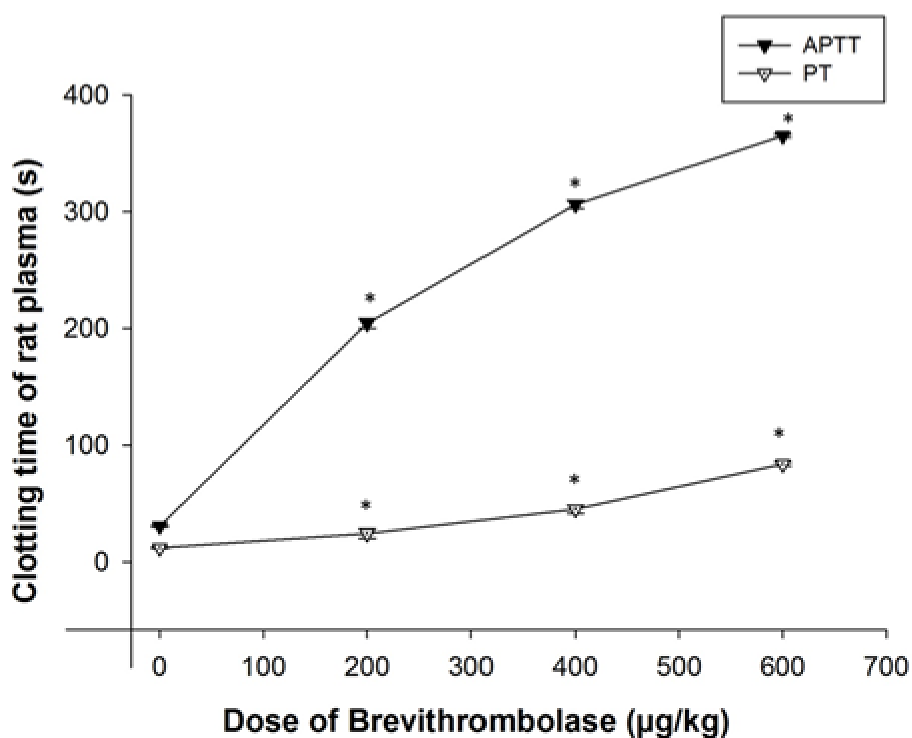


Figure 5.29: Dose dependent activated partial thromboplastin time (APTT) and prothrombin time (PT) of Brevithrombolase

Activated partial thromboplastin time (APTT) and prothrombin time (PT) of Brevithrombolase (200-600 µg/kg) was measured against citrated platelet poor plasma in a coagulometer at 37 °C. **APTT determination:** The C.K.PREST®kit was intended for determination of activated partial thromboplastin time (APTT). The APTT of the treated rat plasma being studied and compared with a reference control. The protocol is described in section 3.2.16.3.1. **PT determination:** The LIQUIPLASTIN® reagent is used for determination of prothrombin time (PT). The PT of the treated rat plasma being studied and compared with a reference control. The protocol is described in section 3.2.16.3.2. The tests were performed in triplicates and average of triplicates result in seconds represents Mean Normal Prothrombin Time (MNPT). Data represent mean ± S.D of triplicate experiments. Significance of difference with respect to control group of rats represent as $*p < 0.001$.

5.12.2 Dose-dependent thrombin time (TT) of Brevithrombolase-treated rats

Brevithrombolase also showed a significant increase in the TT with increasing concentrations in treated rats, in comparison to control rats (Fig. 5.31).

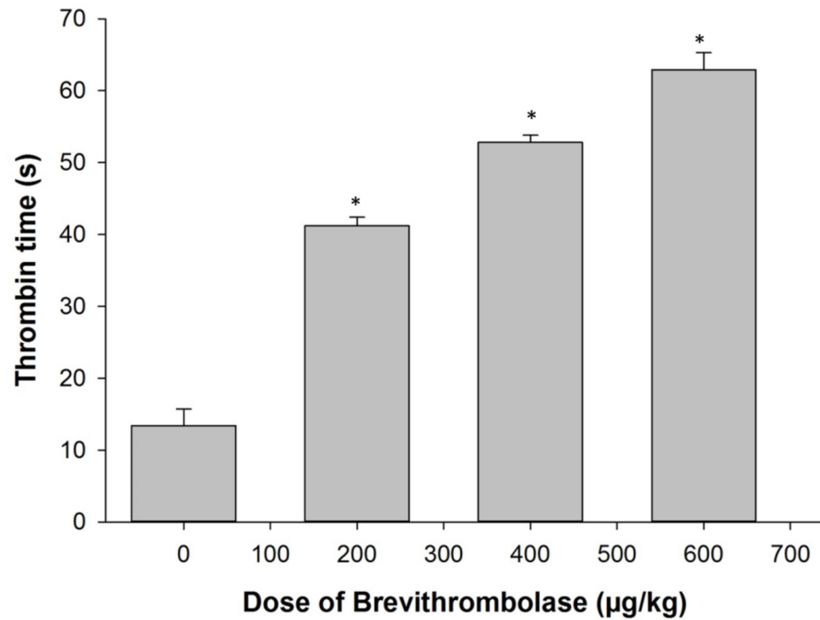


Figure 5.30: Dose-dependent thrombin time (TT) of Brevithrombolase-treated rats.

Plasma (300 µl) was incubated with different doses of Brevithrombolase (200-600 µg/kg) at 37 °C for 2 min. The detail experimental protocol is described in section 3.2.16.3.3. Data represent mean ± S.D of triplicate experiments. Significance of difference with respect to control group of rats represented as*p < 0.001.

5.12.3 *In vivo* dose-dependent fibrinogenolytic activity of Brevithrombolase

The insignificant reduction (p > 0.05) in fibrinogen level in rat plasma suggests that Brevithrombolase poorly degrades fibrinogen (Fig. 5.31)

Figure 5.31: The *in vivo* dose-dependent fibrin(ogen)olytic activity of Brevithrombolase

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

5.12.4 *In vivo* recalcification time of Brevithrombolase

In addition, the defect in fibrin polymerization was not only reflected in the prolonged thrombin and prothrombin times, but also implicated in the prolonged recalcification time by the progressively increased Ca^{2+} clotting of PPP by Brevithrombolase in a concentration-dependent manner (Fig. 5.32).

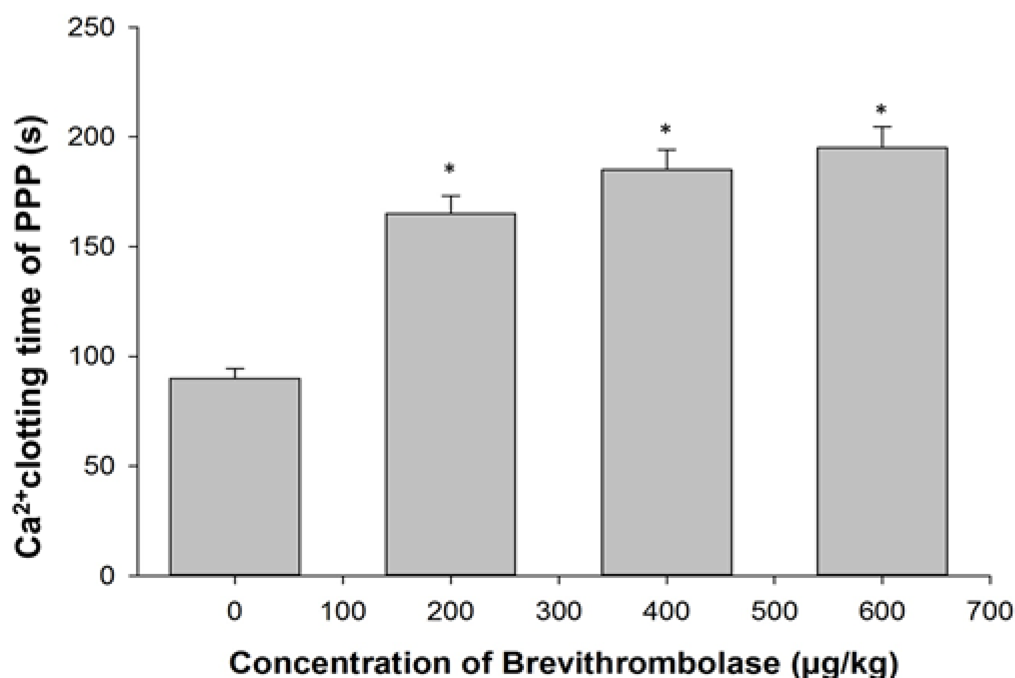


Figure 5.32: Dose-dependent *in vivo* anticoagulant activity of Brevithrombolase.

Determination of re-calcification time of PPP from control and Brevithrombolase-treated groups of rat. The experiment was done as described in the materials and methods, section 3.2.9.2. Data represent mean ± SD of triplicate determinations. Significance of difference with respect to control group of rats represent as *p < 0.001.

5.13 Discussion

To the best of our knowledge, this study is the first report on the characterization and *in vivo* toxicity assessment of a plasmin-like, anticoagulant serine protease purified from *Brevibacillus brevis* strain FF02B. The molecular mass of Brevithrombolase was found to be higher than the molecular mass of fibrinolytic enzymes (18–38 kDa) purified from the genus *Bacillus* [6] and approximately the same as the molecular mass of fibrinolytic enzyme Jerdofibrase and Subtilisin E isolated from venom of *Trimeresurus jerdonii* [161], and *B. subtilis* [162]. But, the biochemical and pharmacological properties of Brevithrombolase differ from fibrinolytic enzymes, Jerdofibrase, Subtilisin E, and *B. subtilis*. This clearly points to the fact that despite identical molecular mass and nearly identical sequence, small

surface residue changes can bring about significant changes in the biochemical and pharmacological properties of serine protease enzymes [107].

Fewer attempts have been made to investigate the secondary structure of the bacterial fibrinolytic enzymes. The secondary structure of Brevithrombolase was found to be identical with Polyproline–II type model [150] and it closely resembles the secondary structure of bovine plasmin at the far UV region [163]. The PMF as well as amino acid composition analyses suggest that Brevithrombolase is a previously uncharacterized, new fibrinolytic serine protease purified from *B. brevis* strain FF02B. Brevithrombolase was found to be glycosylated in nature. The glycosylated proteins show higher stability and activity compared to non-glycosylated proteins [107]. Further, glycosylation probably prevents the aggregation of proteins under native conditions in the extracellular matrix [164].

The optimum temperature and pH requirement of Brevithrombolase for dissolving fibrin clot was comparable to previously reported fibrinolytic enzymes isolated from bacteria such as Bafibrinase [41], Nattokinase [48], and DJ-4 [78]. Further, similar to plasmin, Brevithrombolase displayed less activity for dissolving the heat-treated blood clot compared with unheated blood clot [165]. Since the thrombolytic property of streptokinase is dependent on the activation of plasminogen to plasmin, heat treatment of blood clot completely abolishes the clot lysis activity of streptokinase [165]. These observations provide a fair indication that Brevithrombolase is a plasmin-like, direct-acting fibrinolytic enzyme. Moreover, significantly higher thrombolytic potency of Brevithrombolase compared with streptokinase or plasmin at physiological conditions (37 °C, pH 7.4) reinforces its future therapeutic application as a thrombolytic agent. The higher fibrinolytic/caseinolytic (F/C) ratio (which measures the potency of fibrinolytic enzyme) of Brevithrombolase compared with the other bacterial fibrinolytic enzymes [41, 53, 90, 162, 166, 167] as well as specificity of Brevithrombolase towards fibrin without hydrolyzing the other plasma proteins points to its therapeutic value.

An inhibition study with group-specific reagents or metal chelator predicts the nature of active site in an enzyme and its cofactor requirements [41, 107]. The inhibition study of Brevithrombolase has provided a strong indication of presence of serine, cysteine and histidine in its active site, suggesting it is a trypsin-like serine

protease. Interestingly, the inhibition of protease activity of Brevithrombolase by EDTA is in accordance with a few limited examples of serine proteases, which are inhibited by this metal chelator [40, 78]. Further, Brevithrombolase failed to inhibit DTT, suggesting the presence of no intramolecular and intermolecular disulphide linkage(s) in this enzyme.

On the basis of specificity towards α-chain and β-chain of fibrin/fibrinogen, fibrin(ogen)olytic enzymes are classified as α and /or β fibrinogenase [17, 41]. Based on of fibrin degradation pattern as revealed by SDS-PAGE analysis, Brevithrombolase may be classified as αβ-fibrinogenase [41]. Further, RP-HPLC analysis of fibrin degradation indicates that Brevithrombolase and plasmin probably have the identical cleavage sites in the fibrin chain. Furthermore, a comparison of the K_m value between Brevithrombolase and nattokinase [78] points to the superiority of the former enzyme.

Like many other fibrinolytic enzymes [41, 99], Brevithrombolase significantly prolonged the *in vitro* Ca^{2+} clotting time of PPP as well as demonstrated *in vivo* anticoagulant activity, suggesting it is an anticoagulant enzyme. Reports on the mechanism of anticoagulant action of fibrinolytic enzymes are lacking and only fragmentary data are available to support their anticoagulant mechanisms [39]. Like many of the fibrinolytic enzymes, Brevithrombolase did not inhibit FXa or prothrombin or degraded fibrinogen to exert its anticoagulant activity [17, 107, 168]. Our study suggests that a similar to the anticoagulant mechanism exhibited by a fibrinolytic enzyme isolated from *Bacillus subtilis* strain DC53 [17], Brevithrombolase first binds with the thrombin (which is evidenced by spectrofluorometric analysis) followed by cleavage of subunits A (31 kDa) and B (6 kDa) of thrombin [169] in a dose-dependent and time-dependent manner. This leads to progressive incoagulable blood. The degradation of thrombin by Brevithrombolase may be considered a unique mechanism to exert anticoagulant action, which has a great therapeutics implication. This anticoagulant mechanism of Brevithrombolase, therefore, differs from the currently available anticoagulant drugs such as heparin and warfarin. Heparin binds non-enzymatically to the exosite-II of thrombin and induces a conformational change in antithrombin III to activate it, which in turns inhibits the binding of thrombin with fibrinogen [155]. Conversely, warfarin shows anticoagulant activity by inhibiting the formation of ɣ-carboxyglumate at amino

terminal of prothrombin [156]. Since thrombin catalyzes the final stage of the blood coagulation cascade, thrombin inhibitors such as Brevithrombolase may be considered as an alternative new drug to traditional cardiovascular drugs such as heparin or warfarin [27].

It is worthy to mention that several fibrinolytic enzymes have been purified and characterized; however, except a few examples, data on their *in vivo* toxicity and pharmacological properties is lacking [41]. The lack of cytotoxicity, hemolytic activity, and *in vivo* toxicity of Brevithrombolase in experimental animals is in accordance with our previous observations on Bafibrinase [41]. Further, Brevithrombolase was found to be non-lethal at a dose (i.v.) of 10 mg/kg in rats, which is almost 20 and 5- to 10-fold higher dose than the therapeutic dose of streptokinase [170] and plasmin [171], respectively. This finding clearly points to the suitability of Brevithrombolase for its clinical application as a cardiovascular drug. Taken together, the biochemical and pharmacological properties, thrombolytic potency and strong anticoagulant activity of Brevithrombolase by hydrolysis of thrombin may lead us to anticipate that it is a promising candidate for the peptide-based lead molecule for the treatment and/or prevention of cardiovascular disorders.

The biochemical and pharmacological properties of a fibrinolytic serine protease (Brevithrombolase) were explored and its *in vitro* thrombolytic activity was found to be superior to that of plasmin or streptokinase [89]. Our study was extended to demonstrate that Brevithrombolase also exhibited a potent anticoagulant effect in a carrageenan-induced rat tail thrombus model. However, the *in vivo* thrombolytic effect of Brevithrombolase was comparable to that of streptokinase and plasmin.

A variety of physical and chemical methods have been applied to induce thrombus formation in experimental animals [114]; however, because of the advantages of carrageenan, it has been the chemical of choice [114]. The specific advantages include- accurate continuous measurements of thrombus in a range, without killing the animals, and requirements for fewer animals, which supports their ethical use for *in vivo* experiments [172]. This method also avoids complicated surgery to expose blood vessels in the tail of animals. Moreover, carrageenan may influence the inactivation of Hageman factor, which is followed by endogenous coagulation [114]. The carrageenan-induced thrombus model has been found to be

beneficial in assessing the efficiency of various clinically used thrombolytic agents like heparin, UK,SK, and aspirin [114]. Thus, in the present study, carrageenan-induced rat model was chosen for assessing the *in vivo* antithrombotic potential of Brevithrombolase.

At a dose of 600 µg/kg, the thrombus dissolution properties of Brevithrombolase, SK, and plasmin were found to be identical. Therefore, we assume that, like the currently available thrombolytic drugs (i.e., plasmin and SK), Brevithrombolase also has *in vivo* clot-dissolving properties, making it suitable for use as a thrombolytic drug. Notably, at 600 µg/kg dose, the in vivo thrombus dissolving efficacy of Brevithrombolase surpassed the same activity displayed by nattokinase [117] and fibrinogenase [160] suggests superiority of the former thrombolytic enzyme.

To evaluate the *in vivo* therapeutic potential of Brevithrombolase in managing thrombotic diseases, we examined its anticoagulant activity in the carrageenan-induced rat model. When carrageenan was injected alone, swelling and reddening of the tail was seen first, which was followed by the tail becoming auburn-colored. Brevithrombolase inhibited the carrageenan-induced thrombus formation over 15 min of being intravenously infused (in a rapid dose-dependent manner). The dose-dependent treatment of Brevithrombolase showed a prolongation of the APTT, PT in ex vivo, compared to the placebo-control group of rats. These results suggest that the inhibition of intrinsic pathways are due to a reduction in coagulation factors, such as VIII, IX, XI, and von Willebrand Factor (vWF) [173]; whereas, the delayed PT suggested obstructed extrinsic pathways possibly due to the depletion of coagulation factors such as V, VII, and X [173]. Brevithrombolase may block the activation of the coagulation cascade and the synergistic process of thrombus formation by inhibiting extrinsic and intrinsic pathways.

Like many other fibrinolytic enzymes [92, 174], Brevithrombolase also showed a significant increase in the TT with increasing concentrations in treated rats, in comparison to control rats indicating *in vivo* thrombin degradation by this enzyme. Moreover, the insignificant reduction in fibrinogen level in rat plasma suggests that Brevithrombolase poorly degrades fibrinogen, This suggests that the hydrolysis of thrombin by Brevithrombolase may impair fibrin formation in the coagulation

cascade rather than depletion of the fibrinogen level in Brevithrombolase-treated rat plasma [89]. In addition, the defect in fibrin polymerization was not only reflected in the prolonged thrombin and prothrombin times, but also implicated in the prolonged recalcification time by the progressively increased Ca^{2+} clotting of PPP by Brevithrombolase in a concentration-dependent manner. These findings, taken together, further advocate the in vivo anticoagulant potential of Brevithrombolase.

The application of anticoagulant drugs in treating thromboembolic disease is conceptually attractive and their pharmacological importance has been evaluated based on their mechanisms for preventing thrombus formation. For example, the limited ability of heparin to inhibit thrombin on the thrombus surface and in the surrounding plasma could explain its failure to inhibit the accretion of new fibrin on the clot. Moreover, the addition of intravenous heparin to tissue-plasminogen activator was reported to have an improved patency rate when compared with aspirin[175].While the feasibility of all anticoagulant drugs has been demonstrated, with regards to frequent complications like antigenicity, toxicity, and defective coagulation, Brevithrombolase overcomes these drawbacks, in terms of *in vivo* toxicity. These results suggest that Brevithrombolase may have a dual role in antithrombotic and thrombolytic activities [92, 116].

The results of the present investigation therefore suggests that Brevithrombolase has a thrombolytic function *in vivo* that is as effective as plasmin or SK, and due to lack of toxicity it may be a promising alternative to the commercial thrombolytic drugs.