CHAPTER III

PURIFICATION AND CHARACTERIZATION OF MALABARIN, A THROMBIN LIKE ENZYME FROM TRIMERESURUS MALABARICUS VENOM
Introduction

The pharmacological characterization of T. malabaricus venom clearly indicated that the venom is not lethal but involved in the manifestation of strong local tissue damage, which is not observed in the snakebite of highly lethal snakes such as Naja naja and Daboia russelii. The non-lethality of T. malabaricus venom indicates that the venom is not a well-balanced mixture of systemic toxins and hydrolytic enzymes instead it may be abundant with only hydrolytic enzymes. To substantiate these observations, purification and characterization of enzymes involved in the induction of local tissue damages is necessary.

The hydrolytic enzymes involved in the induction of local tissue damages are hyaluronidase, PLA2 and protease enzymes. Since the T. malabaricus venom does not contain any hyaluronidase enzyme activity the involvement of this enzyme is ruled out completely. The inhibition studies using known PLA2 inhibitors did not neutralize the local tissue damages indicated that PLA2 enzymes are not the main toxic components in inducing local tissue damages. Inhibition studies with protease enzyme of crude T. malabaricus venom by specific resulted in the complete neutralization of edema, hemorrhage and procoagulant activities. As a result protease enzyme from the T. malabaricus venom is targeted for further purification and complete characterization so that its role in the induction of local tissue damages can be established.

Several protease enzymes have been purified from many Viperid snake venoms using conventional chromatographic methods. Based on the molecular size and other structural domains these proteases are classified into four major groups. The molecular size of these protease enzymes varies from small 20 kDa enzymes to a larger 120 kDa enzymes. Irrespective of the size of venom protease all of them are involved in the induction of many pathological symptoms especially local tissue damage. Since no
information is available on the venom composition and the molecular characterization of hydrolytic enzymes, purification has been carried out by conventional gel filtration chromatography, followed by ion-exchange chromatography has been employed. The purified enzyme will be characterized for all the observed pathological symptoms of the crude venom so that one can easily design an inhibitor so that the inhibitor can be effectively used for therapeutic purposes.

Materials and methods

Materials

Sephadex G-75, DEAE Sephadex A-25, fat free casein, bovine serum albumin (BSA), human fibrinogen, TAME, and protease inhibitor PMSF were from Sigma Chemical Company, St. Louis, MO, USA. Other protease inhibitors - 1',10-phenanthroline, EDTA and EGTA were from SRL Chemical Company, Bangalore, India. Molecular weight markers were from Genei Private Limited, Bangalore, India. Calibration kits for isoelectric point determination were from Amersham Pharmacia Biotech, USA. Lactate dehydrogenase, creatine kinase and prothrombin kits were purchased from Auto Span Diagnostic Center, Bangalore, India. Male Swiss Wistar albino mice weighing 20-25 g were obtained from Central Animal House facility at Department of Studies in Zoology, University of Mysore, Mysore, India. The animal care and handling were conducted in compliance with the national regulations for animal research. The animal experiments were carried out after reviewing the protocols by the animal ethical committee of the University of Mysore, Mysore. Fresh human blood samples are collected from healthy volunteers from the Department of Studies in Biochemistry, University of Mysore, Mysore, India. All other reagents and chemicals used were of analytical grade. All the solvents were redistilled before use.
Methods

Sephadex G-75 column chromatography

Lyophilized *T. malabaricus* venom (100 mg in 1 ml) was dissolved in 10 mM Tris-HCl buffer pH 8.0; centrifuged at 5000 g for 5 min. The supernatant was applied to a column (0.8 cm X 120 cm) of Sephadex G-75 equilibrated and eluted with the same buffer at 20 °C. The fractions from column were eluted at a flow rate of 20 ml / hr and 2 ml fractions are collected. Protein elution was monitored at 280 nm using a Shimadzu spectrophotometer (1601A). Alternate tubes were assayed for protease activity. Fractions having enzyme activity were pooled individually, desalted, lyophilized and stored at - 4 °C.

DEAE Sephadex A-25 column chromatography

TM-I sample (28 mg in 2 ml of equilibrating buffer) from Sephadex G-75 column were loaded onto a DEAE Sephadex A-25 column (1.6 cm X 20 cm) equilibrated in 10 mM Tris-HCl buffer pH 8.0. The column was eluted by a stepwise gradient of Tris-HCl buffers and NaCl with respective pH as indicated in Fig. 2.02. Fractions were carried out at 20 °C at a flow rate of 20 ml / hr and 2 ml fractions were collected. Protein elution was monitored at 280 nm using a Shimadzu spectrophotometer. Fractions having enzyme activity were pooled, desalted, lyophilized and stored at - 4 °C.

Electrophoresis

Native PAGE was carried out for *T. malabaricus* venom, TM-I and malabarin fraction on 12.5 % polyacrylamide gel using Tris-Glycine buffer pH 8.3 according to the method of Davis (1964). SDS-PAGE was carried out for the same samples with molecular mass standards (14.3 - 97.4 kDa) according to the method of Laemmli (1970) on 10 % polyacrylamide gel containing 0.1 % SDS. After electrophoresis, all the gels were stained with 0.25 % Coomassie brilliant blue R-250 and proteins were visualized after destaining with methanol, acetic acid and water (30:10: 60 v / v).
High performance liquid chromatography

Purified malabarin was subjected to RP-HPLC on Vydac-C_{18} (5 µm, 0.21 X 25 cm) column. The column was first equilibrated with Solvent A (0.1 % TFA) till the baseline monitored at 280 nm was stable. 40 µg of malabarin was then injected into the column. Elution was carried out with a linear gradient of 0 to 100 % Solvent B (70 % Acetonitrile in 0.1 % TFA). Protein elution was monitored at 280 nm.

Mass spectrometry

The molecular mass of malabarin was determined by Matrix-Assisted Laser Desorption Ionization Time of Flight (MALDI-TOF) mass spectrometry (Voyager Spec # 1 MC) in the positive ionization mode. α-Cyano-4-hydroxycinnamic acid was used as MALDI matrix.

Fluorescence emission spectrum

The fluorescence emission spectrum of malabarin was recorded using Shimadzu spectrofluorophotometer. The fluorescence emission of malabarin, 30 µg in 2.0 ml of 0.1 M Tris-HCl buffer pH 8.0 in 1.0 cm path length quartz cuvette measured between 300 and 400 nm after exciting at 280 nm.

Ultra violet absorption spectrum

Ultra violet absorption spectrum of malabarin was recorded using Shimadzu 1601A spectrophotometer. Malabarin (200 µg in 1.0 ml of 0.1 M Tris-HCl buffer pH 8.0 in 1.0 cm path length quartz cuvette was recorded.

N-terminal sequencing

The terminal sequencing of malabarin was carried out in a fully automated Shimadzu protein sequencer (PSQ-1) system that employs Edman’s degradation reaction for sequential separation of N-terminal amino acids.
pH and Temperature kinetics

The pH optimum of malabarin was determined using different buffers of 0.1 M strength (sodium acetate [pH 3.6 - 5.6], sodium phosphate [pH 6.0 - 8.0] and Tris-HCl [pH 9.0 - 10]) were used. The temperature optimum of malabarin was established by determining the activity at various temperatures (0 - 80 °C) under standard assay conditions.

Isoelectrofocusing

Calibration kit was used for $pI$ determination of malabarin according to the manufacture's protocol. Broad $pI$ calibration standards and the enzyme were run on a 10 % polyacrylamide gel containing Pharmalyte 3 -10. After focusing, the proteins on the gel were fixed with the 20 % TCA solution and stained with Phast Gel Blue R solution. The proteins were visualized after destaining with methanol, acetic acid and water (30:10: 60 v / v).

Protein estimation

Protein concentration was determined according to the method of Lowry et al., (1951) using BSA as standard.

Caseinolytic activity

Caseinolytic activity was assayed according to the method of Murata et al., (1963). The 1.0 ml reaction mixture containing 0.4 ml of casein (2 % in 0.2 M Tris-HCl buffer pH 8.5) was incubated with different concentration of T. malabaricus venom, TM-I and malabarin sample separately at 37 °C for 2 hr. The reaction was stopped by adding 1.5 ml of 0.44 M TCA and allowed to stand for 30 min. The mixture was centrifuged at 1,500 g for 15 min and an aliquot (1.0 ml) was mixed with 2.5 ml of 0.4 M sodium carbonate and 0.5 ml of 1:2 diluted Folin reagents. The color developed was read at 660 nm. One unit of enzyme activity was defined as the amount of enzyme required to
increase an absorbance of 0.01 at 660 nm / hr at 37 °C. Activity was expressed as units / hr at 37 °C.

Zymogram assay

The zymogram assay of caseinolytic and gelatinolytic (Heussen and Dowdle (1980) activity was made by 0.2 % casein and gelatin incorporated as substrate in to the 10 % resolving gel separately. The *T. malabaricus* venom, TM-1 and malabarin were loaded on to SDS-PAGE under non-reducing condition. Electrophoresis was carried out with a constant voltage of 100 V at room temperature. After electrophoresis, SDS was removed by washing the gel with 2.5 % Triton X-100 and the gel was incubated with 50 mM Tris-HCl buffer pH 7.6 containing 10 mM CaCl$_2$ and 150 mM NaCl at 37 °C for 12 hr and the gel were stained with Coomassie brilliant blue. Clear zones in the gel indicated the hydrolysis of casein and gelatin due to enzyme activity.

Human fibrinogenolytic activity

Fibrinogenolytic activity was performed according to the method of Ouyang and Teng (1976). The reaction mixture 40 μl contained 50 μg of human fibrinogen in 10 mM Tris-HCl buffer pH 7.6 incubated at 37 °C with different concentration of malabarin (0 μg - 0.6 μg) and for different time intervals (0 - 2 hr) at 0.3 μg protein concentration. The reaction was terminated by adding 20 μl of denaturing buffer containing 1M Urea, 4 % SDS and 4 % β- mercaptoethanol. The hydrolyzed products are analyzed by 10 % SDS-PAGE carried to the method of Laemmli (1970). The protein pattern was visualized by staining with Coomassie brilliant blue R-250. The inhibition studies were carried out as described above after pre-incubation of 0.6 μg of purified protein with or without 5 mM each of PMSF, EDTA, EGTA and 1, 10 phenanthroline for 20 min at 37 °C with appropriate controls.
Coagulant activity

Re-calcification time: Re-calcification time was determined according to the procedure described by Condrea et al., (1883). Fresh human blood was mixed with 0.11 M trisodium citrate in the ratio of nine parts to one. The mixture was centrifuged for 15 min at 500 g. The supernatant obtained was used as platelet poor plasma (PPP), which was pre warmed to 37 °C before use. Different concentration of *T. malabaricus* venom, TM-1 and malabar in 10 mM Tris-HCl buffer pH 7.4 were separately added to 0.3 ml of PPP and incubated for one min. The clot formation was initiated by adding 30 μl of 0.25 M CaCl2. The time taken for visible clot to appear from the time of addition of CaCl2 was recorded in sec. For control experiments Tris-HCl buffer alone was added instead of the enzyme sample.

Prothrombin time: Prothrombin time was determined according to the method of Quick (1972). Fresh citrated human plasma (0.1 ml) was incubated separately with various concentration of *T. malabaricus* venom, TM-1 and malabar. The plasma clotting time was recorded in sec after the addition of an optima amount (0.2 ml) of brain thromboplastin.

Thrombin like activity

Thrombin like activity was determined as described by Denson (1969). Reaction mixture contained 0.4 ml of 0.5 % human fibrinogen. Clot formation was initiated by adding different concentration of malabar in with continued incubation. The clotting time was recorded in sec.

Hemorrhagic activity

Hemorrhagic activity was determined according to the method of Kondo et al., (1969). Group of mice were injected (intra dermal) with different concentrations of malabar in 30 μl saline. After 3 hr, mice were sacrificed using anesthesia (Barbitone 30 mg / kg i.p.). The dorsal surface of the skin was removed and the inner surface was
measured for hemorrhagic activity. The minimum hemorrhagic dose (MHD) is defined, as the concentration of venom required to induce hemorrhagic spot of 1 cm diameter from the spot of injection. For inhibition studies, similar experiments were carried out after pre-incubation of MHD of malabar in with or without protease inhibitors for 20 min. The same concentrations of protease inhibitors alone were injected and served as controls for these inhibition studies.

**Edema inducing activity**

Edema induction was determined by the method of Yamakawa et al., (1976) as modified by Vishwanath et al., (1987). A Group of five mice were injected in the right footpads with different concentration of malabar in 20 μl saline. After 1 hr mice were sacrificed using anesthesia (Barbitone 30 mg / kg i.p.) and the legs were removed at the ankle joints and weighed individually. The edema ratio was calculated using the formula.

\[
\text{Edema ratio} = \frac{\text{Weight of edematous leg} \times 100}{\text{Weight of normal leg}}
\]

Minimum edema dose is defined as the amount of protein required to cause an edema ratio of 120 %.

**Myotoxicity**

Myotoxic activity was measured by determining creatine kinase (CK) and lactate dehydrogenase (LDH) activities in the serum samples. Mice were injected (i.m.) 1 mg / kg body weight of *T. malabaricus* venom, TM-I and malabar. After 3 hr, mice were anaesthetized and blood samples were collected by cardiac puncture. For inhibition studies, malabar was pre-incubated with various protease inhibitors for 20 min and further assay was carried out as explained earlier with appropriate controls. The serum obtained was diluted 25 times with normal saline and assayed for LDH and CK activities according to the method of Huges (1962) and King (1965) as provided in Auto Span Diagnostics kit. Activity was expressed as units / lt.
Histopathological studies

For histopathological studies, mice were injected (i.m.) with 1 mg/kg body weight of malabarins separately and control mice received saline instead of venom sample. After 3 hr incubation all the animals were sacrificed using anesthesia (Barbitone 30 mg/kg i.p.) and muscle around the site of injection was removed, fixed in 10% formalin solution for 2 to 3 days. The muscle tissue was then washed with distilled water followed by dehydration and incubated for 1 hr in increasing concentration of alcohol (50%, 70%, 90% and absolute alcohol). The tissue was incubated with chloroform to replace the alcohol and impregnated with paraffin for 1 hr and was finally embedded in paraffin block. Tissue along with paraffin blocks was mounted on rider to take 5 μm thick sections using Rotary microtome. Muscle sections were then stained with hematoxylin and eosin. The microphotographs were taken using an Olympus BX40F-3 microscope with an integrated Olympus camera.

Statistics

The data were expressed as the mean ± SEM of at least 3 independent experiments.

Result and discussion

The biochemical and pharmacological characterization of crude venom of T. malabaricus snake revealed that the major manifestation caused by this snake is extensive local tissue damage mainly due to metalloprotease activity. This chapter discusses the isolation and characterization of metalloprotease enzyme from T. malabaricus venom. This study helps in understanding the role of metalloproteases in causing extensive local tissue damage and also in designing new therapeutic drugs useful against such venom toxicities.
Metalloprotease from *T. malabaricus* venom was purified by the combination of gel permeation and ion exchange chromatography. First *T. malabaricus* venom was subjected to fractionation on Sephadex G-75 column. On fractionation the venom components resolved into 4 prominent peaks, which are designated as TM-I to TM-IV (Figure 3.01A). The major peak was TM-I contributing 26% of the total protein and 87.3% of the total protease activity recovered. Since TM-I was not held in the G-75 column indicated that the protease enzyme has a high molecular weight molecule.

The TM-I peak from the Sephadex G-75 fraction was pooled concentrated and desalted using Sephadex G-10 column. The pooled TM-I fraction was further resolved into five peaks on DEAE-Sephadex A-25 column by applying stepwise NaCl gradient (Figure 3.01B). Only the third fraction designated as ‘Malabarin’ showed protease activity and contributed to 59.72% of the total activity loaded. 2.84% of the protein loaded on DEAE A-25 column was recovered in this malabarin fraction. The specific activity of this malabarin fraction was increased by 18 fold compared to the crude activity. Table 3.01 summarizes the purification of malabarin protease enzyme. The zymogram assay of crude *T. malabaricus* venom using the substrate casein and gelatin showed two and three discrete translucent bands suggesting the presence of more than one protease isoforms. However, on column chromatography separation only one isoform was detected. The activity of other isoforms of casein hydrolyzing and gelatin hydrolyzing forms were not able to detect during the column chromatographic separation.

The homogeneity of the malabarin protease was examined by SDS-PAGE and native gel electrophoresis. Malabarin moved as a single homogenous band in SDS-PAGE under reducing and non-reducing condition as well as in native gel electrophoresis under basic conditions (Figure 3.02A and B). These electrophoretic data suggests that malabarin has been purified to homogeneity. The approximate molecular weight based on SDS-PAGE was found out to be 43.5 kDa (Figure 3.02B). The enzyme is monomer and is acidic in nature. Further homogeneity was established by RP-HPLC using C18.
column. The elution buffer contained 0.1 % TFA and was eluted with acetonitrile gradient. Malabarin eluted as a single symmetrical peak with a retention time of 15.59 min suggesting that protein is less hydrophobic in nature (Figure 3.04). The molecular mass as determined by MALDI-TOF mass spectrometry was found to be 45.76 kDa (Figure 3.05). The apparent calculated molecular weight of 43.5 kDa by SDS-PAGE is in very close agreement with that of molecular weight determined by MALDI-TOF. The N-terminal sequence analysis of the malabarin gave the following 11 amino acid sequence; Ile-Ile-Leu-Pro(Leu)-Ile-Gly-Val-Ile-Leu(Glu)-Thr-Thr (Table 3.02). All these data clearly establishes that the malabarin protease is purified to homogeneity.

The malabarin protease gave a broad pH optimum and was optimally active at pH 8.0. The enzyme activity was abolished below pH 6.0 and above pH 10.0. The optimum temperature was found to be 37 °C and malabarin protease undergoes denaturation at > 60 °C. The isoelectric point determined was found to be 6.2. Malabarin showed typical ultra violet absorption maxima between 270 - 280 nm with the greater absorbance in the far ultraviolet region, which is characteristic for protein. The malabarin protease showed two fluorescence emission maxima, one between 310 -320 nm and another between 330 -340 nm when excited at 280 nm indicating the presence of exposed tyrosine and as well as tryptophan residues in the molecule. The biochemical and biophysical characteristics of malabarin protease is summarized in Table 3.02.

Snake venom proteases that are involved in the hemorrhagic activity are known to degrade variety of substrates such as laminin, fibrnectin, collagen IV and fibrinogen in vitro (Baramova et al., 1989; Gutierrez and Rucavado, 2000; Estevao-Costa et al., 2000; Escalante et al., 2004). The zymogram of crude T. malabaricus venom with casein and gelatin exhibited more than one translucent band and also hydrolyses fibrinogen. The purified malabarin protease hydrolyses both gelatin and casein and showed single translucent band (Figure 3.06A and B respectively). These zymogram data also
substantiate the homogeneity of malabarain. The purified malabarain protease also hydrolyses fibrinogen.

Several venom proteases with fibrinogenolytic activity have been reported and they preferentially hydrolyse Aα and Bβ subunits of fibrinogen (Matsui et al., 2000; Braud, et al., 2000; Vieira et al., 2004; Swenson and Markland Jr, 2005). In order to know the action of malabarain in hemostasis, human fibrinogenolytic activity was studied as a function of protein concentration and time using purified human fibrinogen. Most of the metalloproteases are more specific towards Aα subunits and serine proteases towards Bβ subunit of fibrinogen (Markland, 1998). Malabarain is more specific towards Aα subunit, which is not a glycoprotein and hydrolyses preferentially. It hydrolyses Aα subunit of human fibrinogen completely at 0.1 μg of protein concentration with 1 hr period of incubation. As the concentration increases Bβ subunit, which is a glycoprotein is also hydrolysed and it was completely hydrolyzed over 0.6 μg of protein concentration (Figure 3.07A). Figure 3.07B shows the time dependent fibrinogenolysis at 0.3 μg of malabarain. At this concentration Aα chain was hydrolyzed with in 5 min incubation period. As the incubation time increases, the Bβ chain was also hydrolysed and complete hydrolyses was observed within 2 hr incubation period. But γ subunit which is more resistant to most of the venom fibrinogenases was not affected by malabarain even at very high concentration of protein and over 2 hr incubation period (data not shown).

The enzymes that advance the coagulation process are therefore referred as thrombin like enzymes (Markland, 1998; Braud et al., 2000; Lu et al., 2005). These enzymes are mainly serine proteases and metalloproteases that resemble thrombin in their function to trigger the clotting of fibrinogen through fibrinopeptide release. Thrombin hydrolyses fibrinogen from N-terminal disulfide knot of Aα and Bβ subunits and initiate clot formation by releasing fibrinopeptides A and B (Matsui et al., 2000; Lu et al., 2005). Similarly any venom fibrinogenases, which hydrolyses Aα and Bβ subunits of fibrinogen from N-terminal knot to induce clot formation, are called thrombin like enzymes (Stocker
and Barlow, 1976; Komri et al., 1985; Iwasaki et al., 1990; Vieira et al., 2004). Few venom fibrinogenases that cleaves fibrinogen at C-terminal end of $\alpha$, $\beta$, and $\gamma$ chains of fibrinogen into non-clottable fibrinopeptides are known to prolong clot formation and are anticoagulants (Nawarawong et al., 1991). The snake venom fibrinogenases based on the site of cleavage induces either procoagulant or anticoagulant activities (Vieira et al., 2004; Lu et al., 2005). To understand the nature of action of malabarin on hemostasis, its proteolytic action on purified human fibrinogen was determined. Malabarin hydrolyses both $\alpha$ and $\beta$ subunits of fibrinogen and forms fibrin clot within 40 sec by releasing fibrinopeptide A and B (Figure 3.07A and B). This data indicates that malabarin has thrombin like enzyme activity.

Since purified malabarin hydrolyses biologically important substrates like gelatin and fibrinogen its involvement in inducing hemorrhage and procoagulant activity like that of crude $T. malabaricus$ venom is determined. Purified malabarin protease induces strong hemorrhagic activity. The dose dependent hemorrhagic activity is shown in Figure 3.08 and malabarin exhibited MHD at 3.0 $\mu$g protein concentration. In addition to hemorrhage and thrombin like activities, these metalloproteases are also known to be involved in inflammatory reactions (Teixeira et al., 2005; Chen et al., 2005; Farsky et al., 2005). Figure 3.09A shows the dose dependent hemorrhagic edema inducing activity of malabarin. The MED of malabarin is 1 $\mu$g protein concentration. Figure 3.09B shows the dose dependent edematous footpad depicting hemorrhage edema.

The purified malabarin exhibit strong myotoxic activity compared to crude $T. malabaricus$ venom. Elevated levels of serum CK and LDH activities as a function of myotoxic activity is given in Figure 3.10B. At a given concentration, purified malabarin showed increased CK and LDH activities when compared to crude venom and G-75 fraction TM-1. The myotoxic activity of malabarin is further substantiated by histopathological studies (Figure 3.10A). The pattern of histopathological changes induced by malabarin is very much similar to that of crude $T. malabaricus$ venom.
Similar to crude *T. malabaricus* venom purified malabarin also affects blood coagulation system and advances coagulation time. The re-calcification time and prothrombin time of crude *T. malabaricus*, TM-I fraction and malabarin is shown in Figure 3.11A and B respectively. Purified malabarin showed strong procoagulant activity both with re-calcification and prothrombin time compared to TM-I and crude venom fractions.

Venom proteases so far characterized belongs to either serine or metalloproteases (Iwasaki *et al.*, 1990; Matusi *et al.*, 2000; Jagadeesha *et al.*, 2002). In a recent inventory of α and β fibrinogenases, 67 direct acting enzymes have been described (Swenson and Markland Jr, 2005). Majority of these venom fibrinogenases belongs to the metazincin family of zinc dependent metalloproteases with matrix metalloproteinases (MMPs). Among 67 fibrinogenases reported, 47 are metalloproteases and their specificity is directed towards Aa subunit of fibrinogen (Swenson and Markland Jr, 2005). In order to know the nature of the fibrinogenases in malabarin, the inhibition studies were carried out in the presence of specific protease inhibitors using fibrinogen as substrate (Figure 3.12A). The fibrinogenolytic and thrombin like activities were inhibited by metalloproteases inhibitors EDTA, EGTA and 1,10-Phenanthroline but not by the serine protease inhibitors. The activity was not inhibited by PMSF, a serine protease suggesting that malabarin is a direct acting 48th metalloproteases. Most of the metalloproteases are both fibrinogenolytic and fibrinolytic (Gasmi *et al.*, 1997; Zhu and Wu *et al.*, 1999; Estevao-Costa *et al.*, 2000; Swenson and Markland Jr, 2005). However, the malabarin exhibits only a fibringenolytic but did not exhibits fibrinolytic activity. This is an exceptional among metalloproteases.

The hemorrhagic activity of malabarin is also completely abolished by the metalloproteases inhibitors, EDTA, EGTA and 1,10-Phenanthroline (Figure 3.12B). This data correlates well with the observation made by proteases which act on gelatin or casein are usually belongs to zinc containing matrix metalloproteases.
Based on the molecular mass and their domain structure, the snake venom metalloproteases (SVMPs) have been classified into four classes, P-I to P-IV. (Markland, 1998; Lu et al., 2005; Kamiguti, 2005). Metalloproteases of class P-I includes proteins with molecular mass of 20-30 kDa and contain only protease catalytic domain. Class P-II SVMPs comprise proteins with molecular masses of 30-60 kDa and contain protease catalytic domain and disintegrin-like domain. Class P-III SVMPs proteins include proteins with molecular mass of 60-90 kDa and contain a cysteine rich domain, in addition to protease catalytic and disintegrin-like domains. P-IV SVMPs are high molecular masses proteases in the range of 90-120 kDa and posses all the domains of class P-III and in addition it contains lectin-like domains (Markland, 1998; Bjarnason and Fox, 1994; Hati et al., 1999; Lu et al., 2005; White, 2005). Among these groups class P-I and P-IV SVMPs are reported to have low hemorrhagic activity or devoid of any hemorrhagic activity but exhibit strong direct acting fibrinogenolytic activity. P-II and P-III SVMPs is represented by the large hemorrhagins, which are believed to be the most potent hemorrhagins and are also involved in affecting hemostasis (Hati et al., 1999; Fox and Serrano, 2005). The molecular mass of purified malabarin and its strong hemorrhagic activity suggests that it belongs to class P-II.

Most of the pathological symptoms exhibited by crude *T. malabaricus* venom is also exhibited by purified malabarin protease enzyme. In conclusion, the malabarin is the major toxic component in the *T. malabaricus* venom, which induces several pathophysiological symptoms. The inhibition of the catalytic activity of malabarin interns abolishes all the pharmacological activities. A known inhibitor of this enzyme helps in the management of *T. malabaricus* snakebite victims rather than anti-venin.
Table 3.01: Summary of Purification of malabarin from *T. malabaricus* venom

<table>
<thead>
<tr>
<th>Step</th>
<th>Total protein (mg)</th>
<th>Protein recovery (%)</th>
<th>Total activity <strong>(units / min)</strong></th>
<th>Specific activity (mg / min)</th>
<th>Activity yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Whole venom</td>
<td>100</td>
<td>100</td>
<td>2200 ± 0.41</td>
<td>18.3 ± 0.23</td>
<td>100</td>
</tr>
<tr>
<td>G-75 fraction</td>
<td>26</td>
<td>26</td>
<td>1701 ± 0.34</td>
<td>65.41 ± 0.04</td>
<td>87.37</td>
</tr>
<tr>
<td>DEAE A-25 fraction</td>
<td>3.1</td>
<td>2.84</td>
<td>1016 ± 0.07</td>
<td>327.6 ± 0.09</td>
<td>59.72</td>
</tr>
</tbody>
</table>

*One unit of enzyme activity was defined as the amount of enzyme required to increase in absorbance of 0.01 at 660 nm/hr at 37°C. **Values are mean ± S.D. of three independent experiments.
### Table 3.02: Biochemical and Biophysical properties of malabarın

<table>
<thead>
<tr>
<th>Properties</th>
<th>Value/Details</th>
</tr>
</thead>
<tbody>
<tr>
<td>Molecular weight by</td>
<td></td>
</tr>
<tr>
<td>(a) MALDI-TOF (kDa)</td>
<td>45.76</td>
</tr>
<tr>
<td>(b) SDS-PAGE (kDa)</td>
<td>45.5</td>
</tr>
<tr>
<td>UV-absorbance maximum (nm)</td>
<td>270-280</td>
</tr>
<tr>
<td>Fluorescence emission maximum (nm)</td>
<td>310 –320 and 330 - 340</td>
</tr>
<tr>
<td>N-terminal sequence</td>
<td>Ile-Ile-Leu-Pro(Leu)-Ile-Gly-Val-Ile-Leu(Glu)-Thr-Thr</td>
</tr>
<tr>
<td>Isoelectric point</td>
<td>6.2</td>
</tr>
<tr>
<td>Carbohydrate content</td>
<td>Nil</td>
</tr>
<tr>
<td>Optimum pH</td>
<td>8.0</td>
</tr>
<tr>
<td>Optimum temperature (°C)</td>
<td>37</td>
</tr>
<tr>
<td>Substrate specificity</td>
<td>Casein, gelatin, human fibrinogen</td>
</tr>
<tr>
<td>Effect of inhibitors:</td>
<td></td>
</tr>
<tr>
<td>EDTA, PMSF, 1, 10 phenanthroline</td>
<td>Inhibited</td>
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<tr>
<td>PMSF</td>
<td>No</td>
</tr>
<tr>
<td>Esterase activity</td>
<td>Yes</td>
</tr>
<tr>
<td>Lethal potency</td>
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</tr>
</tbody>
</table>
Figure 3.01: Sephadex G-75 elution profile of *T. malabaricus* venom

Sephadex G-75 column (0.8 x 120 cm) was eluted with 10 mM Tris-HCl buffer pH 8.0 at a flow rate of 20 ml/hr and 2.0 ml fractions were collected. The protein elution was monitored at 280 nm (—) and protease relative activity at 660 nm (...) of alternate fractions. The fractions having enzymatic activity (dotted line) were pooled for further fractionation.
The column (1.6 x 20 cm) was pre-equilibrated in 10mM Tris-HCl buffer pH 8.0. The column was eluted by a stepwise gradient of Tris-HCl buffer (10 mM -150 mM) and NaCl gradient (0.01M -2 M) as indicated in Figure. The 2.0 ml fractions were collected and proteins elution was monitored at 280 nm in a spectrophotometer. Fractions showing enzyme activity (dotted line) were pooled and desalted for further analysis.
Figure 3.03: Electrophoretic pattern of malabaricin

Native basic PAGE: 70 µg of *T. malabaricus* venom (A), 40 µg of Sephadex G-75 I peak (B) and 15 µg of malabaricin (C) was resolved on 12.5% polyacrylamide gel, run at pH 8.3 using Tris-Glycine buffer with bromophenol blue as a tracking dye.

SDS-PAGE (10%): Samples containing 60 µg of *T. malabaricus* venom (B), 40 µg of Sephadex G-75 I peak (C), 15 µg of malabaricin under non-reducing (D) and reducing (E) conditions. Molecular weight markers (A) in kDa: Phosphorylase b (97.4), bovine serum albumin (66.0), ovalbumin (43.0), carbonic anhydrase (29.0), soyabean trypsin inhibitor (20.0) and lysozyme (14.3).
Figure 3.04: RP-HPLC elution profile of malabar in
Purified malabar in was run on a Vydac C_{18} RP-HPLC column. Solvent A
was 0.1 % TFA and Solvent B was 70 % acetonitrile in 0.1% TFA. A
gradient of 0 -100 % solvent B was run from 0 to 60 min as indicated in
the figure. Elution was monitored at 280 nm.
Figure 3.05: MALDI-TOP mass spectrum of malabar in
MALDI-TOF mass spectrometry of malabar in the positive ionization
mode using α-Cyano-4-hydroxycinnamic acid as MALDI matrix.
Figure 3. 06: Zymogram of casein and gelatin of T. malabaricus venom, TM -I and malabarin samples

Zymogram of casein (A) and gelatin (B): 0.2 % casein and gelatin was incorporated as substrate into the 10 % resolving gel separately. Venom samples were loaded on to the gel and electrophoresis was carried out under non-reducing condition. Clear zones in the gel indicate the enzymatic activity of venom samples. A - T. malabaricus venom (2 μg), B-TM -I (0.5 μg) and C- malabarin (0.1 μg)
Figure 3.07: Dose and time dependent hydrolysis of human fibrinogen by malabarín

(A) Malabarín was incubated with 50 μg of human fibrinogen at 37 °C for 60 min in presence of 10 mM Tris-HCl buffer pH 7.6. SDS-PAGE (10%) was performed to visualize fibrinogen degradation products. A - Control, B -0.1 μg, C -0.2 μg, D -0.3 μg, E -0.4 μg, F -0.5 μg and G -0.6 μg.

(B) Time dependent: Malabarín (0.3 μg) was incubated with 50 μg of human fibrinogen at 37 °C for different time intervals (0 - 2 hr) in presence of 10 mM Tris-HCl buffer pH 7.6. SDS-PAGE (10%) was performed to visualize fibrinogen degradation products. A -0 min, B -5 min, C -15 min, D -30 min, E -60 min, and F -120 min of incubation.
Figure 3.08: Dose dependent hemorrhagic activity of malabar

Samples were injected at a constant volume of 20 μl in saline by an intra dermal route on the back of mice and hemorrhagic activity was carried out as described in methodology section. The animals were injected with different concentration of malabar. Saline was injected to the control group. A- Control, B- 0.5 μg, C- 1.0 μg, D- 1.5 μg, E- 2.0 μg and F- 2.5 μg.
Figure 3.09: Dose dependent edema inducing activity of malabarin

(A) Mice with various concentration of malabarin injected into intra plantar surface of mouse footpad. Edema ratio was calculated as described in methodology section.

(B) Dose dependent edematous footpad depicting hemorrhage edema. Values are mean ± S.D. of three independent experiments.
Figure 3. 10: Histopathology and serum CK and LDH levels of mice injected i.m.) with *T. malabaricus* venom

(A) Histopathological section: Microphotograph of mice thigh muscle injected with malabar venom. The microphotographs were taken using an Olympus BX40F-3 microscope with an integrated Olympus camera. A- Control, B- 1 mg / kg body weight. H- Widespread hemorrhage, N- Necrotic cells and IS - Intrastial space.

(B) Serum CK and LDH levels: Serum CK and LDH enzymes were assayed using Span diagnostics kit. The activity was expressed in U / Lt. Con: Control, Tm: *T. malabaricus*, TM-I: G-75 first fraction and Mal: Malabar. Values are mean ± S.D. of three independent experiments.
Figure 3.11: Effect of *T. malabaricus* (○), TM-I (■) and malabarin (▲) on human plasma coagulation

(A) Recalcification time: Samples was pre-incubated with 0.3 ml of platelet poor plasma (PPP) at 37 °C for 1 min. 30 μl CaCl₂ (0.25 M) was added to the pre-incubated mixture to observe the clot formation. Plasma devoid of venom served as control.

(B) Prothrombin time: Samples was pre-incubated with 0.1 ml of human plasma and clotting time was recorded in sec after the addition of 0.2 ml of brain thromboplastin. Plasma devoid of venom served as control. Plasma devoid of venom served as control. Values are mean ± S.D. of three independent experiments.
Figure 3.12: Effect of protease inhibitors on human fibrinogen and hemorrhage activity of malabarìn

(A) Hydrolysis of human fibrinogen: Malabarìn (0.6 μg) was pre-incubated with and without 5 mM of protease inhibitors separately for 20 min. Pre-incubated reaction mixture was incubated with 50 μg of human fibrinogen at 37 °C for 60 min in presence of 10 mM Tris-HCl buffer pH 7.6. SDS-PAGE (10 %) was performed to visualize fibrinogen degradation product. A -Control, B -Malabarìn, C -PMSF, D -EDTA, E -EGTA and F -1,10-phenanthroline.

(B) Inhibition of hemorrhagic activity: 3 μg of malabarìn was pre-incubated for 20 min with and without 5 mM each of protease inhibitors. Pre-incubated reaction mixture was injected (intra dermal) separately into groups of 3 mice each. Saline was injected to the control group. A -Control, B -Venom alone, C -EDTA, D -EGTA, E -1,10-phenanthroline and F -PMSF.
Figure 3.13: Effect of protease inhibitors on the serum CK and LDH activities in mice after intramuscular route injection of malabar

1 mg / kg body weight of malabar was pre-incubated for 20 min with and without 5 mM each protease inhibitors. Pre-incubated reaction sample were injected intramuscular route on the right thigh of mice. Serum CK and LDH enzymes were assayed using Span diagnostics kit. The activity was expressed in U / Lt. Values are mean ± S.D. of three independent experiments.