CHAPTER 6

Characterization of Erythrofibrase: A haemostatically active protein from the venom of *Trimeresurus erythrurus*

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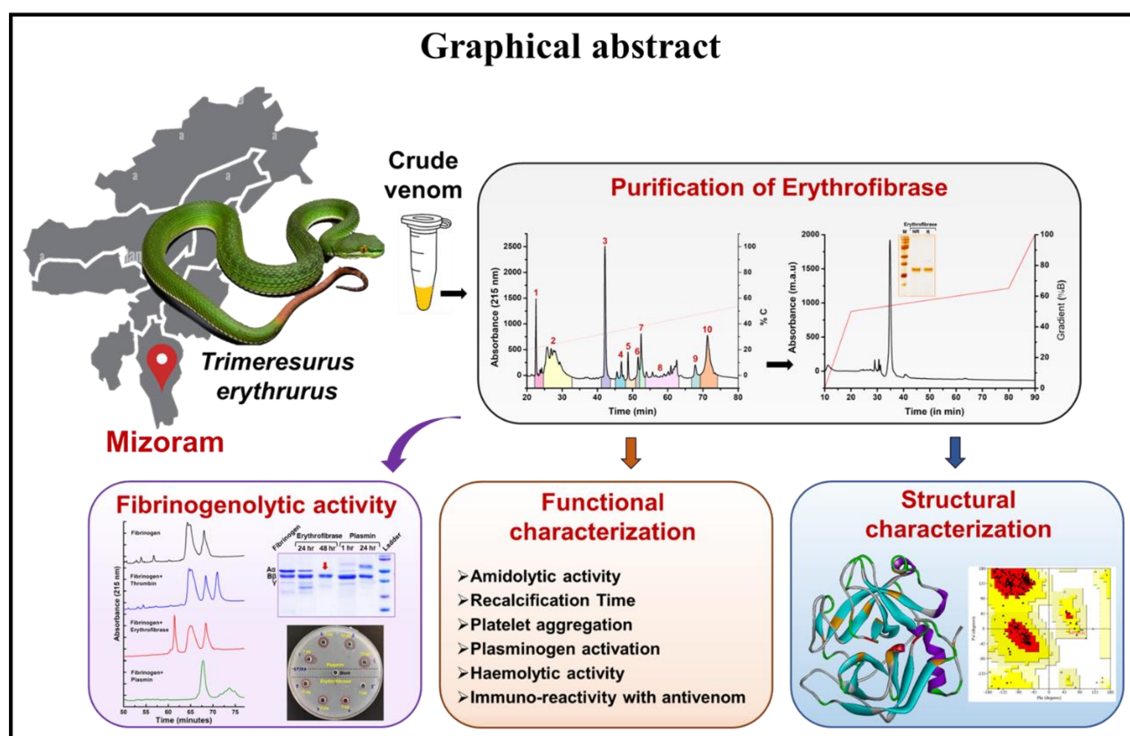

6.1. Introduction

Haemostasis is a highly regulated vital physiological process of body which functions to maintain the normal blood flow preventing excessive blood loss during vascular injury as well as dissolution of unwanted clot [133]. The components of haemostatic system constitute the most preferred physiological targets for snake venom toxins. In particular, the coagulation cascade and fibrinolytic system comprise the foremost target for majority of venom toxins which show either anticoagulant or procoagulant effect in patients [147, 148]. Moreover, a particular type of pseudo-procoagulant effect is observed during pit viper envenomation due to abundance of snake venom thrombin-like enzymes (SVTLEs), a subgroup of serine proteases. SVTLEs functions analogous to thrombin, targeting the fibrin polymerization step of coagulation

cascade to form a thrombus. However, unlike thrombin, SVTLEs cannot activate factor XIII, as a result of which the clot formed does not get stabilized, thereby easily removed by fibrinolytic system [150, 240]. This causes defibrinating syndrome in patients leading to a situation commonly known as venom induced consumptive coagulopathy (VICC). The consumptive coagulopathy and resulting hypofibrinogenemia causes incoagulable blood for several days in the green pit viper envenomated patients [150].

A number of SVTLEs have been isolated and characterized from green pit viper venoms in the past few decades. A few of them like batroxobin, ancrod etc. has also passed the clinical trials and commercialized as therapeutic and diagnostic agents in ischemic heart diseases [148, 156, 241]. However, the major toxic components of Indian green pit vipers causing incoagulable blood in envenomated patients has not been identified. Investigation undertaking excavation of major venom toxins might lead to better understanding of pharmacological profile of venom, its immuno-reactivity as well as identification of potent bioactive molecules which could be explored further as promising therapeutic agents. Therefore, the current study has been undertaken to identify and characterize a haemostatically active protein from the venom of Indian *Trimeresurus erythrurus* with special emphasis on snake venom thrombin-like enzymes.

6.2. Results:

6.2.1. Purification of erythrofibrase from *Trimeresurus erythrurus* venom

Purification of erythrofibrase from *Trimeresurus erythrurus* venom was performed by fractionating crude venom using reverse-phase HPLC with a gradient of 20-65% acetonitrile. The chromatogram revealed a total of ten peaks with varying hydrophobicity and intensities (Figure 6.1A). Each of these Rp-HPLC peaks were then screened for thrombin-like activity and plasmin-like activity.

Screening of Rp-HPLC peaks for thrombin-like activity was done using chromogenic substrate S2238. Among all Rp-HPLC peaks, Peak 6 and Peak 7 showed highest thrombin-like activity of 1.904 µmol/min and 7.204 µmol/min respectively (Figure 6.1C). However, at a dose of 10 µg/ml, Peak 7 shows slightly more thrombin-like activity than crude venom i.e., 6.624 µmol/min. Rp-HPLC peaks were further screened for plasmin-like activity using chromogenic substrate S2251 and Peak 7 was again observed to possess highest plasmin-like activity i.e., 3.064 µmol/min among all

the fractions (Figure 6.1D). The SDS-PAGE profile of Peak 7 showed multiple bands in the gel, depicting Peak 7 to contain a mixture of proteins (Figure 6.1B). The lanes containing non-reduced and reduced samples display presence of 3 faint bands and 1 thick and prominent band in a molecular weight range of 15-50 kDa.

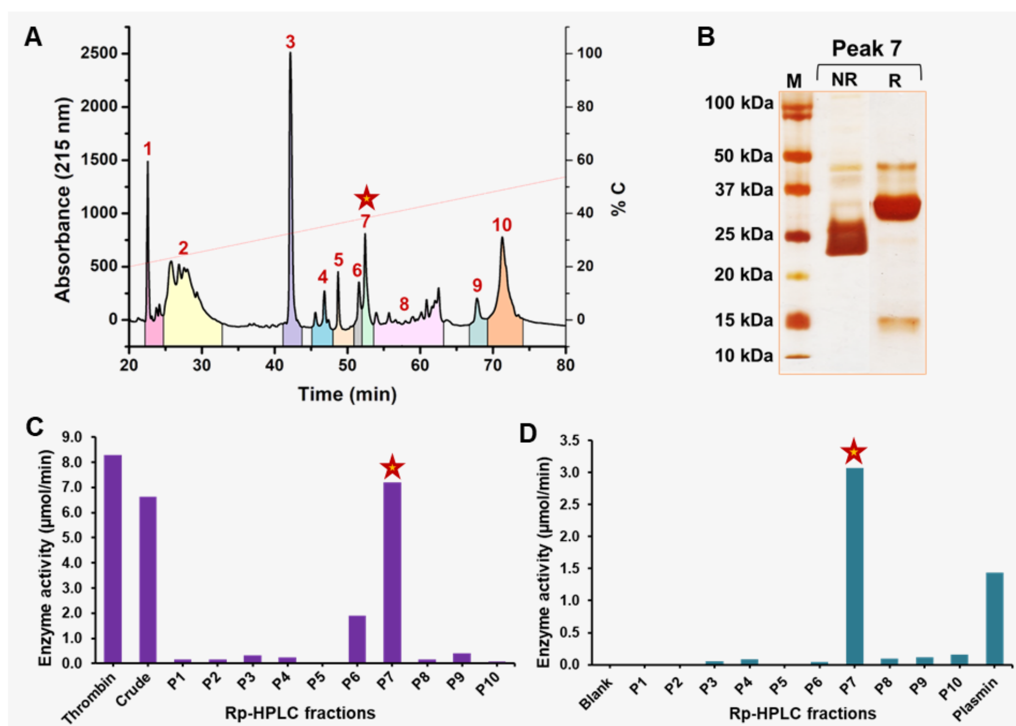

Figure 6.1. Isolation and screening of haemostatically active protein from *Trimeresurus erythrurus* venom (A) Reverse phase chromatogram of crude venom of *Trimeresurus erythrurus*. Crude venom (1 mg) was loaded on Symmetry C18 column and fractionation was carried on with a linear gradient of 20-65% acetonitrile containing 0.1% TFA. **(B) SDS-PAGE of Peak 7.** Five microgram of Peak 7 was loaded on 12.5% Tris-glycine gel and electrophoresis was performed in both non-reducing (NR) and reducing (R) condition. M represents standard protein ladder. **(C) Thrombin-like activity of Rp-HPLC fractions.** Thrombin (0.5 nM in reaction) was taken as positive control. **(D) Plasmin-like activity of Rp-HPLC fractions.** Plasmin (10 nM in reaction) was taken as positive control. One unit of enzyme activity was expressed in µmol of product formed per minute for both the assays.

Since Peak 7 was a mixture of protein, it was subjected to further purification by a second chromatographic step. Re-chromatography of Peak 7 revealed a prominent and sharp peak which was named as **Erythrofibrase** (Figure 6.2A). SDS-PAGE profile showed single prominent band at ~30 kDa in both non-reducing and reducing condition suggesting erythrofibrase to be pure protein (Figure 6.2B). Further, erythrofibrase showed both thrombin-like and plasmin-like activity significantly more than Peak 7 and

crude venom suggesting increase in enzyme activity with the increase in purity (Figure 6.2C and 6.2D).

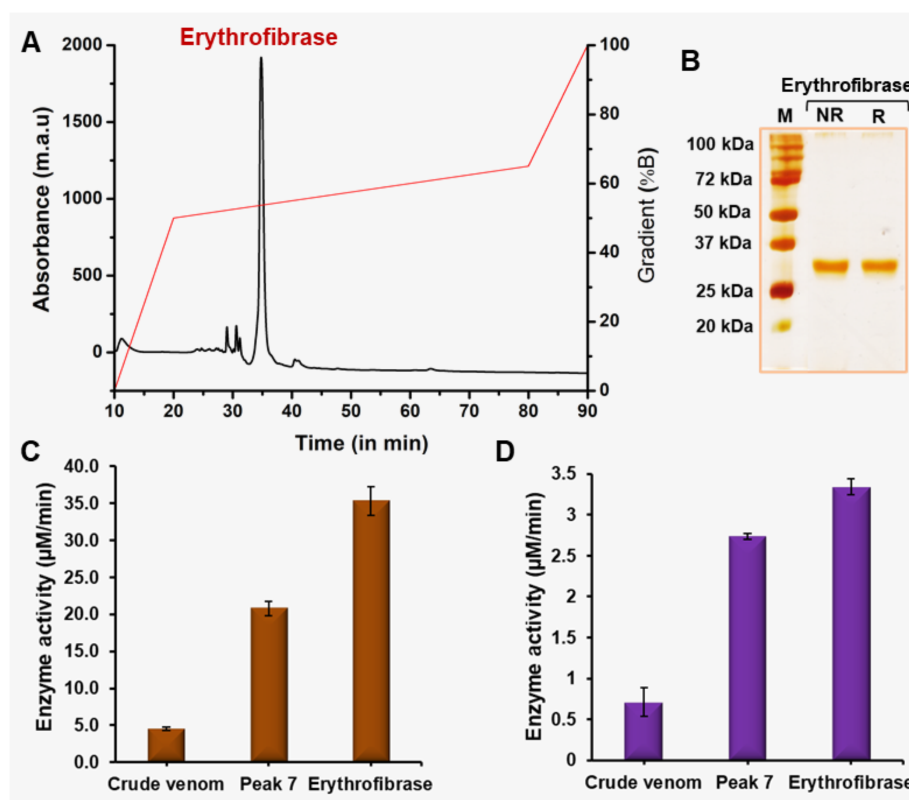

Figure 6.2. Purification of erythrofibrase from *Trimeresurus erythrurus* venom. (A) Re-chromatography of Peak 7. Peak 7 (50 µg) was loaded on Acclaim C18 column and fractionation was carried out with a gradient of 45-65%. **(B) 12.5% tris-glycine SDS-PAGE of erythrofibrase. M** represents standard protein ladder. **NR** and **R** represents non-reducing and reducing conditions respectively. **(C) Thrombin-like activity of erythrofibrase. (D) Plasmin-like activity of erythrofibrase.** Each data point represents mean ± SD of three independent experiments.

In solution trypsin digestion and ESI LC-MS/MS of erythrofibrase resulted in identification of 18 peptide fragments of varying length. The detailed summary of identified peptide fragments of erythrofibrase is enlisted in table 6.1. Based on sequence homology of peptide fragments, erythrofibrase was identified to be homologous to alpha-fibrinogenase **albofibrase** (Accession no. P0CJ41) with a molecular weight of 28 kDa. Albofibrase is a serine protease composed of 258 amino acids which was previously cloned from a venom gland cDNA library of *Trimeresurus albolabris* [219]. Sequence alignment of peptides with the reported sequence of albofibrase (from NCBI) is shown in figure 6.3. It was observed that all the amino acid residue from position 81 to 258 of albofibrase were identified in erythrofibrase. However, no peptide fragment was identified with NxT residue which represents *N*-linked glycosylation site in albofibrase.

Table 6.1. Peptide sequence of trypsin digested fragments of erythrofibrase obtained by LC-MS/MS analysis.

Protein identity	Peptide fragements	Position	No. of peptides	z	MH+[Da]	Score
Alpha-fibrinogenase albofibrase (P0CJ41)	KLLNEDEQIR	81-90	1	3	1257.68	294.83
	KLLNEDEQIRNPK	81-93	2	3	1597.85	
	LLNEDEQIR	82-90	2	2	1129.58	
	LLNEDEQIRNPK	82-93	2	3	1468.78	
	LLNEDEQIRNPKEK	82-95	1	4	1725.91	
	EKFICPNK	94-101	1	2	4344.26	
	KSNEILDKDIMLIK	102-115	2	4	1675.93	
	SNEILDK	103-109	1	2	2764.27	
	SNEILDKDIMLIK	103-115	2	2	1531.84	
	SNEILDKDIMLIKLDSPVSNSAHIAPLSLPSSPPSVGSVCR	103-143	2	5	4344.26	
	DIMLIK	110-115	2	1	732.43	
	DIMLIKLDSPVSNSAHIAPLSLPSSPPSVGSVCR	110-143	2	4	3544.85	
	LDSPVSNSAHIAPLSLPSSPPSVGSVCR	116-143	2	3	2831.44	
	IMGWGSTTPIEVTYPDVPYCANINLLDDAECKPGYPELLPEYR	144-187	3	4	4957.34	
	TLCAGIVQGGK	188-198	2	1	1103.59	
	TLCAGIVQGGKDTCGGDSGGPLICNEK	188-214	2	2	2764.27	
	DTCGGDSGGPLICNEK	199-214	2	2	1679.70	
	LHGIVSYGGHPCGQSHKPGIYTNVFDYNDWIQSIIAGNTDATCLS	214-248	1	5	4963.32	

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P0CJ41:      VVGGDECNINEHHSLVAIFNSTGFFCSGTLINQEWVVTAAHCDSKNFKMKFGAHSKKLLNEDEQIRNPK
This study:  -------------------------------------------------------KLLNEDEQIRNPK

P0CJ41:      EKFICPNKKSNEILDKDIMLIKLDSPVSNSAHIAPLSLPSSPPSVGSVCRIMGWGSTTPIEVTYPDVPY
This study:  EKFICPNKKSNEILDKDIMLIKLDSPVSNSAHIAPLSLPSSPPSVGSVCRIMGWGSTTPIEVTYPDVPY

P0CJ41:      CANINLLDDAECKPGYPELLPEYRTLCAGIVQGGKDTCGGDSGGPLICNEK
This study:  CANINLLDDAECKPGYPELLPEYRTLCAGIVQGGKDTCGGDSGGPLICNEK

P0CJ41:      LHGIVSYGGHPCGQSHKPGIYTNVFDYNDWIQSIIAGNTDATCLS
This study:  LHGIVSYGGHPCGQSHKPGIYTNVFDYNDWIQSIIAGNTDATCLS
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Figure 6.3. Alignment of peptide fragments identified from LC-MS/MS of erythrofibrase with the sequence of alpha-fibrinogenase albofibrase (accession no. P0CJ41). Different colours represents different digested fragments.

6.2.2. Functional characterization of erythrofibrase

Erythrofibrase showed amidolytic activity on both S2238 and S2251 specific for thrombin and plasmin in dose dependent manner inferring thrombin-like and plasmin like activity of erythrofibrase. The thrombin-like and plasmin-like activity was observed to be increasing with the increase in dose of erythrofibrase with highest activity of 35.3 ± 0.924 µmol/min and 3.34 ± 0.096 µmol/min respectively at 10 µg/ml dose (Figure 6.4A and B). Moreover, erythrofibrase did not cause aggregation of platelet rich plasma unlike thrombin and crude venom (Figure 6.4C). Also, the clotting time of platelet poor plasma (PPP) incubated with erythrofibrase was found to be similar to normal clotting time

(Figure 6.4D) indicating that erythrofibrase does not affect the calcium induced coagulation of PPP. Moreover, erythrofibrase showed 12% haemolysis of goat red blood cells at a dose of 1 µg/ml suggesting its potential to rupture cell wall (Figure 6.4E). Further, at a dose of 10 µg/ml, erythrofibrase showed 1.56 ± 0.207 µmol/min plasminogen activation which was significantly higher than plasminogen activation of crude venom i.e., 0.48 ± 0.06 µmol/min (Figure 6.4F).

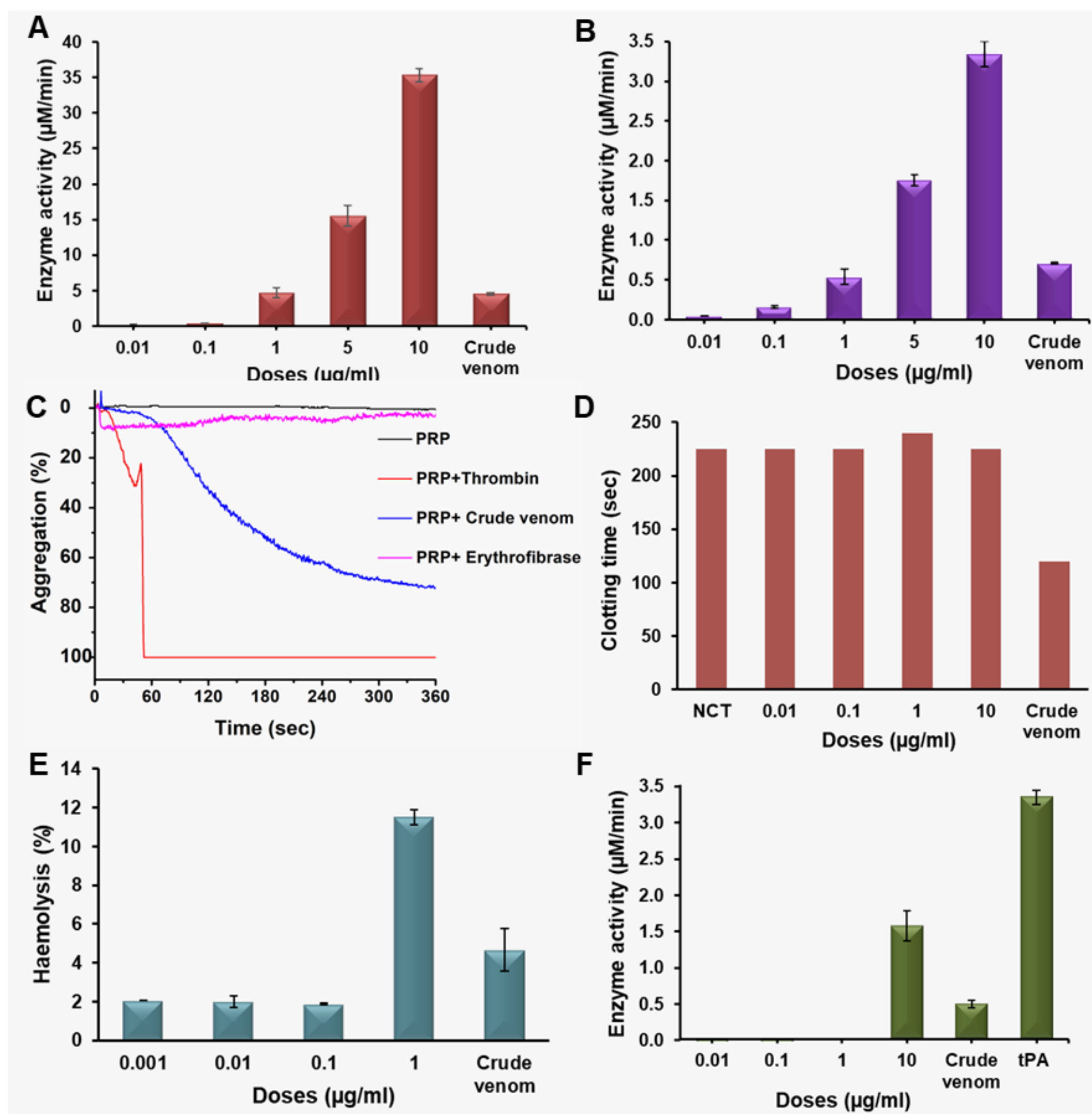

Figure 6.4. Functional characterization of erythrofibrase. (A) Dose dependent thrombin-like activity. (B) Dose dependent plasmin-like activity. **(C) Platelet aggregation assay.** Thrombin (1.34 nM) was taken as positive control. **(D) Recalcification Time (RT).** NCT-Normal clotting Time. **(E) Haemolytic activity assay.** Haemolysis of distilled water was considered as 100%. **(F) Plasminogen activation assay.** tPA (10 nM) was taken as positive control. Crude venom was taken as additional control for each set of experiment.

6.2.3. Effect of erythrofibrase on fibrinogenolytic and fibrinolytic system

Since erythrofibrase shows thrombin-like activity, its effect on the natural substrate of thrombin i.e., fibrinogen was assessed by fibrinogenolytic activity assay. SDS-PAGE profile of untreated fibrinogen shows three prominent bands Aα, Bβ and γ (Figure 6.5). Upon treatment with thrombin, mild shift in the position of Aα and Bβ band was observed pointing out reduction in chain length. (Figure 6.5A). Markedly, fibrinogen treated with 10 µg of erythrofibrase showed partial degradation of Aα band post 24 hours of incubation suggesting mild fibrinogenolytic activity of erythrofibrase (Figure 6.5B). Extended incubation till 48 hour showed complete degradation of Aα band (marked by red arrow in figure 6.5B) which was similar to that of plasmin treated fibrinogen.

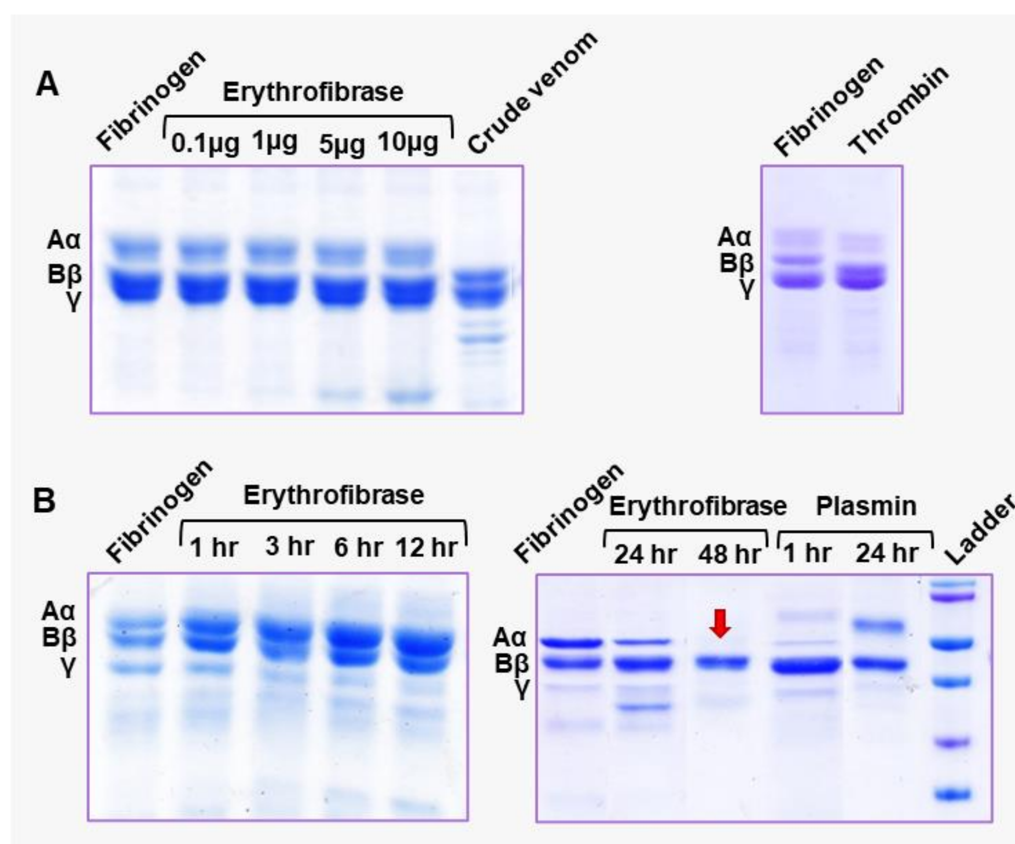

Figure 6.5. Fibrinogenolytic activity of erythrofibrase. (A) Dose dependent (B) Time dependent fibrinogenolytic activity. Thrombin (10 u/ml) and Plasmin (1 µg) was taken as positive control and Fibrinogen without any treatment was taken as negative control. Ladder represents standard protein markers from molecular weight range 20-72 kDa.

The fibrinogenolytic activity of erythrofibrase was further assessed by reverse phase chromatography. Rp-HPLC profile of untreated fibrinogen showed presence of two prominent peak labelled as 1 and 2 (Figure 6.6). Fibrinogen treated with thrombin also showed peak 1 and 2 with same retention time. However, upon treatment of

fibrinogen with erythrofibrase, the intensity of peak 1 was considerably reduced suggesting probable degradation of proteins present in peak 1 while peak 2 remained unchanged (Figure 6.6). Further, Rp-HPLC profile of fibrinogen treated with plasmin showed presence of only peak 2 in the profile indicating complete degradation of proteins present in peak 1. Untreated erythrofibrase, thrombin and plasmin showed peaks away from the zone of fibrinogen peaks. Cumulative results of SDS-PAGE profile and Rp-HPLC profile suggests Aα band degrading activity of erythrofibrase which showed more similarity with plasmin than thrombin. Since the protein was purified from *Trimeresurus erythrurus* venom and showed α-fibrinogenase activity, it was named as **Erythrofibrase.**

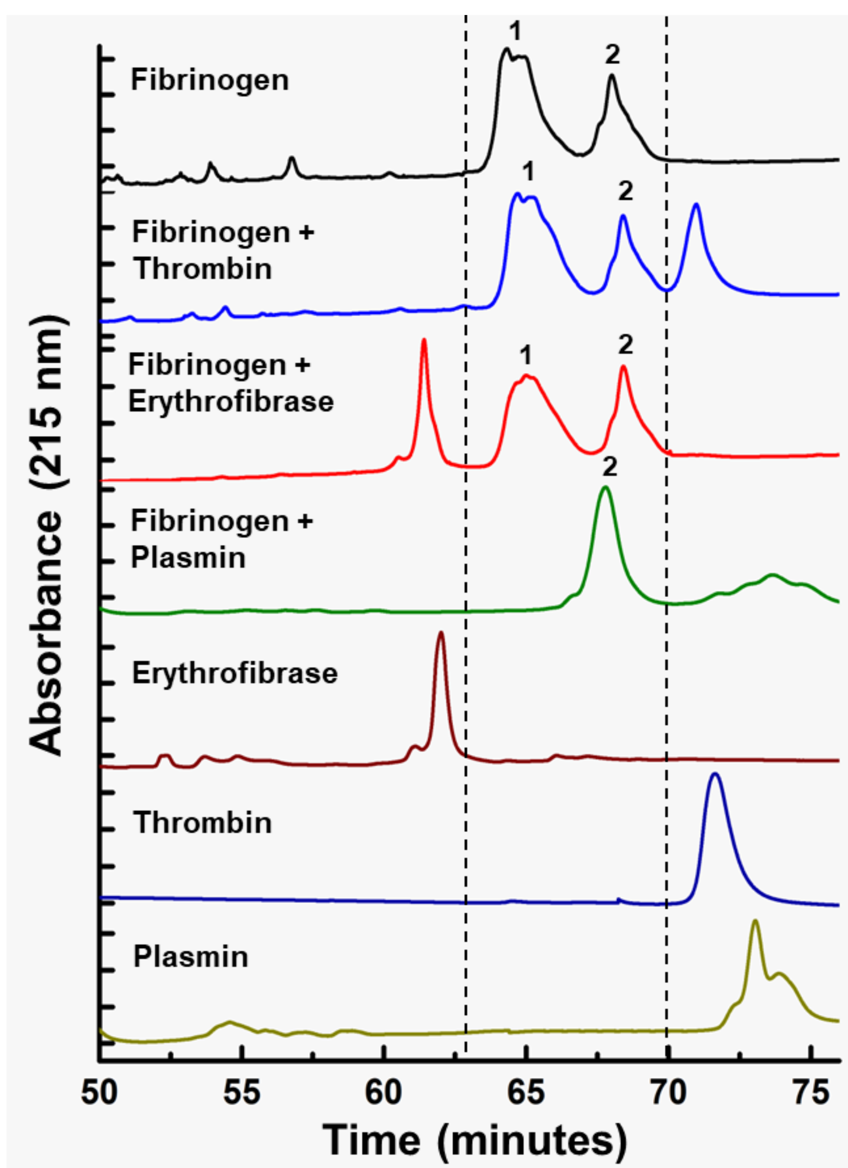

Figure 6.6. Rp-HPLC profile showing fibrinogenolytic activity of erythrofibrase. Cleavage pattern of fibrinogen was analyzed by Rp-HPLC. Chromatographic separation was carried out using a gradient of 20-65% acetonitrile at a flow rate of 0.2 ml/min.

Fibrinolytic activity was assessed by recording zone of clearance in the fibrin plate incubated with erythrofibrase. Appearance of small zone of clearance around the wells (marked by red circles) containing 10 µg and 15 µg of erythrofibrase suggests mild fibrinolytic activity of erythrofibrase (Figure 6.7).

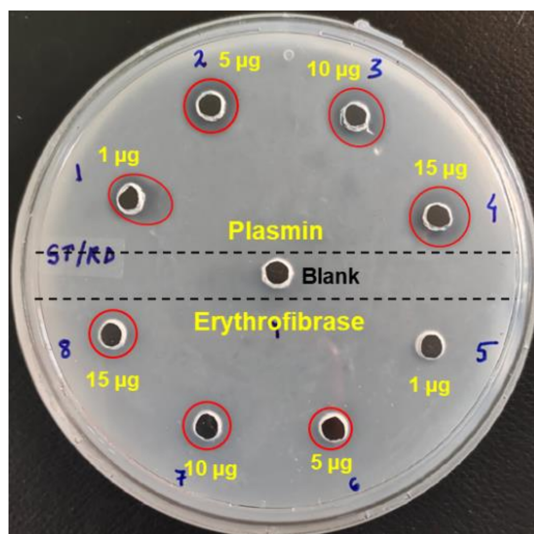

Figure 6.7. Fibrinolytic activity of erythrofibrase. Fibrin plate was prepared with fibrinogen (5 mg/ml) and Thrombin (3 u/ml). Red colour circles around the wells represents zone of clearance.

Since erythrofibrase possesses fibrinolytic activity, its effect on formation and dissolution of plasma clot was evaluated. The coagulation pattern of platelet poor plasma (PPP) induced by various treatments were assessed by manual observation of clotting (Figure 6.8 and Table 6.2). Platelet poor plasma (PPP) incubated with $CaCl_2$ (Normal clotting time) and thrombin resulted in formation of a solid stable thrombus after 7 minutes and 3 minutes respectively (Figure 6.8 A2 and A3). Further, no clot formation was observed in plasma treated with crude *Daboia russelii* venom (Figure 6.8 B1). However, treatment with crude *Trimeresurus erythrurus* venom resulted in continuous formation of a jelly-like unstable clot which gets dissolved readily (Figure 6.8 B2). Similarly, treatment of plasma with erythrofibrase resulted in formation of a soft jelly-like thread which gets dissolved within 5 seconds of formation, with no subsequent clot formation, indicating low plasma clotting/ fibrinogen clotting activity of erythrofibrase.

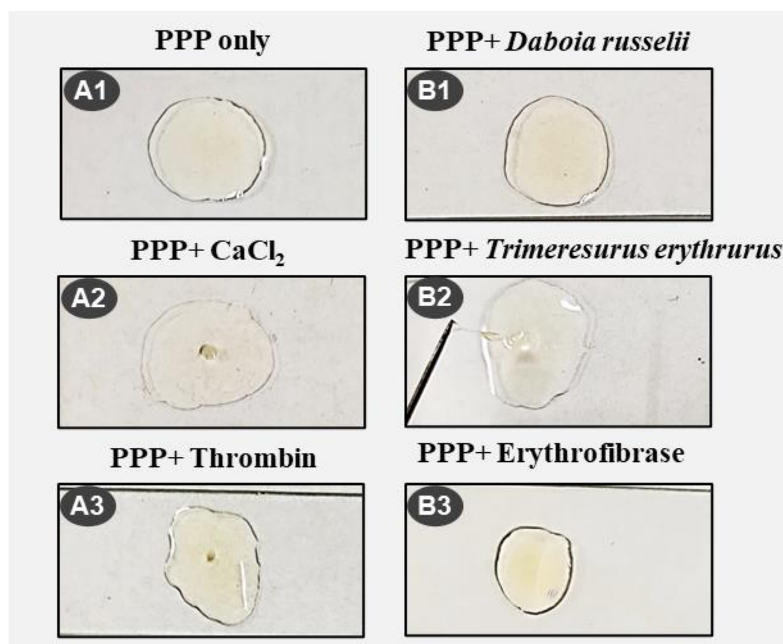

Figure 6.8. Plasma clot formation and dissolution property of erythrofibrase. Coagulation pattern of platelet poor plasma (PPP) upon treatment with crude venoms and purified protein was observed. **A1-A3** represents control set and **B1-B3** represents treatment set of experiment. Coagulation time and nature of clot formed was recorded for each experiment.

Table 6.2. Summary of coagulation pattern of platelet poor plasma (PPP) upon treatment with various components.

Protein Sample	Treatment	Clotting time	Clot dissolution time	Nature of clot	Image
-	$CaCl_2$	7 min	-	Thrombus	A2
Daboia russelii	-	No clot	-	No clot	B1
Daboia russelii	$CaCl_2$	3 min	-	Thrombus	
Trimeresurus erythrurus	-	25 sec 7 min 19 min 23 min	30 sec 15 min 20 min 24.5 min	Continuous formation and dissolution of jelly like clot	B2
Trimeresurus erythrurus	$CaCl_2$	7 min	-	Thrombus	
Erythrofibrase	-	25 sec	30 sec	Thin jelly like clot	B3
-	Thrombin	3 min	-	Small stable clot	A3

6.2.4. Sequence alignment of erythrofibrase and structure prediction:

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Albofibrase     VVGGDECNINEHHSLVAIFN--STGFFCSGTLINQEWVVTAAHCDSK----------NFKMKFGAHSK
GPV-TL1         VIGGDECNINEHRFLVALYDVWSGDFLCGGTLINKEYVLTAAHCETR----------NMYIYLGMHNK
Chitibrisin     VIGGDECNINEHRSLVVLFN--SSGALCGGTLINQEYVLTAAHCDMP----------NMQILLGVHSA
Stejnobin       VIGGDECNINEHRFLVALYDFWSGDFLCGGTLINQEYVLTAAHCKTR----------NMYIYLGMHNK
Stejnifibrase1  IIGGDECNIDEHRFLVALYK--SGRFRCGGTLINQEWVLTAAHCDRR----------NMEIKLGMHSK
Batroxobin      VIGGDECDINEHPFLAFMYY--SPRYFCGMTLINQEWVLTAAHCNRR----------FMRIHLGKHAG
Ancrod          VIGGDECNINEHRFLVALYDSTTRNFLCGGVLIHPEWVITAKHCNKK----------SMVLYLGKHKQ
Thrombin        IVEGSDAEIGMSPWQVMLFRKSPQELLCGASLISDRWVLTAAHCLLYPPWDKNFTENDLLVRIGKHSR
                   *                         **      *  ** **  ---60loop---       * *

Albofibrase     KLLNEDEQIRNPKEK-FI-CPNKKSNEILDKDIMLIKLDSPVSNSAH-IAPLSLPSSPPSVGSVCR
GPV-TL1         NVQFDDEQRRYPKKKYFFRCSN--NFTRWDKDIMLIRLNRPVRNSEH-IAPLSLPSSPPSVGSVCR
Chitibrisin     SVLNDDEQARDPEEKYF--CLSSNNDTKWDKDIMLIRLNRPVNNSVH-IAPLTLPSSPPRLGAICR
Stejnobin       SVQFDDEQRRYPKKKYFFRCRN--NFTRWDKDIMLIRLNRPVRNSAH-IAPLSLPSSPPTVGSVCR
Stejnifibarse1  NVPNEDEQRRVPKEKFF--CDSNKNYTQWNKDIMLIRLNSPVNNSTH-IAPLSLPSSPPIVGSVCR
Batroxobin      SVANYDEVVRYPKEK-FI-CPNKKKNVITDKDIMLIRLDRPVKNSEH-IAPLSLPSNPPSVGSVCR
Ancrod          SVKFDDEQERFPKEKHFIRC-NKP-RTRWGEDIMLIRLNKPVNNSEH-IAPLSLPSNPPIVGSVCR
Thrombin        TRYERNIEKISMLEKIYIHPRYNWRENL-DRDIALMKLKKPVAFSDY-IHPVCLPDRETQAGYKGR
                                              ** *   *   *         * *   **

Albofibrase     IMGWGSTTPI-----EVTYPDVPYCANINLLDDAECKPGYPELLPE-YRTLCAGIVQ---GGK
GPV-TL1         VMGWGTITSP-----NETLPDVPRCANINLLNYTVCRGVFPRLPAR-SRTLCAGVLQ---GGI
Chitibrisin     IMGWGAITSP-----NETYPDASQCANINILRYSLCQAVYRGMPAQ-SRIVCAGILR---GGK
Stejnobin       VMGWGTITSP-----NETLPDVPRCANINLFNYTVCHGVFPWLPAR-SRILCAGVLQ---GGI
Stejnifibrase1  IMGWGTITSP-----NETYPDVPHCANINLFNYTVCHGAHAGLPAT-SRTLCAGVLE---GGK
Batroxobin      IMGWGAITTS-----EDTYPDVPHCANINLFNNTVCREAYNGLPAK---TLCAGVLQ---GGI
Ancrod          VMGWGSINKY-----IDVLPDEPRCANINLYNYTVCRGVFPRIPKK-SKILCAGDLQ---GRL
Thrombin        VTGWGNLKETWTANVGKGQPSVLQVVNLPIVERPVCKDSTRIR-IT-DNMFCAGYKPDEGKRG
                  ***                        *         *                ***

Albofibrase     DTCGGDSGGPLICNEKLH------GIVSYGGHPCGQSHKPGIYTNVFDYNDWIQSIIAGNTDATCLS
GPV-TL1         DTCKRDSGGPLICNGQLQ------GVVFWGPKPCAQPRKPALYTKVFNHLDWIQSIIAGNTTVTCPP
Chitibrisin     GSCKGDSGGPLICNAQLQ------GIVSAGGDPCAQPRVPVLYIRVFDYTDWIQSIIEGNRTVTCPP
Stejnobin       DTCKRDSGGPLICNGQFQ------GIVSWGPKPCAQPRKPALYTKVFDHLDWIQSIIAGNTTVTCPP
Stejnifibrase1  DTCKGDSGGPLICNGQFQ------GIVSWGGHPCAQPREPGVYTKVFDHLDWIQNIIAGSTTATCPL
Batroxobin      DTCGGDSGGPLICNGQFQ------GILSWGSDPCAEPRKPAFYTKVFDYLPWIQSIIAGNKTATCP-
Ancrod          DSCHCDSGGPLICSEEFH------GIVYRGPNPCAQPDKPALYTNIFDHLHWILSIMAGNAT--CYP
Thrombin        DACEGDSGGPFVMKSPFNNRWYQMGIVSWGE-GCDRDGKYGFYTHVFRLKKWIQKVIDQFGE-----
                  *   *****                 *     *     *     #  *          **
                S1                        S2               S3
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Figure. 6.9. Multiple sequence alignment of thrombin-like enzymes. Albofibrase (accession no. P0CJ41) from *T. albolabris*, **GPV-TL1** (accession no. A7LAC6) from *Trimeresurus albolabris*, **chitibrisin** (accession no. P0DJF6) from *T. albolabris*, **stejnobin** (accession no. Q8AY81) from *V. stejnegeri*, **stejnofibrase 1** (accession no. AAN52348.1) from *V. stejnegeri*. **batroxobin** (accession no. P04971.1) from *Bothrops atrox*, **ancrod** (accession no. P47797.1) from *Calloselasma rhodostoma* and heavy chain of **human α-thrombin** (accession no. 494462). Fully consensus residues were marked in pink color along with asterisk (*). Yellow and green colours represents residues with >=75% and >=50% homology respectively. The residues of catalytic triad i.e., Histidine, Aspartate and Serine were highlighted with red colour asterisk (*). Cysteine residues were highlighted in red bold font. Black double headed arrow represents peptide fragments identified in the erythrofibrase. Red double headed arrow represents C-terminal flag.

Since the amino acid residues of erythrofibrase showed 100% similarity with albofibrase and all the residues of erythrofibrase could not be retrieved, multiple sequence alignment and structure prediction was done using sequence of albofibrase. Multiple sequence alignment of amino acid residues of albofibrase with other serine proteases show high sequence similarity with at least 40 consensus residues (Figure 6.9). Albofibrase showed 27.5% similarity with thrombin and about 76.9% similarity with other SVTLEs (GPV-TL1, stejnobin, ancrod and batroxobin). Further, about 64.1% similarity was observed between albofibrase and Stejnifibrase 1, an alpha-fibrinogenase enzyme isolated from *Viridovipera stejnegeri*. The active site residues His57, Asp102 and Ser206 (Chymotrypsin numbering system) forming catalytic triad were conserved in all the sequences. The major difference was obsereved in number of cysteine residues responsible for formation of disulfide bonds. Unlike 7 cysteine residues in thrombin, albofibrase possesess 12 cystein residues which were conserved in all the SVTLEs (Figure 6.9). S1, S2 and S3 conataing Asp189, Gly216 and Gyl/Ala 226 represents the subsites which binds to substate. Na^{2+} binding Tyr255 of thrombin is replaced by Pro255 in all SVTLEs. Also, all the SVTLE has a C-terminal extension of 5 amino acid residues which is absent in thrombin.

The modeled tertiary structure of albofibrase showed typical β/β hydrolase fold similar to thrombin and plasmin (Figure. 6.10). The catalytic site lies at the junction of β barrels and is consisted of His67, Asp102 and Ser195 residues which are marked in red (Figure. 6.10A). Quality of the generated structure was validated by generating Ramachandran plot which shows the statistical distribution of the φ-ψ torsion angles of the protein backbone (Figure 6.10B). It is evident from the Ramachandran plot of albofibrase that 88.6% amino acid residues reside in most favoured region and 10.9% residues remain in additional allowed regions with no residues in disallowed region (Figure. 6.10B). Albofibrase contains 12 cysteine residues at conserved positions forming disulfide bridges between pairs of cysteine residues 31-163, 50-66, 98-256, 142-210, 174-189 and 200-225 which are highlighted in orange (Figure 6.10A). Further, the structure of human thrombin and superimposed structure of albofibrase with thrombin is shown in figure 6.10C and D respectively for comparison.

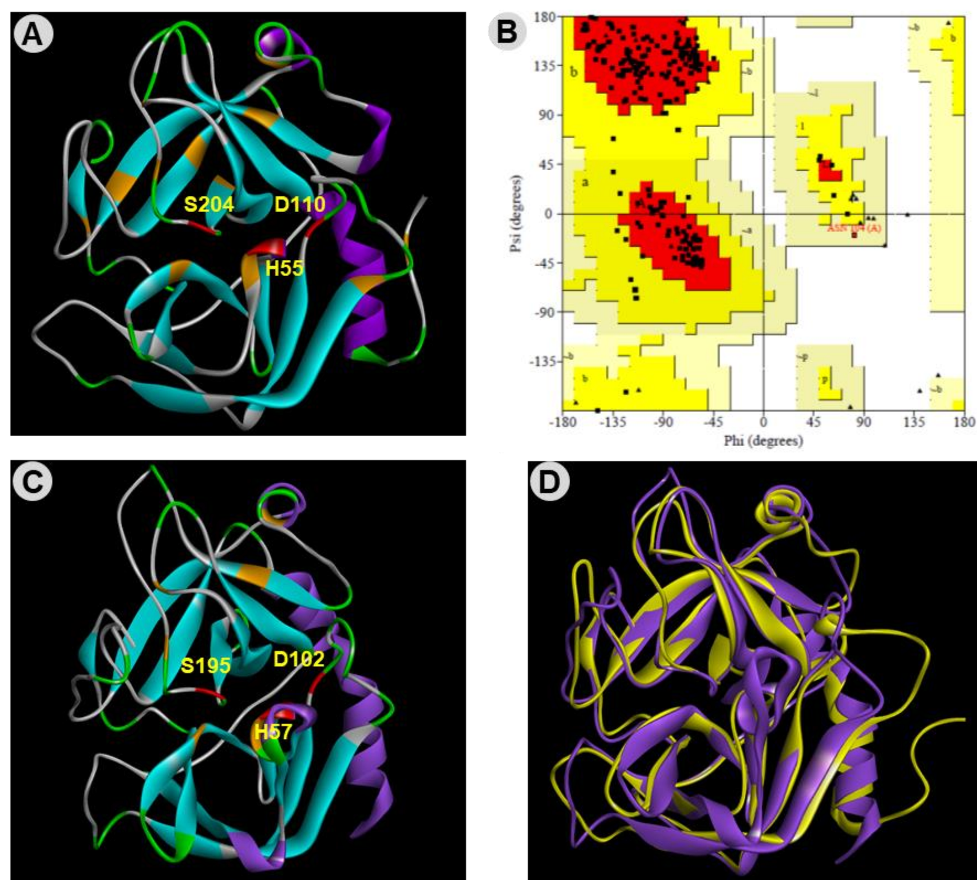

Figure. 6.10. Homology modelling and structure prediction of albofibrase. (A) Modelled structure of albofibrase. (B) Ramachandran plot of albofibrase to show quality of modelled structure. (C) Structure of human alpha-thrombin (PDB ID: P00734). Alpha-helices, β-sheets, turns and loops are highlighted in purple, turquoise blue, white and green color respectively. Catalytic residues His (67), Asp (102), Ser (195) are highlighted in red. Disulfide residues are marked in orange colour. **(D) Superimposed structure of thrombin with albofibrase.** Yellow and purple ribbon represent albofibrase and thrombin respectively.

6.2.5. Immuno-reactivity of erythrofibrase with Antivenom:

The immuno-reactivity of erythrofibrase with Indian polyvalent antivenom was assessed by neutralization experiments. At a ratio of 1:100 (w/w), Indian polyvalent antivenom showed 35% inhibition of thrombin-like activity (65% residual activity) of erythrofibrase (Figure 6.11A). However, polyavlent antivenom did not show any neutralization of plasmin-like activity of erythrofibrase (Figure 6.11B) at any dose indicating reduced efficacy of polyvalent antivenom in neutralizaing the toxic activities of erythrofibrase. Moreover, Indian polyvalent antievnom showed complete recognition of erythrofibarse band in western blotting (Figure 6.11C).

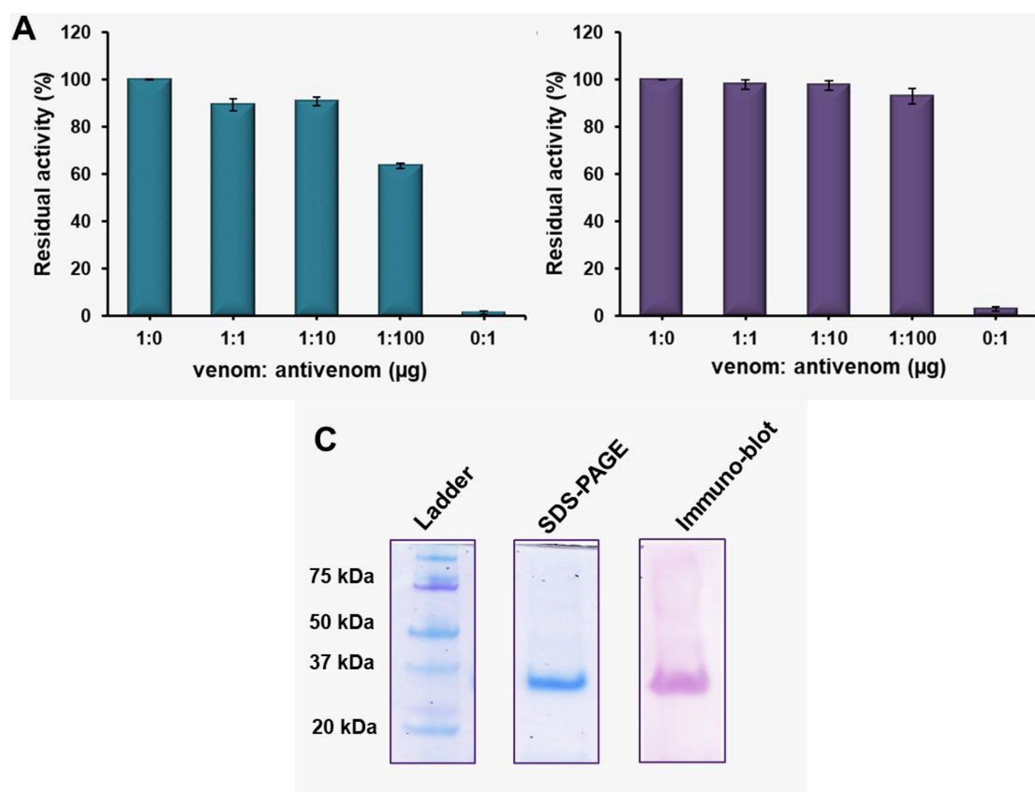

Figure 6.11. Immuno-reactivity of erythrofibrase with Indian polyvalent antivenom. (A) Neutralization of thrombin-like (B) Neutralization of plasmin-like activity. Erythrofibrase (1 µg) and Premium serum polyvalent antivenom was incubated for 1 hour at 37 ºC in different ratios, followed by respective enzymatic assays. Each data point represents mean ± SD of three independent experiments. **(C) Immuno-blotting of erythrofibrase.** Western blotting of erythrofibrase was performed using Indian polyvalent antivenom as primary antibody.

6.3. Discussion

Trimeresurus erythrurus is one of the most prevalent green pit viper in north-eastern India [123] which causes painful swelling and incoagulable blood for many days in envenomated victims (discussed in chapter 4). The envenomation mediated pathophysiological interventions might be the results of toxicity exerted by some potent toxin molecules or a combined effect of various snake venom toxins. With the help of proteomics studies, all the major and minor toxins present in the venom has been identified (discussed in chapter 5). The study revealed phospholipases A_2 and snake venom serine proteases as the most abundant protein families of the venom. However, the most potent toxin responsible for haemotoxic interventions in the victims and their mechanism of action is yet to be explored. Green pit vipers are widely reported to possess thrombin-like enzymes (TLEs) in their venom causing consumptive coagulopathy,

however, their mechanism of action shows considerable variation across species. Purification and characterization of haemostatically active proteins from *T. erythrurus,* in particular thrombin-like enzymes might provide with detailed understanding of pathophysiological abnormalities caused in patients along with shedding lights on their cross-reactivity with antivenom.

Thrombin and plasmin are the key components of haemostatic system targeting fibrin polymerization and fibrinolysis respectively to maintain normal haemostasis. Green pit viper envenomated patients show abnormal fibrinogen and fibrin expanse in the blood which might be associated with the peculiar functioning of venom proteins targeting fibrinogenolytic and fibrinolytic systems [242]. As such, the purification of haemostatically active protein was undertaken on the basis of thrombin-like and plasmin-like potential of the proteins. Erythrofibrase was purified to homogeneity from *Trimeresurus erythrurus* after a two-step reverse phase chromatography venom showing a single prominent band at ~30 kDa in SDS-PAGE profile. In solution trypsin digestion of erythrofibrase and LC-MS/MS analysis of generated peptide fragments resulted in identification of a protein Alpha-fibrinogenase albofibrase with a molecular weight of 28 kDa. Albofibrase is a thrombin-like serine protease composed of 258 amino acids which was previously cloned from a venom gland cDNA library of *Trimeresurus albolabris* [219]. In a subsequent study, albofibrase was expressed in *Pichia pastoris* system and the recombinant albofibrase protein showed molecular weight of 30 kDa with 2.2 kDa glycosylation [243]. Erythrofibrase isolated from *T. erythrurus* also showed high similarity in molecular weight and peptide fragments, therefore was further characterized.

Snake venom thrombin-like enzymes are named after serine protease thrombin because of their functional similarities [160]. Thrombin is a multifunctional enzyme which plays central role in the functioning of haemostasis [143]. One of the significant functions include activation of platelets for the formation of platelet plug in the primary haemostasis. Thrombin activates PAR-1 and PAR-4 receptors of platelet surface by N-terminal proteolytic cleavage, ultimately leading to thrombin-induced platelet aggregation [213, 214]. Snake venom thrombin-like enzyme, TA-2 isolated from *Trimeresurus albolabris* have also reported to induce platelet aggregation via ADP and collagen receptors [164]. However, erythrofibrase did not show any effect on aggregation

of platelet poor plasma unlike the crude venom of *T. erythrurus*. Further, erythrofibrase was assessed for its amidolytic activity on small synthetic substrates. Erythrofibrase showed dose dependent amidolytic activity on chromogenic substrates S2238 (H-D-Phe-Pip-Arg-*p*NA) along with S2251 (H-D-Val-Leu-Lys-*p*NA) which are specific for thrombin and plasmin respectively suggesting their thrombin-like and plasmin-like activity. Furthermore, erythrofibrase did not alter the recalcification time of platelet poor plasma indicating no effect in Ca^{2+} induced coagulation of plasma. This points out the functional dissimilarity of erythrofibrase with thrombin which along with Ca^{2+} ions, plays an important role in coagulation cascade by activation of factor V, VIII, XI and XIII ultimately promoting coagulation. Mild haemolytic activity of erythrofibrase can be correlated with the cell wall rupture potential and initial bleeding of bite site in envenomated patients [109]. Further, erythrofibrase showed mild activity in plasminogen activation assay which is often observed in thrombin-like enzymes [243].

Although thrombin is known to function at various points in haemostatic system, its primary function is fibrin polymerization and clot formation at the end of secondary haemostasis. It is the last proteolytically activated enzyme of the coagulation cascade which cleave Aα and Bβ chain of fibrinogen releasing fibrin monomers and fibrinopeptides A (FpA) and B (FpB) [142, 143]. The fibrin monomers spontaneously polymerize over the loose platelet plug to form a thrombus. Thrombin also activates factor XIII, which further aid in cross-linking of fibrin monomers, thereby stabilizing the clot [142]. Snake venom thrombin-like enzymes (SVTLEs) are serine endopeptidase which also cleave Arg-Lys bond releasing FpA or FpB or sometimes both from the fibrinogen, however, unlike thrombin, they do not usually activate factor XIII [148, 150, 161]. The fibrin clot formed as a result does not get stabilized and therefore, readily gets dissolved by endogenous fibrinolytic system [145, 150]. Such thrombin-like enzymes show high fibrinogen-clotting activity *in vitro*. Some examples of fibrinogen-clotting enzymes isolated from green pit vipers include albolabrase, purpurase, stejnobin etc. [165, 167, 244]. Fibrinogenolytic activity of erythrofibrase on bovine fibrinogen was assessed and it was observed that erythrofibrase could completely degrade the Aα band of bovine fibrinogen (at 48 hours incubation) without affecting Bβ and γ chains indicating it to be an **α-fibrinogenase enzyme**. Fibrinogenases are direct fibrinogenolytic enzymes which degrade fibrinogen into small fragments rendering it unclottable by thrombin [245]. Unlike fibrinogen-clotting enzymes, they do not convert fibrinogen to fibrin,

instead decrease the coagulability of fibrinogen. Most of the isolated fibrinogenase are α-fibrinogenase with only few which preferentially cleave Bβ chains. Stejnefibrase 1 and 3 isolated from *Trimeresurus stejnegeri*, alpha-fibrinogenase isolated from *Agkistrodon rhodostoma* are to name a few α-fibrinogenase and stejnefibrase 2 is a β-fibrinogenase [245, 246]. Recombinant albofibrase also shows α-fibrinogenolytic activity along with showing plasminogen activation similar to erythrofibrase [243]. Moreover, recombinant chitibrisin, GPV-TL1 and TA-2 shows fibrinogenolytic activity on both Aα and Bβ chains of plasminogen-free human fibrinogen along with showing variable extent of fibrinogen-clotting activity [164, 166, 247]. The Aα band degradation activity of erythrofibrase was comparable to that of plasmin, however, with very low potency. Further, the effect of erythrofibrase on fibrinolytic system was checked by fibrin clot dissolution ability which resulted in mild fibrinolytic activity of erythrofibrase.

The effect of erythrofibrase on plasma clotting was evaluated by manual observation of clot formation. Unlike the stable thrombus formation in case of thrombin treatment, erythrofibrase showed an initial formation of a thread-like structure which gets dissolved within 5 seconds with no subsequent clot formation. This can be correlated with the fibrinogenolytic activity of erythrofibrase which causes depletion of fibrin monomers, as a result of which clot formation could not be completed. On the other hand, crude venom showed repeated formation and dissolution of jelly-like unstable clot indicating presence of fibrinogen-clotting proteins in the venom. Fibrinogen-clotting enzymes causes quick conversion of fibrinogen into insoluble fibrin clots; however, the unstable clot readily gets dissolved by fibrinolytic system [145]. The process of clot formation and dissolution continues until the all the fibrinogen is consumed in the process leading to consumptive coagulopathy [154]. Fibrinogenolytic enzymes like erythrofibrase might be responsible for further acceleration in the exhaustion of fibrinogen causing fibrinogen depletion leading to hypofibrinogenemia. Thus, consumptive coagulopathy and hypofibrinogenemia together can be suggested to causes defibrination syndrome and incoagulable blood observed in the *Trimeresurus erythrurus* envenomated patients.

Based on above experimental outcomes, a schematic model has been proposed for erythrofibrase mediated fibrinogen digestion and its subsequent effect on clot formation (Figure 6.12).

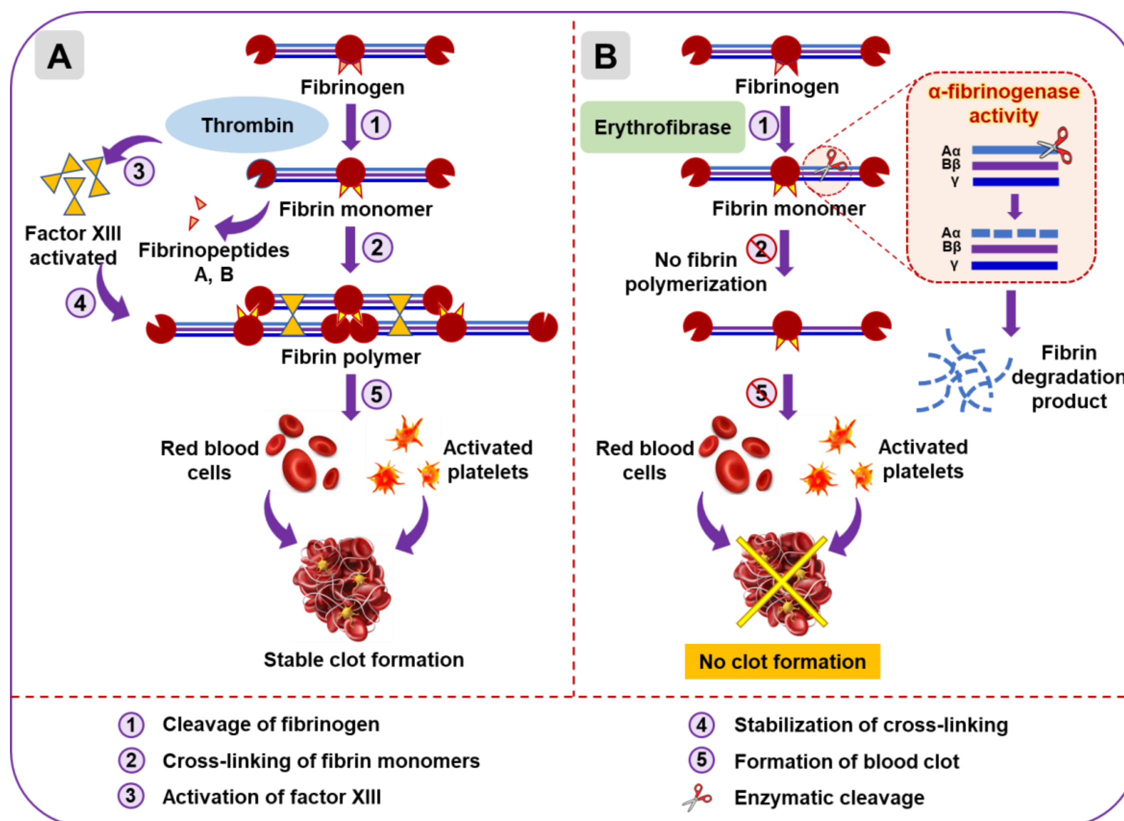

Figure 6.12. Schematic model showing alpha-fibrinogenase activity of erythrofibrase. (A) Cascade of reaction showing Thrombin mediated fibrinogen cleavage and clot formation under normal physiological condition. Thrombin cleaves fibrinogen releasing fibrin monomers which cross-links over platelet plug and RBCs to form blood clot. **(B) Effect of Erythrofibrase on fibrinogen cleavage and clot formation.** Erythrofibrase digests Aα band of fibrinogen, leaving it unavailable for fibrin polymerization and clot formation, thereby, rendering the blood incoagulable.

The envenomation mediated pathology of green pit vipers cannot be reversed with the administration of Indian polyvalent antivenom available in hospitals (discussed in chapter 3). Inefficient neutralization potential of toxic activities of erythrofibrase further justifies the inefficacy of Indian polyvalent antivenom. However, western blotting studies showed complete immuno-recognition of erythrofibrase by antivenom. This suggests presence of immunogenic epitopes, structurally similar to some portion of erythrofibrase molecule in the venom pool used to raise the antivenom. The discrepancy in immuno-blotting and neutralization studies can be discussed in light of the presence of multiple pharmacological sites in a single molecule which might be immunogenic to a varying extent. Presence of multiple pharmacological sites in *Daboia russelii* PLA_2 molecules other than catalytic site has been reported previously [248]. Such

immunogenic pharmacological sites, although can be recognized with specific antibody, does not interfere with the catalytic activity of the molecule.

Structurally, snake venom thrombin-like enzymes (SVTLEs) are serine endopeptidases which belong to trypsin-like serine protease superfamily and peptidase family S1 [249]. They have a typical β/β hydrolase fold, which consists of two β-barrels similar to S1 peptidase serine protease. Each β-barrels contains six beta strands with the insertion of short beta strands and α-helices [150]. Modelled structure of albofibrase also showed similar structural features. The overall three-dimensional structure is divided in two sub-domains N and C, both of which consists of helices and beta strands. The catalytic cleft lies at the junction of two beta barrels (N and C domain) and it is surrounded by various loops. The active site residues His67, Asp102 and Ser195 is conserved in all S1 family proteases [240]. Apart from active site, subsites S1 to S3 containing residues Asp189, Gly216 and Gly/Ala226 (chymotrypsin numbering system) is conserved in all SVTLEs which additionally binds to substrate. Despite the name, thrombin-like enzymes show very less structural similarity with thrombin (29-36%) and with other SVTLEs (57-85%) [150]. Unlike 3 disulfide bridges in thrombin, presence of 6 disulfide bonds contributes to stabilize the native structure of the SVTLEs [150]. Thrombin also possesses an additional three-residue S1 loop which allows substrate with larger side chains to access the catalytic cleft which is absent in SVTLEs [150]. Further, shorter length of 60 loop in SVTLEs leads to formation of narrower groove, unlike the deeper groove in thrombin which facilitates substrate binding. Multiple sequence alignment of albofibrase showed 27.5% similarity with thrombin and 77% similarities with other SVTLEs. Regardless of having high sequence similarity, there exists large functional variation among SVTLEs categorizing them in group A, B and AB [161, 240]. The functional diversity between SVTLEs and thrombin and within SVTLEs is attributed to the variation in both amino acid composition, charge distribution and length of the loops surrounding the active site providing diverse substrate specificity to them [152, 219, 250]. Because of their conserved structure and functional diversity, SVTLEs have been a potential candidate for therapeutic molecules. They are widely used as defibrinogenating agents and anticoagulants for treating patients with thrombosis, cerebral and myocardial infarction, peripheral vascular diseases and acute ischemia [161]. The commercially available SVTLEs are Arvin® from *Calloselasma rhodostoma* and Defribrase® from *Bothrops atrox* [148].

In conclusion, the toxic component of *Trimeresurus erythrurus* targeting haemostatic system of prey leading to incoagulable blood is observed to be a snake venom serine protease erythrofibrase which shows high similarity with alpha-fibrinogenase albofibrase. The characterized protein from our study erythrofibrase acts on the fibrinogen to cleave Aα band causing substantial depletion in coagulable fibrin monomers. The resulting hypofibrinogenemia leads to incoagulable blood in patients which cannot be treated with Indian polyvalent antivenom. This suggests an urgent need for an alternative of current antivenom therapy for green pit viper envenomation such as a region-specific polyvalent/ monovalent antivenom.