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'Partitagin' a hemorrhagic metalloprotease from *Hippasa partita* spider venom: Role in tissue necrosis

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Abstract

The poisonous bite by *Hippasa partita*, a funnel web spider from the Indian subcontinent has been demonstrated to give rise to severe dermo- and myonecrosis. In this work a hemorrhagic metalloprotease, Partitagin was purified from *H. partita* venom by successive chromatography on Sephadex G-100, DEAE Sephadex A-50 and Biosep DEAE columns. SDS-PAGE, reversed phase HPLC on a C_4 column, N-terminal amino acid sequencing and MALDI-TOF mass spectrometry confirmed the homogeneity. Partitagin was assayed using fat free casein as substrate. EDTA, 1,10-phenanthroline and cyanide, inactivated it irreversibly while, EGTA, PMSF, leupeptin, pepstatin and aprotinin did not inhibit. The presence of Zn^{+2} was confirmed by atomic absorption spectrometry. Partitagin caused hemorrhage when tested in a mouse model. Light microscopy of skin tissue sections at the site of injection revealed extensive damage of extracellular matrix (ECM) in which the basement membrane surrounding blood vessels and capillaries showing signs of extensive destruction and also loss of vessel wall integrity. Similar intense damage was also noticed in the ECM of muscle tissue sections but with no damage caused to myocytes. Partitagin showed specificity of action on the components of ECM and degraded collagen type-IV and fibronectin but not collagen type-I. Partitagin was devoid of edema, myotoxicity and lethality. This is the first report on the isolation and characterization of a toxin from spider venom in the Indian subcontinent.

Keywords: Spider venom; *Hippasa partita*; Partitagin; Hemorrhagic metalloprotease; Extracellular matrix; SVMPs

1. Introduction

Hemorrhage and an eventual necrosis of the tissue at the envenomed region are reported in several spider bites [1–5]. Predominantly, zinc dependent metalloproteases of the 'metzincin' family enzymes of the venom are found responsible for this toxic property [6–8]. Hemorrhage is caused due to the destruction of structural components of basement membrane surrounding blood vessels and capillaries. Basement membrane is a highly specialized type of extracellular matrix and plays a key scaffolding role in endothelium and other cell types. It holds several structural components of the extracellular matrix such as collagen type-IV, fibronectin, laminin, entactin and some glycosaminoglycans. However, the molecular composition of the basement membrane tends to vary among different tissues and cell types [9,10]. Fibronectin molecule present in the basement membrane bridges the cells and the extracellular matrix and also adjacent cells in a tissue. The binding specificity is achieved by the RGD sequence at the interaction sites [11]. Degradation of basement membrane components predominantly by matrix degrading metalloproteases is found responsible for the venom induced hemorrhage and tissue necrosis at the bitten region. Several such proteases have been isolated and studied extensively from spider venoms [12–16] and as well as from snake venoms [17,8].

In our earlier study on the comparative examination, *Hippasa partita* venom induced hemorrhage in mice, while the other two, *Hippasa agelenoides* and *Hippasa lycosina*

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venoms did not [18]. Therefore, *H. partita* venom was investigated further for the hemorrhagic activity, and isolated a 29.129 kDa Zn^{+2} dependent metalloprotease. The protease was found to cause hemorrhage in mice and showed *in vitro* degradation of several structural proteins of the extracellular matrix and the results of which are presented in this manuscript.

2. Materials and methods

H. partita female spiders were collected from Irpu falls region, Madikeri district (Western Ghats in Karnataka) during February to April. The spiders were identified based on Tikader and Malhotra [19] and confirmed by Dr. Manju Siliwal, Wildlife Information and Liaison Development Society, Zoo Outreach Organization, Coimbatore, Tamilnadu. Sephadex G-100, DEAE Sephadex A-50, fat free casein, gelatin, collagen type-IV (from Engelbreth–Holm–Swarm murine sarcoma basement membrane), fibronectin (from human plasma), collagen type-I (from rat tail tendon), ethylene diamine tetraacetic acid (EDTA), potassium cyanide (KCN), phenyl methyl sulfonyl fluoride (PMSF), aprotinin, ethylene glycol-*N*,*N*,*N*′,*N*′-tetracetic acid (EGTA), 1,10-phenanthroline, pepstatin and leupeptin were purchased from Sigma Chemicals Co. St Louis, USA. Molecular weight markers were from Biorad Company, USA. LDH and CK diagnostic kits were from AGAPEE (India). *Daboia russelii* venom was purchased from Hindustan Park, Kolkata, India. All other chemicals used were of analytical grade. Swiss Wister albino mice weighing 20–25 g (from the central animal house facility, Department of Zoology, University of Mysore, Mysore, India) were used for pharmacological studies. Animal care and handling complied with the National Regulation for Animal Research.

2.1. Preparation of venom gland extract

Venom was extracted from wild caught spiders (around 200 in number) that were kept for five days without food but with water. The venom gland extract was prepared as previously described [15]. Briefly, adult female spiders were anaesthetized using chloroform inhalation, dissected and a pair of glands was collected into ice cold phosphate buffered saline (PBS). The glands were washed in PBS in order to remove possible contaminants and venoms were harvested in PBS by gentle compressing of the glands. The suspension was clarified by centrifugation at 8000 *g* and the venom gland extract was stored at −20 °C until use.

2.2. Sephadex G-100 column chromatography

The lyophilized *H. partita* venom gland extract sample: 100 mg in 1.0 ml of 0.1 M NaCl was applied on to a Sephadex G-100 column (1.5 × 75 cm) that was equilibrated with 0.1 M NaCl. The column was eluted using 0.1 M NaCl with a flow rate of 22 ml/h and 2.2 ml fractions were collected. Protein elution was monitored at 280 nm using Shimadzu spectrophotometer (1601 Å). Alternative tubes were assayed for proteolytic activity using fat free casein as substrate. Fractions having enzyme activity were pooled and concentrated.

2.3. DEAE Sephadex A-50 column chromatography

The proteolytic fraction recovered from the previous step: 8 mg in 2 ml of the equilibrating buffer was loaded onto the DEAE-Sephadex A-50 column (1.6 × 25 cm) that was equilibrated with 10 mM Tris–HCl buffer pH 7.4. Subfractions were then eluted stepwise using 10 mM Tris–HCl buffer pH 7.4 containing different molarities of NaCl (0.02–0.4 M). Fractionation was carried out at a flow rate of 20 ml/h and 2 ml fractions were collected. Finally fractions showing proteolytic activity were pooled and concentrated.

2.4. High-pressure liquid chromatography

The proteolytic fraction recovered from the previous step: 0.65 mg in 0.3 ml equilibrating buffer was loaded on to the Vydac Biosep DEAE-P column (7.55 × 75 mm) that was equilibrated with 10 mM Tris–HCl buffer pH 7.4 containing 30 mM NaCl. Subfractions were eluted using linear gradient of 10 mM Tris–HCl buffer pH 7.4 containing 30–120 mM NaCl. Flow rate was 0.5 ml/min and 1 ml fractions were collected. Proteolytic fractions were pooled and concentrated to obtain Partitagin.

2.5. Proteolytic activity

Proteolytic activity was assayed according to the method of Satake et al. [20]. Fat free casein (0.4 ml, 2% in 0.2 M Tris–HCl buffer pH 7.6) was incubated with 50 μg of Partitagin in 1 ml of the total volume at 37 °C for 2 h 30 min. Adding 1 ml of 0.44 M trichloroacetic acid and left to stand for 30 min stopped the reaction. The mixture was then centrifuged at 2000 *g* for 10 min. Sodium carbonate (2.5 ml, 0.4 M) and Folin ciocalteus reagent (diluted to 1/3 of the original strength) were added to 1 ml of the supernatant and the color 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 37 °C. The specific activity was expressed as the units/min/mg of protein. For inhibition studies, similar reaction was performed independently after pre-incubation of Partitagin for 30 min with 5 mM each of EDTA, 1,10-phenanthroline, EGTA, PMSF, aprotinin, and 2.5 mM of pepstatin and leupeptin. In all the cases appropriate controls were kept.

2.6. SDS-polyacrylamide gel electrophoresis

SDS-PAGE was carried out according to the method of Laemmli [21]. Molecular weight standards from 14.3 to 200 kDa were used. Electrophoresis was carried out using Tris (25 mM), glycine (192 mM) and SDS (0.1%) for 3 h at 90 V at room temperature. After electrophoresis, gels were stained with 0.1% Coomassie brilliant blue R-250 for detection of the protein bands and destained with 25% ethanol and 8% acetic acid.

2.7. PAS staining

SDS-PAGE was carried out as mentioned above. PAS staining was done according to the method of Leach et al. [22].

2.8. Zymogram experiments

SDS-PAGE (10%) was carried out according to the method of Laemmli [21] and polymerized at a final concentration of 0.2% with gelatin/casein. Partitagin (10 μg protein) was prepared under non-reduced condition. Electrophoresis was carried out using Tris (25 mM), glycine (192 mM) and SDS (0.1%) for 3 h at 90 V at room temperature. After electrophoresis, gels were washed with 10 mM sodium phosphate buffer pH 7.6 containing 2.5% of Triton X-100 with shaking for 1 h to remove SDS and incubated overnight at 37 °C in Tris–HCl buffer (50 mM) pH 7.6 containing 10 mM $CaCl_2$ and 150 mM NaCl. Gels were then stained with Coomassie brilliant blue R-250.

2.9. Reversed phase-HPLC (RP-HPLC)

Partitagin was subjected to RP-HPLC using Vydac-C_4 column (5 μm, 0.21 × 25 cm) column, which had been pre-equilibrated with 0.1% TFA in water. The column was eluted using linear gradient from solution A (0.1% TFA in water) to 100% solution B (0.1% TFA in acetonitrile) for 40 min. the protein was eluted at a flow rate of 1 ml/min and monitored at 280 nm.

2.10. Mass spectrometry

The molecular mass of Partitagin was determined by mass spectrometry using Bruker Daltonics Matrix-Assisted Laser Desorption Ionization Time Of Flight (MALDI-TOF) machine in positive ionization mode. α-Cyano-4-hydroxycinnamic acid was used as MALDI matrix.

2.11. Metal analysis

Metal content in Partitagin was determined by atomic absorption spectrophotometry on an AA-670, Shimadzu spectrophotometer. Sample was dissolved in deionized water and parallel water control was also analyzed for metal content.

2.12. N-terminal sequencing

N-terminal sequence of Partitagin was analyzed by automated Edmann degradation method on Applied Biosystems Procise 494 protein sequencer connected to HPLC.

2.13. Hemorrhagic activity

Hemorrhagic activity was assayed as described by Kondo et al. [23]. Partitagin was injected (intradermally) in different concentration (5, 10, 15 and 20 μg) in to groups of six mice each in 50 μl saline. Group receiving saline alone served as negative control. After 3 h mice were anaesthetized using diethyl ether and sacrificed. Dorsal patch of skin surface was carefully removed, without stretching, and the diameter of the hemorrhagic spot on the inner surface of the skin was measured. The minimum hemorrhagic dose (MHD) was defined as the amount of the hemorrhagin capable of producing 10 mm of hemorrhage in diameter.

2.14. Myotoxicity

Myotoxicity was determined according to the method of Gutierrez et al. [24]. Cytoplasmic marker enzymes, lactate dehydrogenase (LDH, EC no: 1.1.1.28) and creatine phosphokinase (CK, EC no: 2.7.3.2) levels were determined in the serum. Partitagin (2.5 mg/kg body weight) and *D. russelii* venom (1.25 mg/kg body weight, i.e., 1/2 LD_{50}) in 50 μl saline were injected intramuscularly into the right thigh of a separate group of mice ($n = 6$) and served as positive control. The group receiving 50 μl saline alone served as negative control. Mice were anaesthetized after 3 h by diethyl ether inhalation. Abdominal cavities were opened and blood was drawn from the abdominal vena cava. The 1:25 diluted serum was assayed for LDH and CK activities using AGAPEE diagnostic kits. LDH and CK activities were expressed as units/l.

2.15. Histopathology studies

Partitagin was injected intradermally at the dose of 20 μg (2 MHD), and intramuscularly at a concentration of 2.5 mg/kg body weight into different group of six mice each, in a total volume of 50 μl of saline. Control mice received saline alone. After a period of 2, 4, 6, 8 and 10 h the animals were sacrificed. Skin and muscle tissues dissected out from site of toxin injection were fixed in Bouin's solution, and subjected to dehydration by processing the tissues through different grades of alcohol and chloroform: alcohol mixture. The processed tissue was embedded in paraffin and cut into 4-μm thick sections. The sections were stained with hematoxylin–eosin staining for microscopic observations. The sections were observed under Leitz wetzlar Germany type-307-148.002 microscope and photographs were taken using Photometrics colorsnap CF camera (made Roper Scientific Photometrics) attached to the microscope.

2.16. Degradation of extracellular matrix molecules

Collagen type-I and type-IV and fibronectin were incubated with Partitagin in the ratio of substrate:enzyme (6:1, w/w) in a total volume of 40 μl of Tris–HCl buffer (10 mM) pH 7.4 containing 10 mM NaCl at 37 °C. Aliquots of the reaction mixtures were taken out at different time intervals of 2, 4, 6, 8 and 10 h and the Partitagin was inactivated by the addition of 20 μl denaturing buffer containing 1 M urea, 4% SDS and 4% β-mercaptoethanol. It was then analyzed on 7.5% SDS-PAGE. The gels were then quantified using densitometry as a function of time.

2.17. Protein estimation

Protein concentration was determined according to the method of Lowry et al. [25] using bovine serum albumin (BSA) as standard.

2.18. Statistical analysis

All the data were presented as mean ± SD.

3. Results

3.1. Purification and properties of Partitagin

Partitagin, a hemorrhagic metalloprotease has been purified to the extent of 26-folds from *H. partita* venom based on molecular sieving and ion exchange properties. The venom when fractionated on a Sephadex G-100 column, four peaks were resolved. The proteolytic activity was concentrated in the II-peak (Fig. 1A). Further fractionation of the protease fraction on a DEAE Sephadex A-50 column indicated the recovery of proteolytic activity in the III-peak (Fig. 1B). This peak upon HPLC on a Biosep DEAE-P column, Partitagin showing the proteolytic activity is eluted as a major peak (Fig. 1C). The total protein and proteolytic activities loaded and recovered in each step are summarized in Table 1. Throughout the purification, alternative tubes were assayed for proteolytic activity and the pooled fractions were tested for hemorrhagic activity. In all the cases, there observed overlapping of proteolytic and hemorrhagic activities. Partitagin moved as a sharp band to the same extent in SDS-PAGE under both non-reduced and reduced conditions (Fig. 2A). Similar purity was also suggested by activity staining when a single transluscent activity band was seen both in casein and gelatin zymograms (Fig. 2B and C). Partitagin eluted as a sharp peak with the retention time of 23.6 min in reversed phase HPLC on a C_4 column (Fig. 1D). It revealed a single symmetrical sharp peak at *m/e* of 29.129 in MALDI-TOF mass spectrometry. It did not reveal any pink color band when subjected for periodic acid Schiff's base staining. The N-terminal sequence analysis of the enzyme gave the sequence of AYDPDPYK*RY*SA*E*HTFFLL. It was optimally active at pH 7.6 and at 37 °C when assayed in the range between pH 4–9 and 4–60 °C, respectively. Aprotinin, leupeptin, pepstatin, PMSF and EGTA, each at 5 mM concentration did not inhibit the caseinolytic activity of Partitagin. In contrast, 1,10-phenanthroline, EDTA and cyanide abolished the activity of Partitagin. Further, neither extensive dialysis nor addition of 15 mM $ZnCl_2$ restore the activity. The presence of Zn^{+2} in Partitagin was confirmed by atomic absorption spectroscopy. The molar ratio for protein to Zn^{+2} calculated was found to be 1:3. Partitagin induced hemorrhage in mice and the effect was found to be dose dependent (Fig. 3). The minimum hemorrhagic dose determined was 10 ± 1 μg. Partitagin was found to be non-lethal to mice up-to the tested dose (ip) of 10 mg/kg body weight.

3.2. Histopathological study

Dermonecrotic effect of Partitagin was evaluated by injecting 2 MHD (20 μg) intradermally to groups of mice ($n = 6$). Mice were anaesthetized and sacrificed after 2, 6 and 10 h of injection to remove the skin tissue sample for histological studies. The tissues were independently processed and 4-μm thick sections were made for observation under light microscope. Time dependent graded degradation of skin tissue is shown in Fig. 4. At 2 h of Partitagin injection, initiation of damage of ECM (Fig. 4Ab), while at 6 h, extensive damage of basement membrane surrounding blood vessels resulted in intense hemorrhage and massive infiltration of inflammatory leukocytes were observed (Fig. 4Ac). However, at 10 h, the integrity of ECM of dermis and the blood vessels were lost (Fig. 4Ad). In contrast, intact ECM and the basement membrane surrounding the blood vessels and also no infiltration of inflammatory leukocytes (Fig. 4Aa) were observed in saline injected tissue sections. Light microscopic observation of longitudinal section of muscle tissue treated with Partitagin is shown in Fig. 5. Intramuscular injection of Partitagin (2.5 mg/kg body weight) caused disorganization of myofibrils in which the myocytes were spaced apart and the distance between myocytes progressively increased with the time of treatment (Fig. 5b–e). However, throughout the study the myocytes were found intact and not damaged and, further, no infiltration of inflammatory leukocytes was found. Partitagin treated muscle sections lost the striated appearance, this is in contrast to the saline injected tissue section in which the tissue displayed the normal striated appearance of myocytes (Fig. 5a). Further, the creatine kinase (CK) and lactate dehydrogenase (LDH) levels in the serum of mice injected with Partitagin did not increase and were in good agreement with the saline injected mice. While injection of ½ LD_{50} (1.25 mg/kg body weight) of *D. russelii* whole venom showed destruction of the muscle tissue in which extensive damage of ECM and the myocytes (Fig. 5f) was observed and, in addition, the serum of mice recorded increased levels of CK and LDH activities (data not shown).

3.3. Degradation of components of extracellular matrix

The ability of Partitagin to degrade the cementing materials of extracellular matrix was examined. Partitagin degraded collagen type-IV, the control intact collagen type-IV revealed three bands corresponding to two α1(IV) (220 kDa and 180 kDa) and one α2(IV) (120 kDa) bands (Fig. 6A). Following incubation with Partitagin there observed reduction in the intensity of both the α1(IV) (220 kDa and 180 kDa) bands and as well as α2(IV) (120 kDa) band with the subsequent generation of additional new low molecular weight (about 60 kDa) band in SDS-PAGE under reduced condition. There observed initial preferential degradation of α1(IV) (220 kDa band) over α1(IV) (180 kDa) band while, α2(IV) band was the least preferred band. At 6 h of incubation, α1(IV) (220 kDa), α1(IV) (180 kDa) and α2(IV) (120 kDa) bands were degraded to the extent of about 40%, 30% and 10%, respectively. At 10 h of

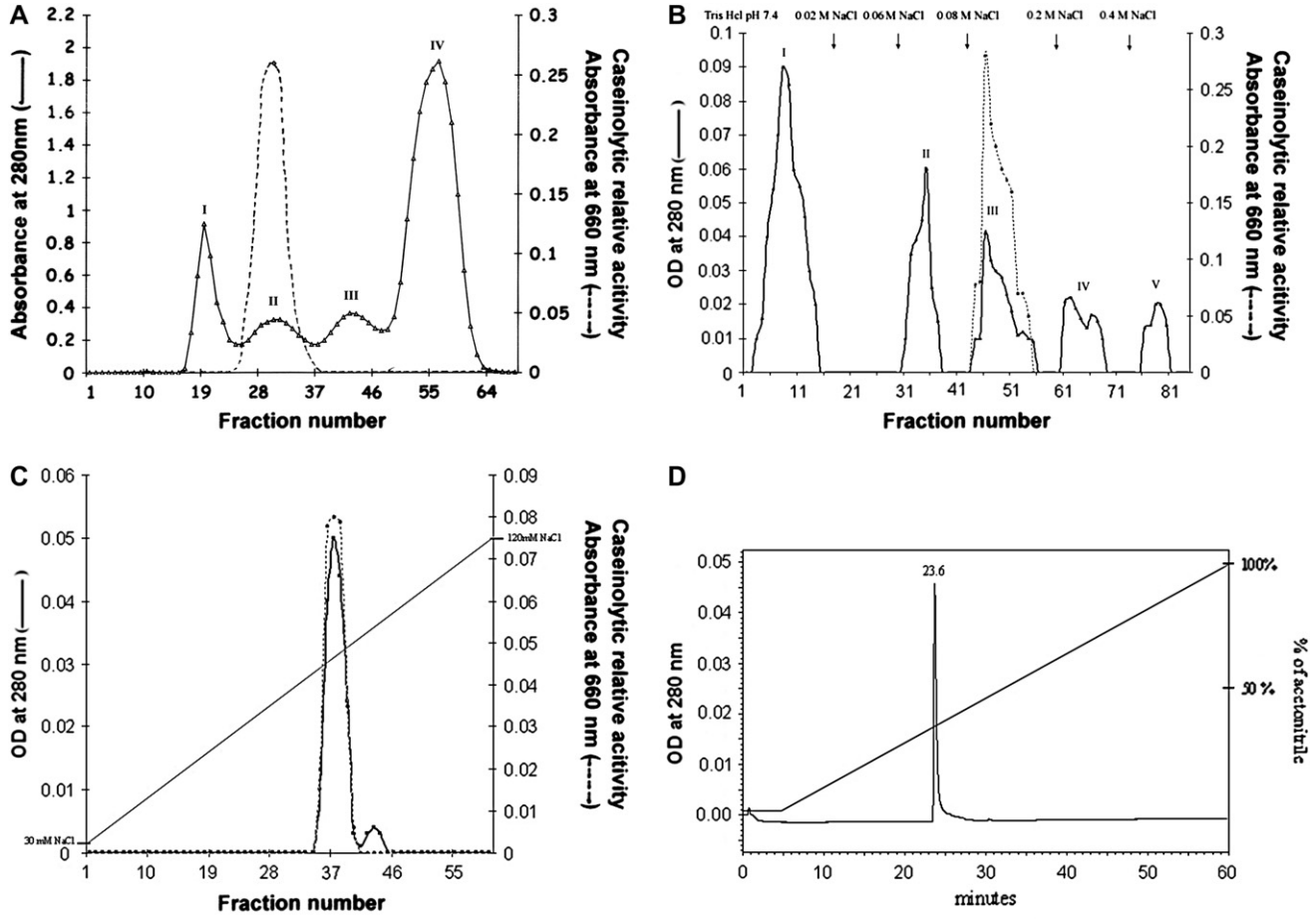

Fig. 1. Purification of Partitagin. (A) Sephadex G-100 column chromatography; *Hippasa partita* venom gland extract sample (100 mg in 1 ml of 0.1 M NaCl) was loaded on to the column (1.5 × 75 cm). It was eluted with 0.1 M NaCl at a flow rate of 22 ml/h and 2.2 ml fractions were collected. Protein elution was monitored at 280 nm (—). Proteolytic activity was determined using casein in alternate fractions at 660 nm (- - - - -). Proteolytic activity was concentrated in peak II. (B) DEAE Sephadex A-50 column chromatography; peak II obtained from the previous step (8 mg in 1 ml of equilibrating buffer) was loaded on to the column (1.6 × 25 cm) that was equilibrated with 10 mM Tris–HCl buffer pH 7.4. The column was eluted stepwise using 10 mM Tris–HCl pH 7.4 buffer containing different molarities of NaCl (0.02–0.4 M). Flow rate was adjusted to 20 ml/h and 2 ml fractions were collected. Protein elution was monitored at 280 nm (—). Alternate tubes were assayed for proteolytic activity on casein at 660 nm (- - - - -). Peak III showed proteolytic activity. (C) Biosep DEAE-P column chromatography; peak III obtained from the previous step (0.65 mg in 0.3 ml of equilibrating buffer) was loaded on to the column (75 × 7.55 mm) that was pre-equilibrated with 10 mM Tris–HCl buffer pH 7.4 containing 30 mM of NaCl. The column was eluted stepwise using 10 mM Tris–HCl buffer pH 7.4 containing different molarities of NaCl (30–120 mM). Flow rate was adjusted to 0.5 ml/min and 1 ml fractions were collected. Protein elution was monitored at 280 nm (—). Tubes were assayed for proteolytic activity on casein at 660 nm (- - - - -). (D) RP-HPLC profile on a Vydac-C_4 column (5 μm, 0.21 × 25 cm) that had been pre-equilibrated with 0.1% TFA in water. Protein was eluted using a linear gradient from solution A (0.1% TFA in water) to 100% solution B (0.1% TFA in acetonitrile) over 60 min. Protein was eluted at a flow rate of 1 ml/min and monitored at 280 nm.

incubation, α1(IV) (220 kDa), α1(IV) (180 kDa) and α2(IV) (120 kDa) bands were degraded to the extent of about 100%, 80% and 20%, respectively as revealed by densitometry (Fig. 6A). Further, progressive increased intensity of additional low molecular weight band generated also suggests collagen type-IV degradation.

Fibronectin is also susceptible for Partitagin activity; Fig. 6B is showing the time dependent degradation of fibronectin in which both A (270 kDa) and B (250 kDa) bands were degraded. The intensity of the bands is progressively diminished as a function of time. However, there observed initial preference for A band over B band. At 6 h of incubation A band was degraded to an extent of 80% while, B band was degraded to the extent of 40%. At 10 h of incubation both the bands were degraded completely and form a wide range of low molecular weight bands ranging from 97 to 18 kDa. Among these, bands in the range of 45–18 kDa were found to be prominent. In contrast, Partitagin did not degrade any of the bands in collagen type-I (Fig. 6C). Neither change in intensity of existing bands nor appearance of new bands seen throughout the incubation period.

4. Discussion

This study describes the isolation and characterization of Partitagin, which is a zinc dependent hemorrhagic metalloprotease from *H. partita* spider venom. Partitagin has been purified to the extent of 26-folds with the final protein and caseinolytic activity yield of 0.6% protein and 19.5% caseinolytic activity (Table 1). Purity has been adjudged by reversed phase HPLC on a C_4 Vydac column, MALDI-TOF mass

Table 1
Summary of purification of Partitagin from *H. partita* venom

Procedure	Total protein (mg)	Protein recovery (%)	Specific activity (units/mg/min)	Total activity[a]	Activity yield (%)	Fold purity
Sephadex G-100 column						
Whole venom gland extract	100	95	0.49	46.55	100	1
Peak-II	9	9	22	19.8	45.53	4.5
DEAE Sephadex A-50 column						
Peak-II from G-100 column	8	100	22	17.6	100	1.0
Peak-III	0.72	9	12.74	9.17	52.10	5.79
Biosep DEAE column						
Peak III from DEAE	0.65	100	12.74	8.28	100	1.0
Sephadex A-50 column						
Partitagin	0.6	92	12.8	7.68	92.75	1.0

Values are average of two independent experiments.
[a] One unit of activity is defined as the amount of enzyme required to cause the increase in OD of 0.01 at 660 nm/min at 37 °C.

spectrometry and N-terminal sequence analysis. Partitagin is a single chain protein as it revealed single band in SDS-PAGE under both reduced and non-reduced conditions. Partitagin is not a glycoprotein, as it did not answer for periodic acid staining. The molecular weight was found to be 29.129 kDa in MALDI-TOF mass spectrometry which agrees well with the metalloprotease of *Loxosceles intermedia* venom [26,27]. Partitagin contained Zn^{+2}, which is not easily dissociable by dialysis, suggesting the tight interaction between the protein and the metal ion. Metal chelating agents such as EDTA, 1,10-phenanthroline and cyanide independently inactivated the Partitagin irreversibly, as addition of Zn^{+2} did not restore the activity lost. EDTA and cyanide independently found to form stable complex with the Zn^{+2}, hence inactivation of Partitagin. Therefore, Zn^{+2} appears to play a role in functioning of Partitagin. However, its role in maintaining the structural integrity cannot be ignored. The metal content appears to be bit more than that reported for several SVMPs, however, agrees well with the metal content of *Lachesis muta muta* [27], *Agkistrodon acutus* [28], *Crotalus horridus horridus* [29]. SVMPs wherein the protein to metal (Zn^{+2} and or Ca^{+2} or both) ratio has been reported to be over 1:3 [30].

Zinc dependent 'metzincin' family metalloproteases are primarily responsible for both local and systemic hemorrhagic effects of envenomation [31]. Several metalloproteases of wide molecular weight range from 22 to 100 kDa have been studied extensively from various venoms [32], albeit only few such proteases from spider venoms are included in to the growing list [33]. Transverse section of hemorrhagic area of mice skin injected with Partitagin showed progressive dermonecrotic effect with the degradation of basement membrane surrounding blood vessels. In the initial 2 h of injection, Partitagin damaged the ECM surrounding blood vessels leading to extravasation in the hypodermis and also caused initiation of infiltration of inflammatory PMNLs (polymorphonuclear leukocytes) observed. Following 6 and 10 h of injection, massive degradation of ECM surrounding blood vessels and capillaries and subsequent damage of vessel walls, irregular and broken vessel wall morphology were seen compared to intact and circular vessel wall morphology of control tissue sections

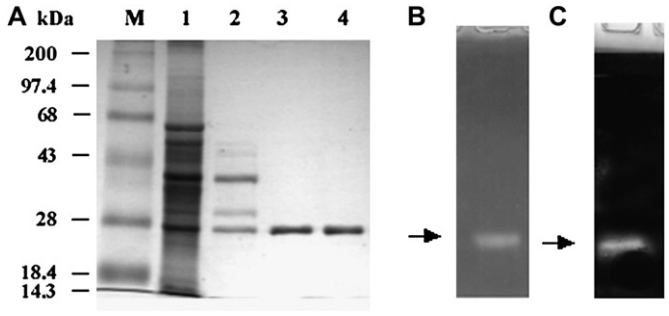

Fig. 2. SDS-PAGE and activity staining pattern of Partitagin. (A) Purification of Partitagin as shown in SDS-PAGE (10%). Samples containing 75 μg of venom gland extract (1), 50 μg of Peak II of Sephadex G-100 column (2), 15 μg of Partitagin under non-reduced (3) and reduced condition (4). M represents the molecular weight markers in kDa (from top to bottom: myosin-H-chain (200), phosphorylase b (97.4), BSA (68), ovalbumin (43), carbonic anhydrase (29), β-lactalbumin (18.4) and lysozyme (14.3). (B) Caseinolytic activity of Partitagin (10 μg) resolved by SDS-PAGE (10%) under non-reduced condition. (C) Gelatinolytic activity of Partitagin (10 μg) resolved by SDS-PAGE (10%) under non-reduced condition.

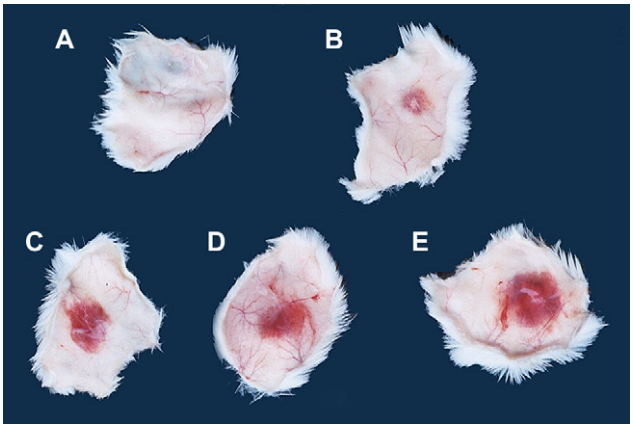

Fig. 3. Dose dependent hemorrhagic activity of Partitagin. Different concentrations of Partitagin was independently injected intradermally in to groups of mice in a total volume of 50 μl saline. A, B, C, D and E represents the samples containing saline alone and 5, 10, 15 and 20 μg of Partitagin in saline, respectively. After 3 h, mice were sacrificed, dorsal patch of skin surface was removed, and diameter of the hemorrhagic spot on the inner surface of the skin was measured in mm.

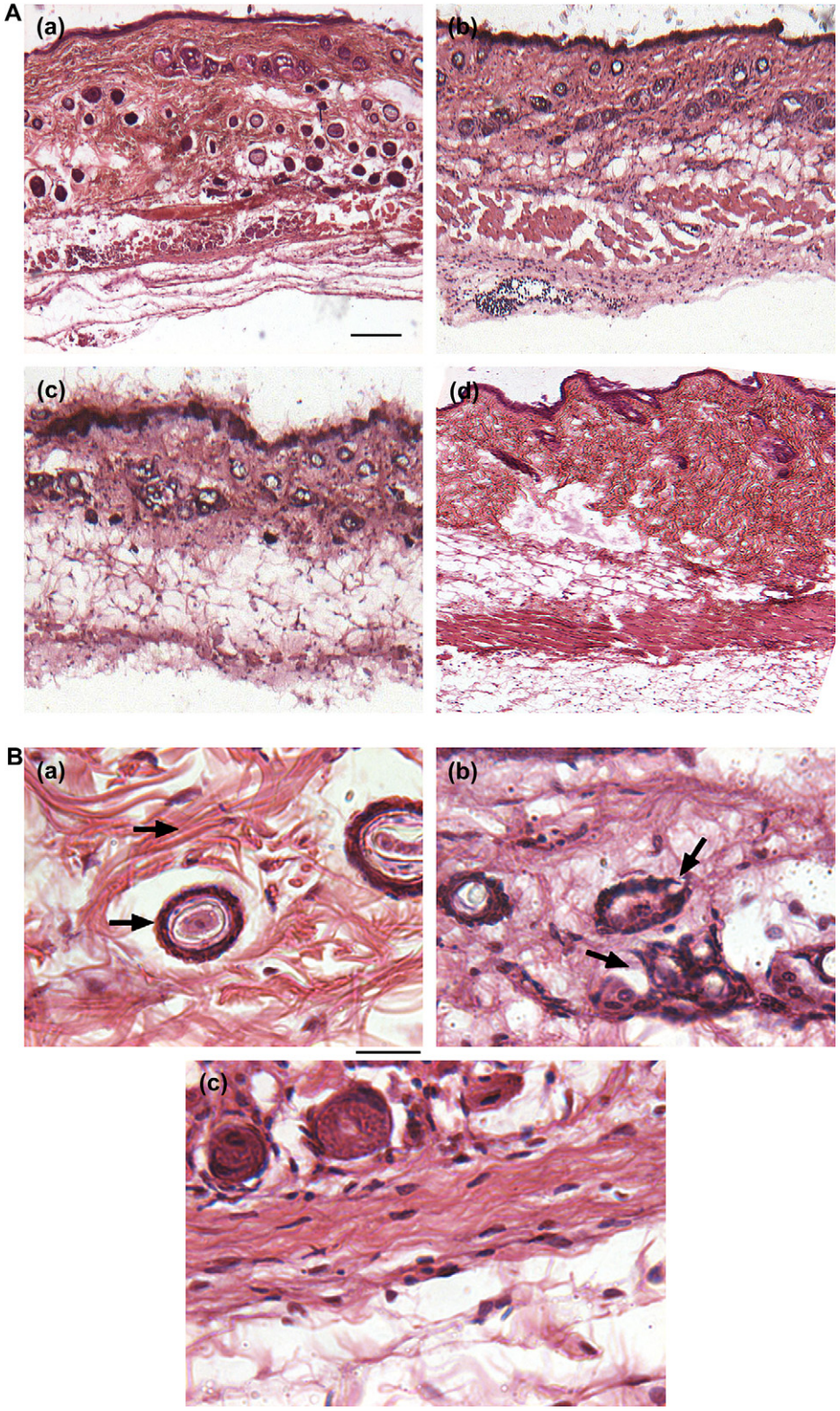

Fig. 4. Light micrograph of transverse section of mouse skin. (A) Light micrograph of transverse section of mouse skin taken at various time intervals following intradermal injection of Partitagin (2 MHD, 20 μg). (a) Control section injected with saline, (b) (c) and (d) are tissue sections taken after 2, 6 and 10 h after injection of Partitagin, respectively. Indicating intact ECM and blood vessels (a), inflammatory reaction in dermal blood vessel (b), destruction of ECM (c), and destruction of blood vessels (d). Bar represents 100 μm. (B) A high power view of transverse section taken at 6 h after intradermal injection of Partitagin. (a) Control section showing the intact blood vessel wall and surrounding tissue, (b) Partitagin treated skin section showing the damaged blood vessel wall and destruction of surrounding tissue, and (c) section showing the infiltration of polymorphonuclear leukocytes. Bar represents 50 μm.

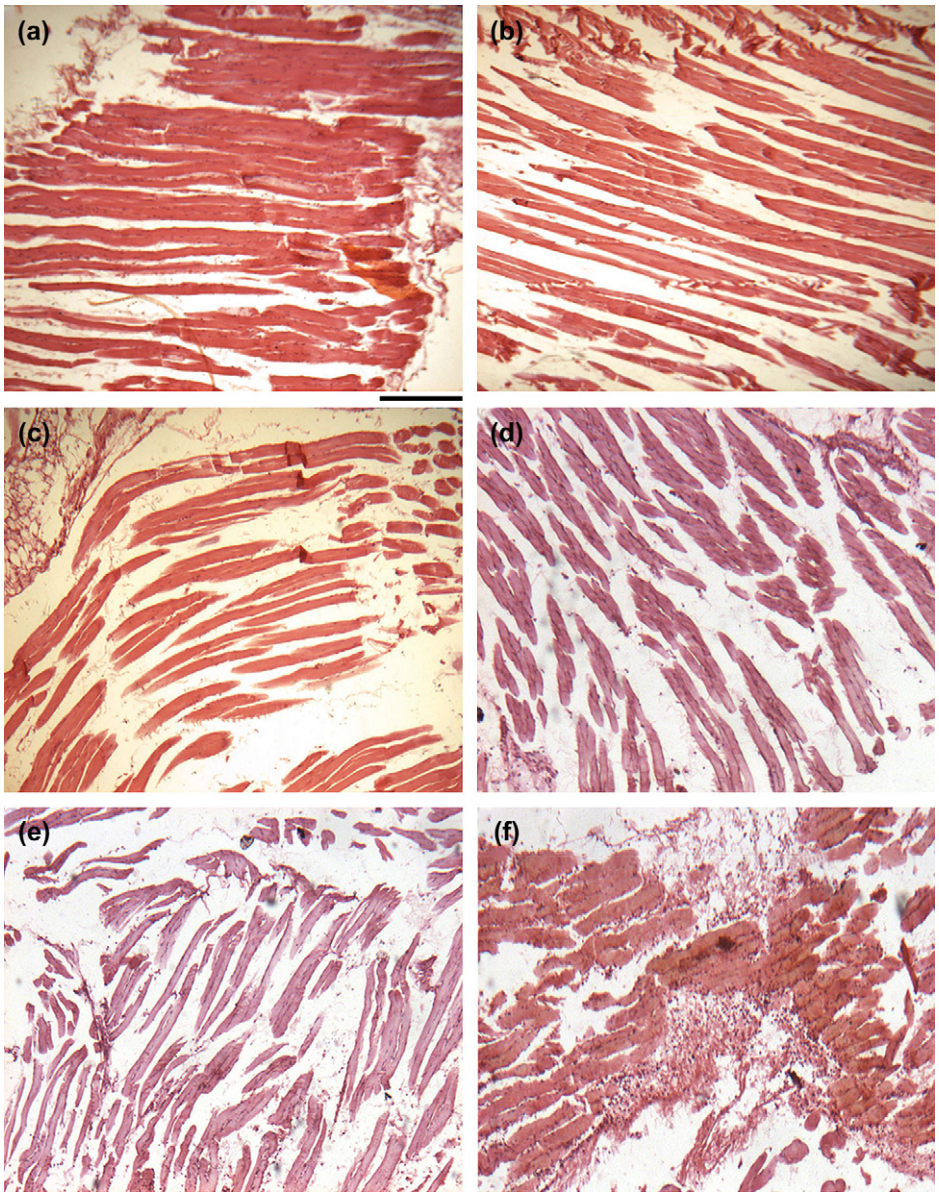

Fig. 5. Light micrograph of longitudinal section of mouse skeletal muscle. (a) Section from control mice injected with saline, note the characteristic striation and intact myoblasts, sections (b) (c) (d) and (e) are 2, 4, 6 and 8 h after the injection of Partitagin (2.5 mg/kg body weight). There observed the progressive disorganization of myoblasts, but with less infiltration of PMNLs. (f) Section injected with *Daboia russelii* whole venom (1.25 mg/kg body weight) showing the destruction of myoblasts with extensive infiltration of PMNLs. Bar represents 100 μm.

(see Fig. 4Ba). Selected and/or limited cleavages of key peptide bonds of basement membrane proteins of blood vessel endothelium appear to weaken the capillary wall leading to extravasation. Similar observations were made with the studies using *L. intermedia* venom [13]. Infiltration and confluence of PMNLs are a natural response for tissue destruction, and this could be due to the action of Partitagin resulting in generation of PMNLs recruiting proteolytic peptide fragments either from collagen type IV or fibronectin or from both.

Partitagin was also evaluated for muscle tissue destruction property. Longitudinal section of muscle tissue showed no signs of destruction of myocytes, however, clear and noticeable progressive disorganization of the striated myofibrils seen with the increased time of treatment but with no infiltration of inflammatory PMNLs (Fig. 5b–e). While, no increased serum CK and LDH activities were recorded as the activity of these enzymes agrees well with the values of saline injected control mice. Thus, Partitagin is devoid of myotoxicity, while specifically act on the ECM of tissues. Further, less or no infiltration of PMNLs possibly suggests little or no generation of PMNLs recruiting proteolytic peptides, which, otherwise seen abundantly in case of skin tissue sections. This could probably explain the varied specificity of action of Partitagin in different tissues or varied relative abundance of different ECM components. In contrast, mice injected (im) with the *D. russelii* whole venom showed extensive necrosis, disorganized ECM and damaged myocytes and flocks of PMNLs; also, the serum recorded the increased levels of serum CK and LDH enzymes.

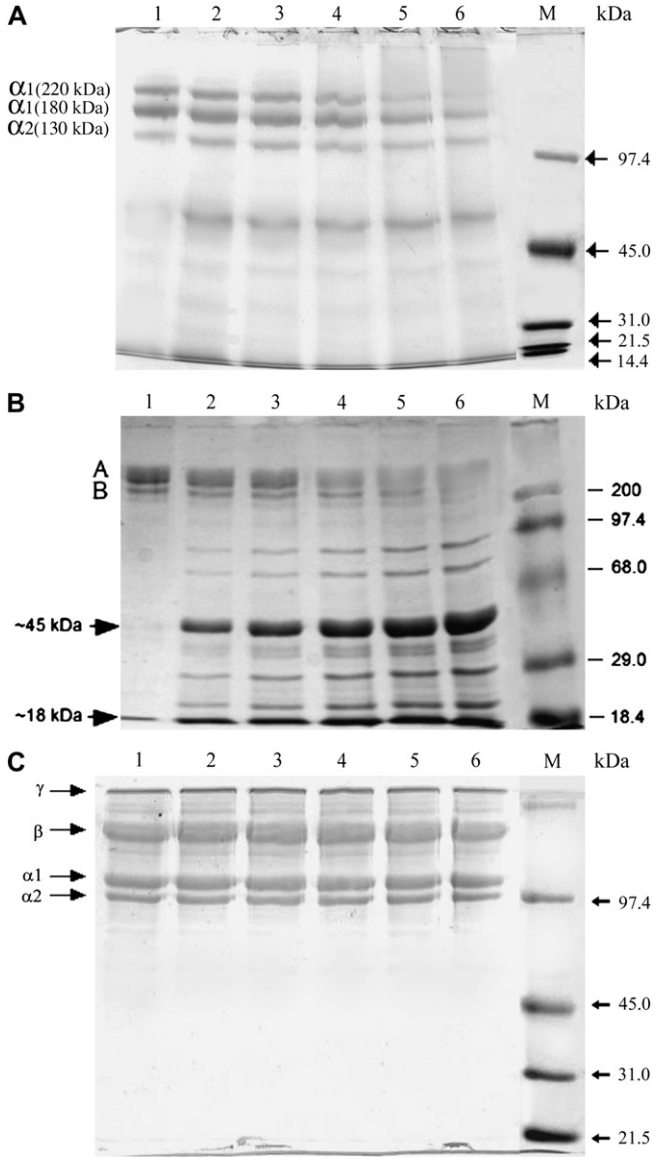

Fig. 6. Degradation of extracellular matrix components by Partitagin. (A) Collagen type-IV (B) fibronectin and (C) collagen type-I. Respective ECM components were incubated independently with Partitagin in the ratio of 6:1 (substrate:enzyme, w/w) at 37 °C for different time intervals and analyzed on SDS-PAGE (7.5%) under reduced condition. In respective cases; lane 1: Intact molecule, and lane 2: 2 h, lane 3: 4 h, lane 4: 6 h, lane 5: 8 h and lane 6: 10 h of Partitagin incubation and lane M represents the standard molecular weight markers in kDa (from top to bottom) myosin-H-chain (200) phosphorylase b (97.4), ovalbumin (45.0), carbonic anhydrase (31.0), trypsin inhibitor (21.5), β-lactalbumin (18.4) and lysozyme (14.4).

BjussuMP-I, a hemorrhagic metalloprotease isolated from *Bothrops jararacussu* venom showed hemorrhagic activity and hydrolysed collagen type IV but devoid of myotoxicity [34]. The precise mechanism of hemorrhagic function has not been fully elucidated for metalloproteases, however, it is widely accepted that proteolytic degradation of basement membrane components of capillary vessels is a key step [8]. *B. jararacussu* metalloprotease, BjussuMP-I, has been suggested to degrade basement membrane of capillary vessels, and hence becoming thinner and eventually developing gaps in their continuity, leading to extravasation [35]. In general, hemorrhage seems to be the early symptom due to the action of metzincin family enzymes; continued action of these enzymes would result in necrosis of the local tissue. Nevertheless, local tissue destruction during natural envenomation is due to cumulative effect of ECM degrading metalloproteases and hyaluronidases and myonecrotic property of myonecrotic phospholipase A_2 enzymes of venom [36,37]. In addition, victim's secondary activated extracellular matrix degrading proteases following envenomation cannot be ignored.

Partitagin selectively degraded collagen type-IV (Fig. 6A) and fibronectin (Fig. 6B), while it did not degrade collagen type-I (Fig. 6C). Collagen type-IV is the major component of basement membrane surrounding blood vessels. It forms network with laminin via nidogen/entactin of basic basement membrane scaffold with which other proteins and proteoglycans interact to form mature basement membrane. Thus, degradation of collagen type-IV might lead to structural collapse/impairment in basement membrane integrity [8]. Collagen type-IV degradation has been associated with the ability to induce hemorrhage in many snake venom metalloproteases [38]. Immunofluorescence staining studies indicated the association of fibronectin with the subendothelial extracellular matrix. Degradation of fibronectin will render the structure of the endothelium more susceptible for damage [12,13,16]. Thus, degradation of basement membrane collagen type-IV and fibronectin molecules in the blood vessels appears to be the key process for the hemorrhage caused by Partitagin and probably whole venom as well. Fibronectin is implicated in such physiological functions as tissue repair, blood clotting and cell migration/adhesion, it serves as adhesion molecule by anchoring cells to collagen or proteoglycan substrates [39]. The defective wound healing observed in some cases of *L. intermedia* envenomation was ascribed to the fibronectinolytic activity of the venom, since fibronectin–integrin interaction is necessary to bridge adjacent cells in a tissue for wound healing [11]. The fact that limits further discussion is the lack of information on structure–function, and systematic classification of spider venom metalloproteases. However, the molecular size of Partitagin agrees well with the molecular size 20–30 kDa of P-I class of snake venom hemorrhagic metalloproteases [6].

In summary, a 29.129-kDa Zn^{+2} dependent hemorrhagic metalloprotease Partitagin has been isolated from *H. partita* spider venom. Partitagin appears to play role in the venom induced hemorrhagic activity and efforts have been made to understand the possible molecular mechanism of its hemorrhagic activity through *in vitro* selective degradation of components of the basement membrane surrounding blood vessels and capillaries.

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