

Chapter 5

PARTIAL PURIFICATION AND CHARACTERIZATION OF A β - FIBRINOGENASE FROM INDIAN KING COBRA (*OPHIOPHAGUS HANNAH*) VENOM

Chapter 5

INTRODUCTION:

Cobra (*Naja naja*), Krait (*Bungarus caeruleus*), Russell's viper (*Vipera/Daboia russelli*) and saw-scaled viper (*Echis carinatus*) are considered to be the major poisonous snakes of Indian the sub-continent and their venom has been extensively characterized for biochemical and pharmacological properties including isolation and extensive characterization of several toxins. However, the king cobra (*Ophiophagus hannah*) venom has been greatly ignored. *Ophiophagus hannah*, commonly known as king cobra is the largest and most lethal snake belonging to the family *Elapidae*. A single bite of king cobra can deliver up to 400-500 mg of venom that is several folds higher than its lethal dose, so that one bite may contain up to 15,000 LD₅₀ mice doses (Broad et al., 1979). Although no reports of mortality and morbidity due to king cobra bite has been reported, it is important to note the fact that due to increased confrontation of humans with snakes in recent years attributed by large scale deforestation, the possibility of intervention with king cobra habitat becomes more frequent. In this context, the eventual survival of the victim after the king cobra bite if not treated in time, is practically impossible as it can inject the venom at exceedingly high dose which will ensure killing with in few minutes.

In recent years, many bioactive protein and non-protein, enzymatic and non-enzymatic, regional specific components have been isolated and characterized from the king cobra venom of south-east Asian countries (Weissenberg et al., 1987; Yamakawa & Omori-Satoh, 1988; Tan & Saifuddin, 1990; Chang et al., 1993; Li et al., 1994; Zhang et al., 1994; Chiou et al., 1995; Lee et al., 1995; Huang & Gopalakrishnakone, 1996; Peng et al., 1997; Lin et al., 1999; Chang et al., 2001; Chang et al., 2002; Du et al., 2002; Zhang et al., 2002; He et al., 2004; Chang et al., 2006; Pung et al., 2006; Guo et al., 2007; Rajagopalan et al., 2007; He et al., 2008; Roy et al., 2010; Lee et al., 2011). However, from the Indian subcontinent, king cobra venom is very less explored and only a few low molecular weight non-enzymatic components have been isolated and characterized so far (Gomes & De, 1999; Gomes et al., 2001; Saha et al., 2006).

Enzymes that might exhibit active interference in the process of hemostasis which is one of the important manifestations of post snakebite symptoms have not been studied and characterized. Hence, this study aims at isolation and biochemical characterization of a β -fibrinogenase from the Indian king cobra venom.

MATERIALS AND METHODS

MATERIALS:

King Cobra Venom was obtained from Shri. Dipak Kumar Mitra, Hindustan Snake Park, Kolkata, India. Sephadex G-100 Coarse Gel, CM-Sephadex- C-25, fat free casein, human fibrinogen, epinephrine, ADP and thrombin were obtained from Sigma Chemicals Pvt. Ltd., St. Louis, USA. Fresh blood samples were collected from healthy human volunteers with the permission and clearance from Human Ethics Committee. All other chemicals used otherwise were of analytical grade.

METHODS:

Sephadex G-100 column chromatography

King Cobra (*Ophiophagus hannah*) venom (100 mg) was dissolved in 1 ml of distilled Water, centrifuged at 1000 rpm, supernatant was taken and loaded on to a Sephadex G-100 column (1.8×115 cm) that was pre-equilibrated with phosphate buffered saline, pH 7.2. The protein was eluted at a flow rate of 15 ml per hour and 1.5 ml fractions were collected per 6 min and the protein elution pattern was monitored at 280 nm using a spectrophotometer.

CM-Sephadex C-25 column chromatography

The pooled protein sample (8 mg) of peak-V obtained from the previous step was loaded on to a CM-Sephadex C-25 column that was pre-equilibrated with Tris-HCl buffer, pH 7.2, 10 mM. The column was then eluted with Tris-HCl buffer with increasing pH (range: 7.2 to 8.4) and molar concentrations (range: 10mM to 200mM). Protein was eluted at a flow rate of 15 ml per hour and 1.5 ml fractions were collected.

The protein elution was monitored at 280 nm. Alternate tubes were tested for protease activity using fat free casein. Peak III which showed proteolytic activity was pooled, desalted and lyophilized. This was designated as OH-Protease III.

SDS- Polyacrylamide gel electrophoresis

SDS-PAGE (10%) was carried out for according to the method of Laemmli, (1970). 75µg of king cobra venom and 50µg of peak III sample under non-reduced condition were loaded. Molecular weight standards from 3.5 to 205 kDa were used. Electrophoresis was carried out using Tris (25 mM), glycine (192 mM) and SDS (0.1%) for 3 h at 90V at room temperature. After electrophoresis, gels were stained with 0.1% Coomassie brilliant blue R-250 for detection of the protein bands of four venoms and destained with 25% ethanol in 8% acetic acid and water (30:10:60 v/v).

Proteolytic activity and casein zymogram:

Substrate gel assay for proteolytic activity was performed as described by Laemmli et al., by incorporating 2% fat free casein into 10% SDS polyacrylamide gel. Protein sample of peak III (25µg) under non reducing conditions were loaded into the wells. After electrophoresis, the gels were soaked in 3% Triton X-100 for 30 minutes. Later, gel was repeatedly washed in distilled water and was incubated at 37⁰ C for 24 hours, immersed in incubation buffer. After incubation, the gel was stained with Coomassie brilliant blue stain solution overnight and then destained (using Water, ethanol and acetic acid solution, 50:45:5) to visualize the activity band.

In test tube assay, varying concentration range (5-25 µg) of protease in 1 ml volume was pre-incubated independently with PMSF, EDTA and 1,10-phenanthroline 5 mM each and incubated for 2hr 30 mins. A positive control containing only protease sample (25 µg) was also taken. 1 ml of 0.44 M Trichloroacetic acid was added to stop the reaction and left to stand for 30 mins. The mixture was then centrifuged at 2000g for 10 min. Sodium carbonate (2.5 ml, 0.4 M) and Folin-Ciocalteu's reagent (1:2 dilutions with water) were added to 1ml of the supernatant and the colour developed was read at 660 nm. One unit of enzyme activity was defined as the amount of the

enzyme required to cause an increase in OD of 0.01 at 660 nm/min at 37⁰C. The specific activity was expressed as units/min/mg of protein.

Plasma re-calcification time

The method of Quick et al., (1935) was followed. Normal human citrated plasma (0.2 ml) and different concentrations (0 to 6 µg) of the protease sample were taken in glass tubes and mixed with Tris-HCl Buffer 10 mM, PH 7.5 to make up the final volume to 250 µl. Samples were incubated at 37⁰C for 5 min. The clotting time was determined after adding 0.1 ml of 0.25 M CaCl₂. The clotting time of Citrated human plasma in the absence of venom sample formed the control of the experiment.

Fibrinogenolytic activity

Fibrinogenolytic activity was tested according to Gao et al., (1998) by incubating protease (0.5 µg to 5 µg) with human plasminogen-free fibrinogen (50 µg) in 50 mM Tris-HCl buffer, 0.1 M NaCl, pH 7.6. Both dose dependent and time dependent activities of the protease sample were analyzed. For dose dependent activity, the protease with increasing concentration (0-5 µg) was incubated for 120 min at 37⁰C and for time dependent activity protease (2 µg) was incubated at 37⁰C with different incubation time varying from 0 to 120 min. The reaction was stopped by adding 20 µl of denaturing buffer containing 1 M urea, 4% SDS and 4% β-mercaptaethanol. The samples were boiled for 5 min, the fibrinogen degradation products were then analyzed by 10% SDS-PAGE.

Platelet aggregation

Turbidometric method of Born and Cross, (1963) was followed, using a dual channel *Chrono-log* model 700-2 aggregometer (Havertown, USA). Briefly, 235 µl of PRP suspension was maintained at 37⁰C in a siliconized glass cuvette and pre-incubated with different doses (ranging from 0 to 15 µg) of protease for 3 min and the aggregation was initiated by adding the agonist ADP (10 µM/ml), Epinephrine (10 µM/ml) and thrombin (1 U/ml) in independent experiments. The aggregation was followed for 6 min with constant stirring at a speed of 1200 rpm. Aggregation induced by agonist alone was considered as 100% aggregation in each case. The aggregation

trace was the plot of light transmission between platelet rich plasma (PRP) and platelet poor plasma (PPP) base line, which represent 0% and 100% aggregation respectively.

Protein estimation by Lowry's method

Protein concentration of king cobra venom and isolated protease was determined according to the method of Lowry et al., (1951) using bovine serum albumin as standard.

Statistical analysis

All the quantitative values were presented as Mean \pm SD using SPSS software.

Results

King cobra venom upon fractionation on a Sephadex G-100 column, resolved in to 6 distinct peaks (Fig. 5.1). Peak-V showed proteolytic activity on casein. Hence, fractions of peak-V were pooled, processed and concentrated. This was further subjected to fractionation on CM-Sephadex C-25 cation exchange column.

The column was eluted by changing molarity of the eluting buffers ranging from 10 mM to 200 mM and pH ranging from 7.2 to 8.4. The elution profile showed 5 distinct protein peaks (Fig. 5.2). Alternate tubes from each peak were tested for proteolytic activity using fat-free casein as the substrate. The peak with the proteolytic activity was pooled, processed and concentrated.

Electrophoresis of the king cobra venom of on a 10% SDS-PAGE under non-reduced condition showed distinct protein banding patterns that lie in the wide molecular mass range from 3.5 kD to 205 kD. Protein banding pattern of the protease was also analyzed on 10% SDS-PAGE under non-reduced condition. It showed a single protein band in a range of around 20 kDa. Further, the protein was subjected for substrate gel assay using fat-free casein. The protein produced a distinct proteolytic activity band in casein zymogram (Fig. 5.3).

The venom was tested with specific protease inhibitors such as PMSF (para-methyl sulphonyl fluoride), EDTA (Ethylene diamine tetra acetate) and 1,10-phenanthroline. The proteolytic activity was abolished by PMSF while EDTA and 1,10-

phenanthroline did not show any significant effect when compared with the positive control of the experiment (Table. 5.1).

Figure 5.4 depict the effect of protease on recalcification time of citrated human plasma. It significantly decreased the recalcification time in dose dependent manner.

The protease degraded human fibrinogen. The effect was found to be both dose (Fig. 5.5A) and time (Fig. 5.5B) dependent. It specifically degraded the B β chain, while the A α and γ chains remained resistant to cleavage when incubated for 2h at varying concentrations. In time dependent assay, the B β chain was completely degraded in 5 min of incubation period when used 2 μ g of protease incubated at 37^o C. Hence, the protease was named as β -fibrinogenase.

The protease sample showed inhibitory activity on human platelet aggregation process induced by agonists such as ADP (Fig. 5.6A), epinephrine (Fig. 5.6B) and thrombin (Fig. 5.6C). It showed > 90% inhibition of agonists induced platelet aggregation at a concentration of 15, 12 and 8 μ g respectively.

Discussion

β -fibrinogenase was found to be insensitive to protease specific inhibitors such as EDTA and 1,10-phenanthroline. However, it was sensitive to PMSF indicating that it is a serine protease. It was found to be pro-coagulant in nature as it reduced the plasma recalcification time of citrated human plasma. The B β -chain of human fibrinogen was sensitive towards the proteolytic activity as it was degraded both in dose and time dependent manner by the protease while the A α and γ chain remained resistant. Many α -chain and γ -chain fibrinogenases have been reported from snake venoms. A number of these types of endoproteinases also possess hemorrhagic activity and are members of metalloproteinase family. However, very few reports claim fibrinogenases that specifically cleave the B β -chain of fibrinogen. The thrombin induced aggregation of human platelets was inhibited efficiently by the β -fibrinogenase enzyme at lesser concentration than compared with the ADP and epinephrine induced platelet aggregation.

Figures and Tables

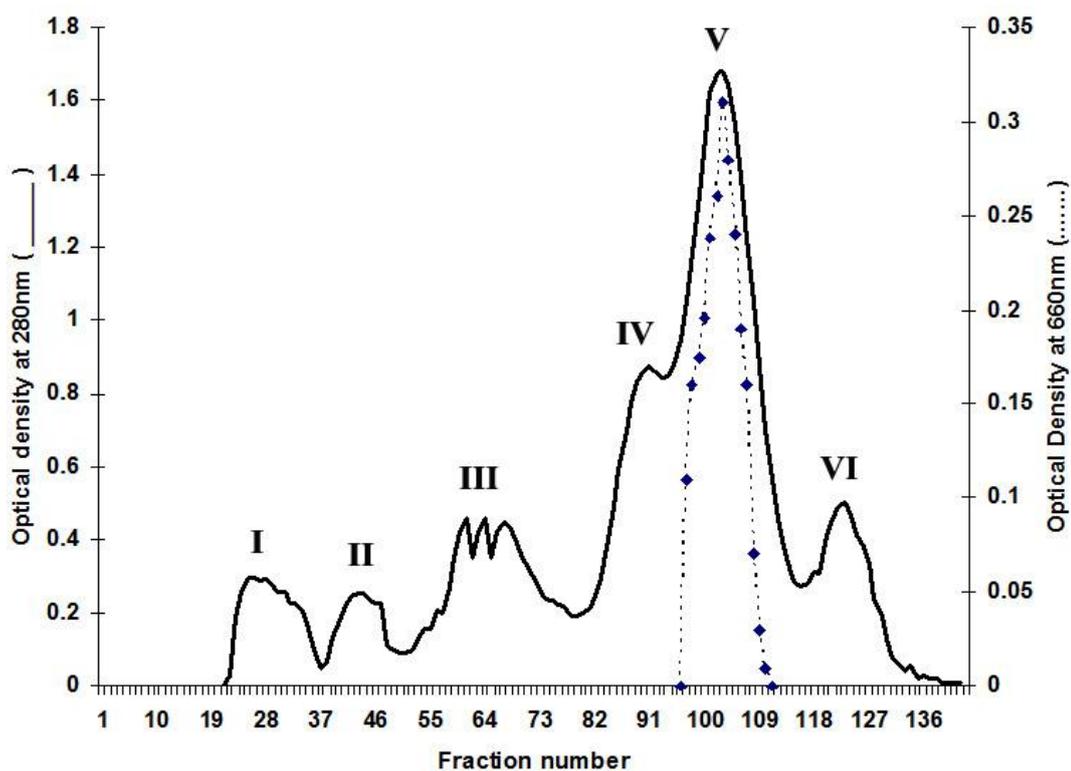


Fig. 5.1: Sephadex G-100 column chromatography: King cobra venom (100 mg) was loaded on to a 1.8×115 Sephadex G-100 column which was pre-equilibrated with phosphate buffered saline, pH 7.2. The protein was eluted with PBS at a flow rate of 15 ml per hour (1.5 ml fraction/6min) and monitored at 280 nm. Peak with dotted line indicates proteolytic activity which was monitored at 660 nm.

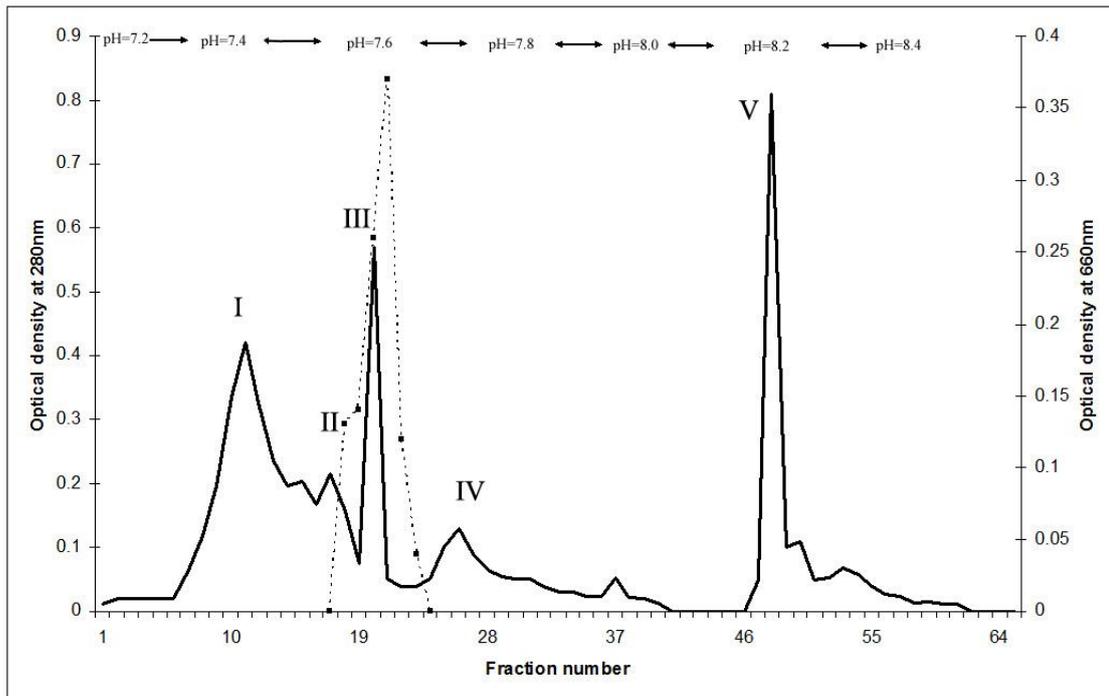


Fig. 5.2: CM-Sephadex C 25 column chromatography: Peak V obtained from previous step (13 mg of protein in equilibrating buffer) was loaded on to the column (1.2 × 25 cm) that was pre-equilibrated with Tris-HCl buffer, pH=7.2, 10mM. The protein was eluted at a flow rate of 15 ml per hour (1.5 ml/min) stepwise using different molarities and pH values of Tris-HCl buffer. Protein content was monitored by absorbance at 280 nm. Alternate tubes were assayed for proteolytic activity using casein as substrate monitored at 660nm.

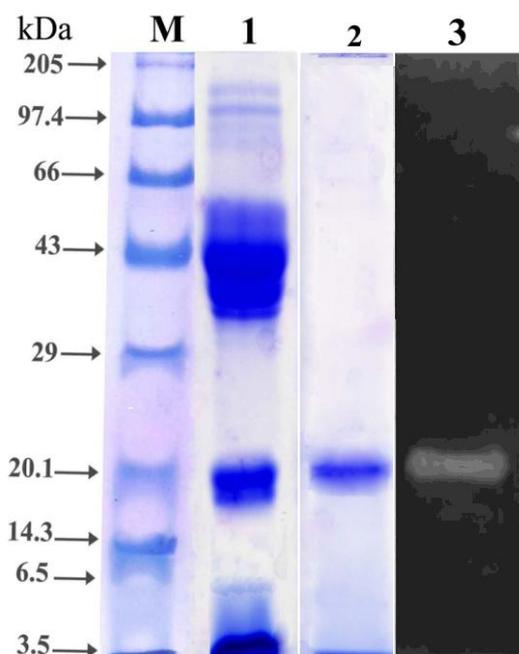


Fig. 5.3: Protein banding patterns of *O. hannah* venom, isolated protease component and protease zymogram: Lane M represents protein band pattern of molecular weight markers in kDa: rabbit muscle myosin (205), phosphorylase B (97.4), Bovine serum albumin (66), ovalbumin (43), carbonic anhydrase (29), soyabean trypsin inhibitor (20.1), lysozyme (14.3), aprotinin (6.5), insulin (3.5). Lane 1 represents protein banding pattern of king cobra venom under non-reducing condition, lane 2 represents CM-Sephadex C-25 isolated protease and lane 3 represents activity staining of zymography of the isolated protease resolved by 10% SDS-PAGE under non-reduced condition.

Protease Inhibitors	Protease activity in Units
Protease alone	2.9 ± 0.08
Protease+PMSF	0.22 ± 0.06
Protease+EDTA	2.8 ± 0.08
Protease+1,10-phenanthroline	2.7 ± 0.12

Table 5.1: Effect of protease inhibitors (5 mM concentration each) on the activity of protease.

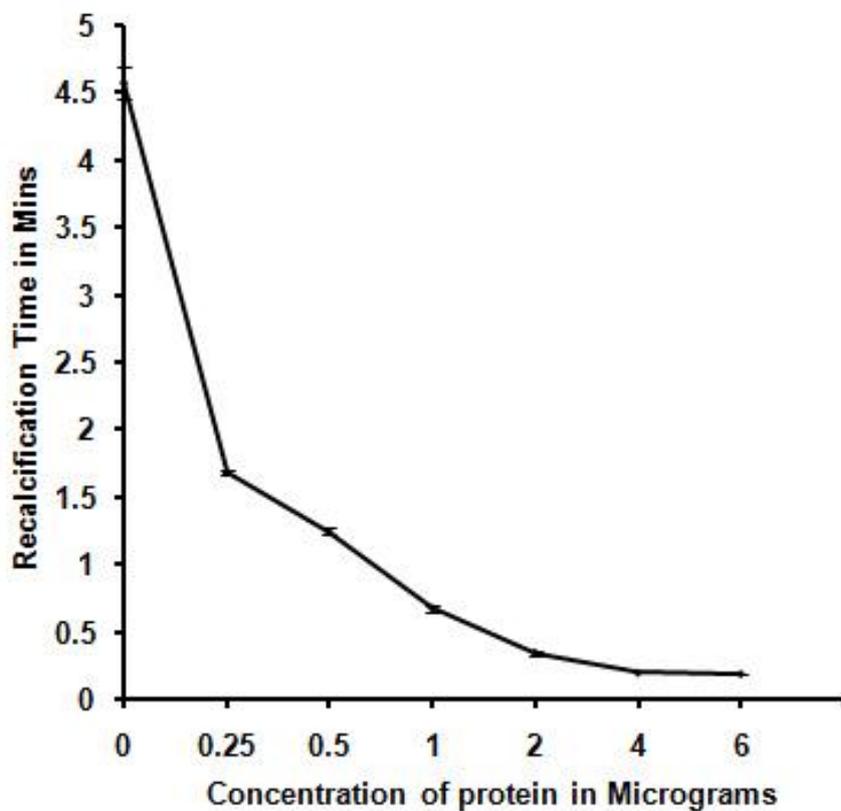


Fig. 5.4: Effect of protease on recalcification time of citrated human plasma: Protease showing decreased clotting time when 0.2 ml of citrated human plasma was incubated with different concentrations of the protease ranging from 0 to 6 μg for 5 min at 37°C . Clotting was initiated by adding 0.25M CaCl_2 and time recorded in min.

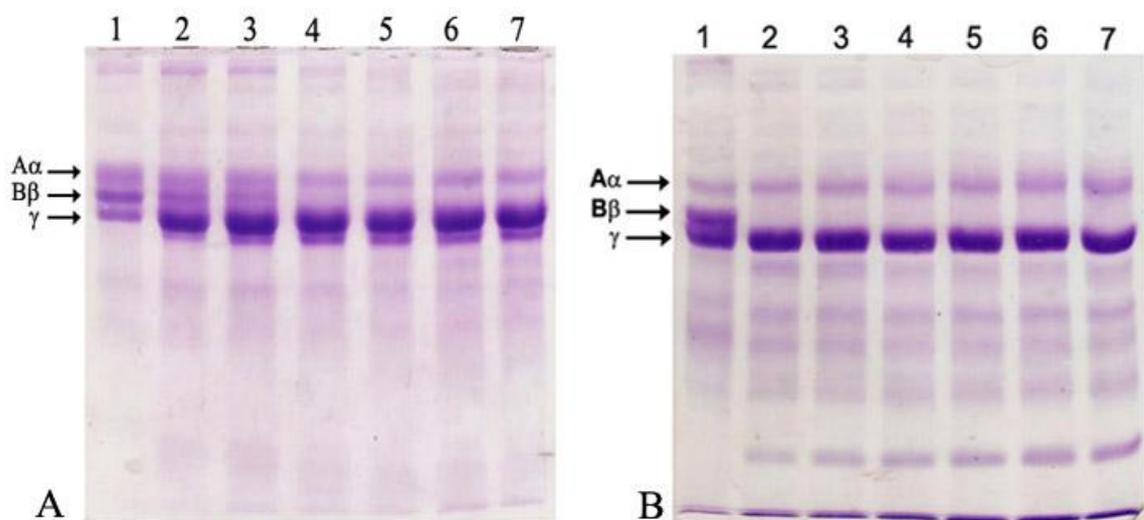


Fig. 5.5: Fibrinogenolytic activity of protease: **A:** Lane 1 showing Fibrigen (50µg) alone that served as control, lane 2, 3, 4, 5, 6 and 7 containing fibrinogen (50 µg) + 0.5, 1, 2, 3, 4 and 5 µg of protease incubated for 120 min at 37⁰C and the degradation products resolved on 10% SDS-PAGE under reduced condition. **B:** Lane 1 containing fibrinogen (50 µg) alone, lane 2, 3, 4, 5, 6 and 7 containing fibrinogen (50 µg) + 2µg of protease incubated for 5, 10, 20, 40, 60 and 120 min respectively at 37⁰C and the degradation products resolved on 10% SDS-PAGE under reduced condition.

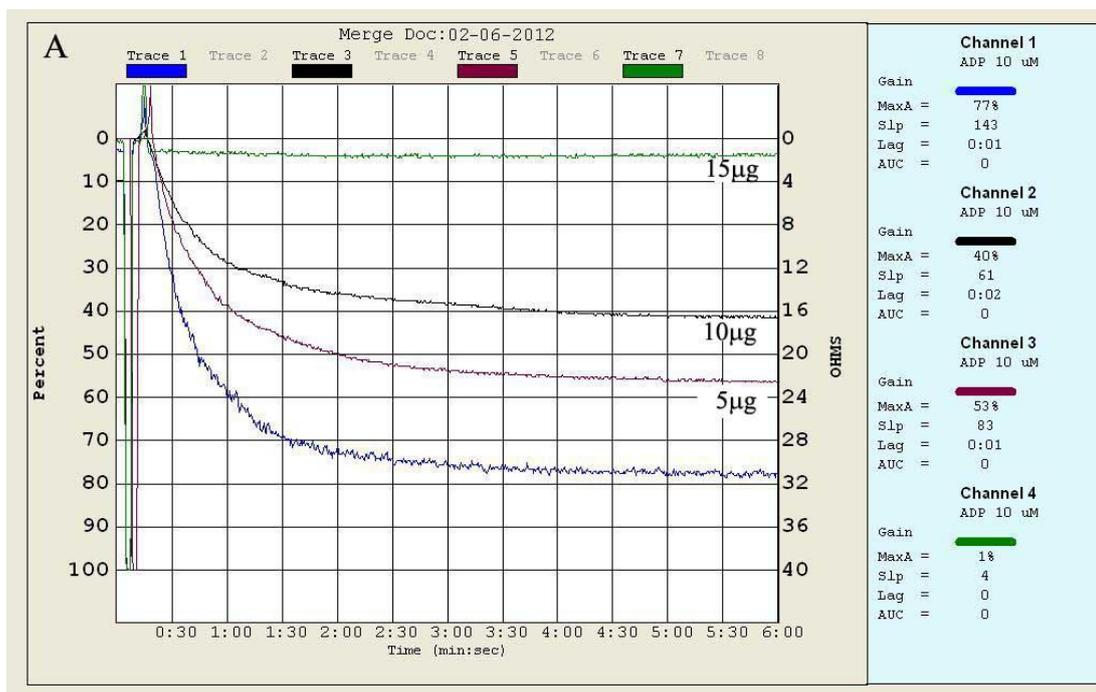


Fig. 5.6A: Effect of β -fibrinogenase in increasing concentration (5 to 15 μ g) on ADP induced platelet aggregation. Aggregation was initiated by adding the agonist ADP (10 μ M/ml) and the experiment was carried out as described in methods. Blue trace was considered as 100 % aggregation, taken as control of the experiment.

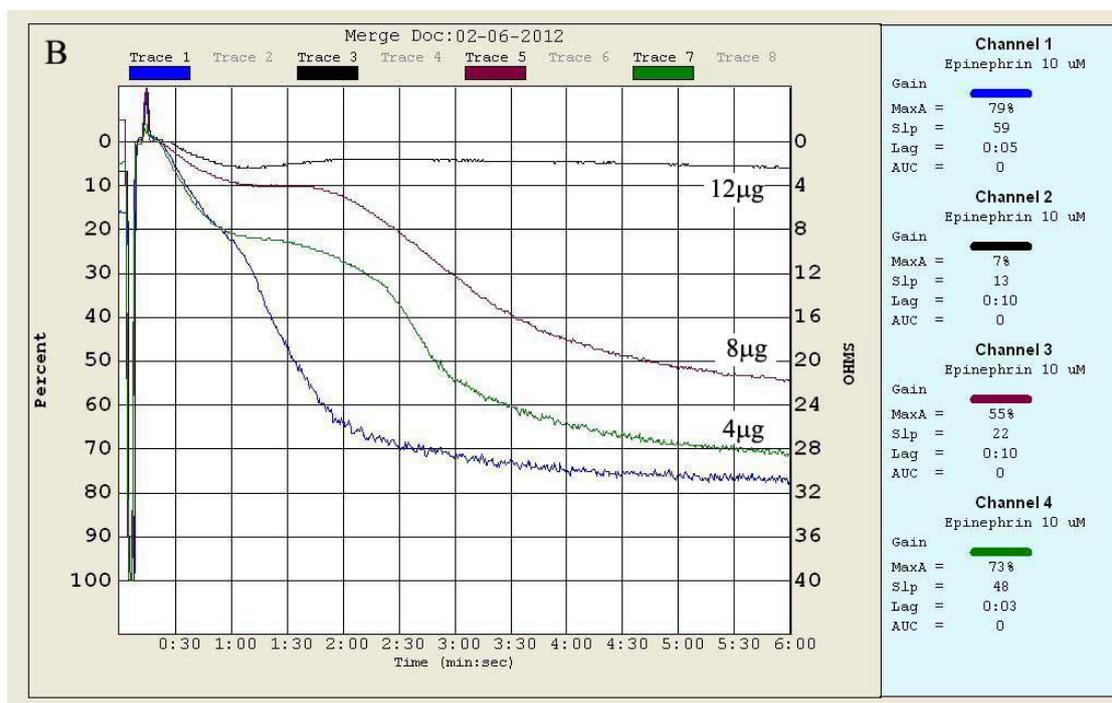


Fig. 5.6B: Effect of β -fibrinogenase in increasing concentration (4 to 12 μ g) on epinephrine induced platelet aggregation. Aggregation was initiated by adding the agonist Epinephrine (10 μ M/ml) and the experiment was carried out as described in methods. Blue trace was considered as 100 % aggregation, taken as control of the experiment.

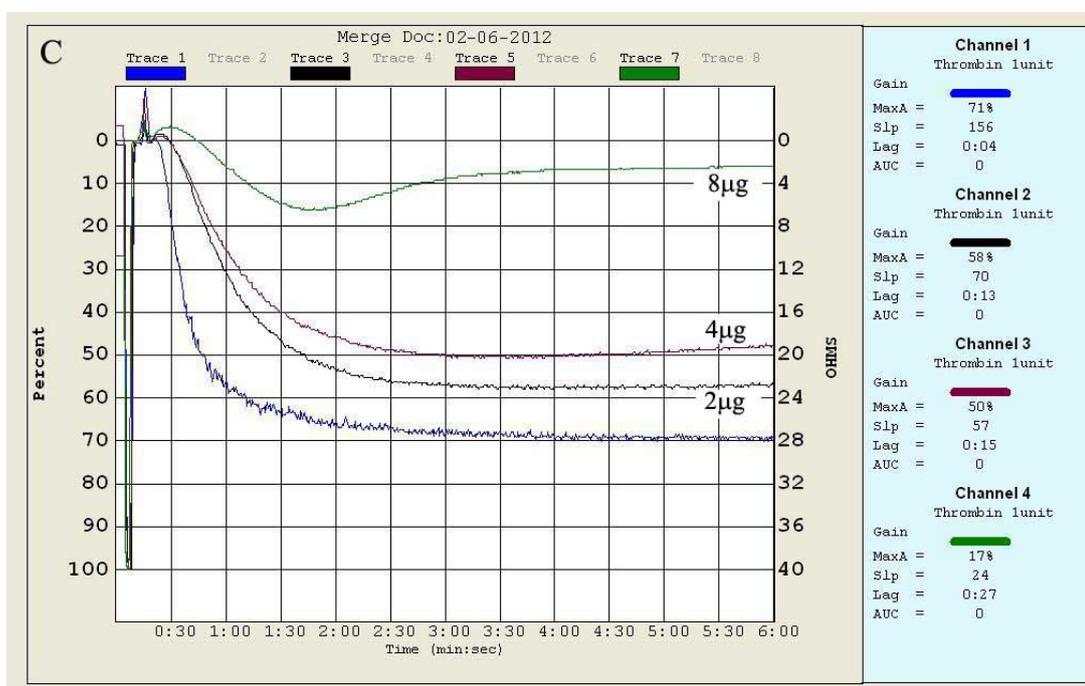


Fig. 5.6C: Effect of β -fibrinogenase in increasing concentration (2 to 8 μ g) on thrombin induced platelet aggregation. Aggregation was initiated by adding the agonist thrombin (1 U/ml) and the experiment was carried out as described in methods. Blue trace was considered as 100 % aggregation, taken as control of the experiment.