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Serine proteinases from *Bothrops* snake venom activates PI3K/Akt mediated angiogenesis

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ABSTRACT

The discovery of rapid acting and powerful angiogenic proteins are of significant interest in the treatment of various human disorders associated with insufficient angiogenesis such as ischemia, menorrhagia and delayed wound healing. Snake venoms consist of a mixture of bioactive proteins and polypeptides and are rich sources of pharmacologically important molecules. Serine proteinases are one of the abundant proteins present in *Bothrops* snake venoms and possess multiple biological functions including the regulation of the blood coagulation cascade. In this study, serine proteinases from *Bothrops atrox* (*B. atrox*) and *Bothrops brazili* (*B. brazili*) that modulate angiogenesis were purified and characterized. Molecular size exclusion chromatography, affinity chromatography followed by ion exchange chromatography of the serine proteinases indicated molecular masses of around 32 kDa. Serine proteinases from both the species exhibited diverse catalytic activities such as the ability to induce amidolytic, fibrinogenolytic, gelatinolytic activities and also coagulated plasma with a minimal coagulation concentration of 2.4 μg/mL. Serine proteinases facilitated the sprouting of human umbilical vein endothelial cells (HUVEC) in three-dimensional culture systems and induced tubule formation in monolayer culture systems. Serine proteinase stimulated Akt^{ser473} and $eNOS^{ser1177}$ phosphorylation in endothelial cells and addition of PI3K inhibitor LY294002 abrogated the effects of serine proteinases on sprout formation of endothelial cells in 3D collagen gels, suggesting that serine proteinase facilitated angiogenesis was mediated by PI3K/eNOS signaling axis. We also show in agarose plug assays using a mouse model, serine proteinases from *Bothrops* venoms significantly enhanced neovascularization. Our data suggests pro-angiogenic activity by the serine proteinases from *B. atrox* and *B. brazili* venom and further studies are warranted to explore the therapeutic applications.

1. Introduction

Snakes belonging to the *Bothrops* genus are pit vipers specific to Central and South America and exhibit numerous intraspecific differences in their morphological and ecological traits (McDiarmid et al., 1999; Carrasco et al., 2012). Venoms from *Bothrops* species contain highly toxic and bioactive substances and cause myonecrosis, cardiovascular alterations such as hemorrhage and hypovolemic shock, edema, coagulation disorders and renal alterations (Gutiérrez and Lomonte, 1989). *Bothrops* snake venoms constitute mixtures of biologically active substances, comprising proteins, lipids, steroids, aminopolysaccharides, amines and quinines (Vyas et al., 2013). Metalloproteinases, phospholipases A_2, serine proteinases and C-type lectins present in *Bothrops* venoms have been demonstrated to interfere with the hemostatic system (Markland, 1998; Matsui et al., 2000). Snake venom serine proteinases (SVSPs) that are present abundantly in venoms of Viperidae, Crotalidae, Elapidae and Colubridae form a group of comprehensively studied toxins affecting several physiological processes (Serrano and Maroun, 2005). Serine proteinases form a part of the largest family of peptidases including trypsin family S1 of clan SA (Halfon and Craik, 1998). Diverse biological functions of proteinases have been reported such as the regulation of cytokines and signaling

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receptors leading to the modulation of intracellular signaling pathways (caspase-like enzyme activity) (Ramachandran and Hollenberg, 2008). One of the targets of SVSPs for cleavage are mainly the proteins involved in the coagulation cascade of fibrinolytic and kallikrein–kinin systems which can significantly influence hemostatic systems (Serrano, 2013).

Angiogenesis (neovascularization) is the physiological process that involves growth of new blood vessels (Folkman, 1984). It has long been recognized that several pathological conditions are associated with the component of either excessive angiogenesis or insufficient angiogenesis (Carmeliet, 2003). Pathological angiogenesis is responsible for more than 70 life-threatening diseases. Accordingly, much effort has been devoted to identify target molecules that can be exploited for therapeutic strategies by inhibiting or promoting angiogenesis (Noel et al., 2004; Ribatti and Djonov, 2011). Pathological conditions such as ischemia in ischemic heart diseases, menorrhagia and delayed wound healing are associated with insufficient angiogenesis. Several pre-clinical studies have reported the use of various proteins such as vascular endothelial growth factor, platelet derived growth factor and fibroblast growth factor-2 (bFGF) to induce angiogenesis for the treatment of diseases associated with the impaired angiogenesis (Said et al., 2012). Serine proteinases and other proteolytic enzymes such as matrix metalloproteinases have been implicated in modulating angiogenesis (Pepper et al., 1996). These enzymes degrade extracellular matrix (ECM) proteins to facilitate remodeling of ECM leading to release of matrix bound growth factors thereby stimulating endothelial cell migration and proliferation (Pepper, 2001; Sottile, 2004). Interestingly, multiple functions of serine proteinases akin to thrombin, urokinase plasminogen activator, tissue plasminogen activator, kallikreins, tryptase and chymase have been demonstrated including induction of angiogenesis apart from their key role in facilitating the coagulation cascade (Tsopanoglou and Maragoudakis, 2004). This prompted us to investigate whether serine proteinases from *Bothrops* snake venoms have any influence on modulating angiogenesis. In this study, we have extensively characterized serine proteinases from *B. brazili* and *B. atrox* for their molecular and functional properties and subsequently demonstrate that serine proteinases exert robust pro-angiogenic activities *in vitro* and *in vivo* systems.

2. Materials and methods

2.1. Chemicals

Sephacryl S-200 and benzamidine sepharose 4 fast flow columns were obtained from GE healthcare (Little Chalfont, UK), Nα-benzoyl-DL-arginyl-p-nitroanilide (BApNA), methylcellulose and fibrinogen were procured from Sigma-Aldrich (St Louis, USA). Collagen R solution was procured from SERVA electrophoresis (Heidelberg, Germany), basic fibroblast growth factor (bFGF) was obtained from R & D systems (Minneapolis, USA) and matrigel was purchased from BD biosciences (San Jose, USA). Antibodies that recognize total and phosphorylated Akt^{ser473}, phosphorylated $eNOS^{ser1177}$ and β-actin were procured from Cell Signaling (Danvers, MA, USA). The crude venom samples were obtained from San Maru Serpentarium, which is authorized by the Instituto Brasileiro do Meio Ambiente e dos Recursos Naturais Renováveis (Ibama).

2.2. Cell culture

Endothelial cells (HUVEC) and fibroblasts were isolated from human umbilical cord and foreskin respectively after obtaining institutional ethical clearance from Kasturba Medical College, Manipal University, Manipal, India. Briefly, endothelial cells from veins were dislodged using collagenase Type IV for 30 min, followed by washing with calcium and magnesium free phosphate buffered saline (PBS pH 7.4). Cells were seeded on gelatin coated plates and further cultured until confluency. Endothelial cells were characterized by cell specific markers such as CD31, VE-cadherin and CD44 using flow cytometry (BD Biosciences, USA). Endothelial cells were cultured in Endothelial Cell Growth Medium (ECGM) (Promo cell GMBH, Germany) containing 5% Fetal Bovine Serum (FBS). Fibroblast cells were isolated from skin epidermis, characterized based on typical fibroblast phenotype and were cultured in DMEM with 10% FBS (Himedia, Mumbai, India). Hepatocarcinoma (HepG2), human transformed embryonal kidney cells (HEK293), human transformed epithelial cells (HaCaT), Madin-Darby Canine Kidney Cells (MDCK) and neuroblastoma (IMR32) cell lines were procured from ATCC and cultured in DMEM containing 10% FBS. Peripheral blood mononuclear cells (PBMC) were isolated from healthy volunteers and cultured in RPMI+2% fetal bovine serum.

2.3. Purification of serine proteinases from B. brazili and B. atrox

2.3.1. Molecular size exclusion chromatography

Serine proteinase from venoms of *B. atrox* and *B. brazili* were isolated using chromatographic techniques. A total of 150 mg of the crude venoms of *B. brazili* and *B. atrox* were separately dissolved in 1.5 mL of a 0.02 M Tris–HCl buffer pH 8.0 containing 0.15 M NaCl and were centrifuged at 10,000 × g for 10 min. The clear supernatant was subjected to molecular size exclusion chromatography on a Sephacryl S-200 column previously equilibrated with the aforementioned buffer. The proteins were eluted at a flow rate of 0.2 mL/min, the absorption was monitored at 280 nm and fractions of 1 mL each were collected.

2.3.2. Affinity chromatography on benzamidine sepharose 4 Fast Flow

The fractions corresponding to the peaks containing serine proteinases were pooled and then applied to a benzamidine sepharose 4 fast flow (high sub) column (5 mL bed volume) using a superloop (50 mL) at a flow rate of 0.5 mL/min. The unbound proteins were washed out with 0.02 M Tris-HCl, pH 8.0, 0.15 M NaCl (buffer A). The nonspecifically bound proteins were eluted with the above mentioned buffer, which additionally contained 0.5 M NaCl. Once the baseline was stabilized, the bound proteins were eluted by rapidly changing the pH to 3.0 using a 0.05 M glycine-HCl (buffer B) (step gradient) at a flow rate of 0.5 mL/min, UV absorption was monitored at 280 nm and fractions of 1 mL/tube were collected. The pH of the eluted samples was immediately adjusted to pH 7.0 by adding a buffer containing 1 M Tris pH 9.0.

2.4. Peptide mass fingerprinting of serine proteinases

We determined peptide fingerprints of serine proteinases from *B. atrox* and *B. brazili* using liquid chromatography/mass spectrometry as described previously (Philippova et al., 2008). The HPLC purified protein was run on 10% SDS PAGE and stained with Coomassie Brilliant Blue. The band was sliced from the gel and was treated with 5 mM DTT in 25 mM ammonium bicarbonate (NH_4HCO_3) and incubated at 60 °C for 15 min. Subsequently 5 mM DTT was added and further incubated for 15 min and centrifuged. To the tube, 55 mM iodoacetamide in 25 mM NH_4HCO_3 was added and incubated for 30 min in dark. The gel slice was washed twice with 50 mM NH_4HCO_3 and again washed twice with acetonitrile. The gel slice was chopped into smaller fragments and washed twice with acetonitrile, dried under vacuum and rehydrated with 50 mM NH_4HCO_3 containing trypsin (5 μg/mL) and incubated overnight at

37 °C in a water bath. Next day, the supernatant was collected and stepwise extraction of peptides was performed with (a) 30% acetonitrile with 1% trifluoro acetic acid (TFA) (b) 1% TFA in water (c) 70% acetonitrile with 1% TFA. During each step, 30 min vortex was followed by 3 min sonication prior to collection. Supernatant from each step was collected, pooled, dried in vacuum and was reconstituted in 5% acetonitrile with 0.1% TFA. The LC/MS/MS of digested peptides was performed on a HPLC coupled with Agilent 6520 accurate-mass Q-TOF LC/MS using a reverse phase eclipse plus C18 5 μm 4.6 × 150 mm (Agilent Technologies, Santa Clara, US). 8 μL of reconstituted sample was injected to mass spectrometry and peptides were eluted in gradient form with 3–70% acetonitrile at flow rate of 0.4 μL/min for 50 min. MS/MS spectra were aligned in Qualitative Mass Hunter (Agilent Technologies, Santa Clara, US) and converted into mascot generating file (.MGF) and processed in Mascot database version 2.3 (Matrix Science Limited, London, UK). Proteins were identified using following parameters: Enzyme as trypsin, carbamidomethylation as fixed modification and methionine oxidation as variable modification and with peptide charges more than 3+, and blasted against SwissProt database.

2.5. Functional characterization of serine proteinases

2.5.1. Amidolytic activity of serine proteinases

Serine proteinases were characterized based on its amidolytic activity (Erlanger et al., 1961). Serine proteinase (1 μg) was incubated with 1 mM Nα-Benzoyl-DL-Arginyl p-nitroanilide (BApNA) in 250 μL solution containing buffer (10 mM Tris-HCl, pH 8.0, 10 mM $CaCl_2$ and 100 mM NaCl) at 37 °C for 1 h. The activity was measured every five minutes by recording absorbance at 410 nm. Further characterization of serine proteinases were performed upon inclusion of either protease inhibitors (1,10-phenanthroline (10 mM), PMSF (10 mM)) or chelators (EDTA (10 mM), EGTA (10 mM)) or reducing agents (β-mercaptoethanol) (10 mM) and divalent metal ions ($CaCl_2$, $MgCl_2$, $ZnCl_2$ and $HgCl_2$) (all 10 mM).

2.5.2. Coagulation assay

Coagulation activity was measured using human plasma as explained earlier (Theakston and Reid, 1983). Serine proteinase in a volume of 10 μL containing different concentrations (0.3, 0.6, 1.2, 2.4, 3.2 (μg/mL) was incubated with 140 μL of human plasma at 37 °C. The time needed to form fibrin networks was measured by a semi-automated coagulometer (CoaDATA 4001, UK). Minimum Coagulant Dose (MCD) was defined as the amount of serine proteinase (μg/mL) that clotted plasma in 60 seconds.

2.5.3. Fibrinogenolytic activity

The fibrinogenolytic activity (Zaqueo et al., 2014) was observed by incubating 10 μg of fibrinogen with 1 μg/mL of serine proteinase in 100 mM Tris pH 7.4 and incubated at 37 °C for 2 h. The reaction was terminated by adding Laemmli buffer (100 mM Tris pH 6.8, 20% glycerol, 4% SDS and 200 mM β-mercaptoethanol). The sample was loaded onto 10% SDS PAGE with negative control and observed for the degradation of sub-units of fibrinogen.

2.5.4. Gelatinolytic activity

The gelatinolytic activity was performed as previously described (Zaqueo et al., 2014). Serine proteinase (2 μg) was separated on SDS PAGE containing 0.3% gelatin as copolymerized substrate under non-reducing conditions. After electrophoresis, the gel was incubated with renaturation buffer (0.5% Triton X-100) for 90 min, followed by incubation with developing buffer (5 mM $CaCl_2$, 10 mM Tris and 100 mM NaCl) for 16–20 h. Further, the gel was stained with Coomassie brilliant blue G-250 and the presence of clear zones in the gel was indicative of gelatinolytic activity.

2.5.5. MTT cytotoxicity assay

MTT based cytotoxicity assay was performed using snake venom proteins on cells such as HepG2, HUVEC, fibroblast cells, HEK293, HaCaT, MDCK, IMR32 and PBMCs. Each cell type was seeded at the density of 10,000 cells/well in a 96 well plate. The cells were treated with or without serine proteinase (100 ng/mL). Mitomycin (100 μg/mL) was used as the positive control. Cells were incubated for 48 h, followed by the addition of 20 μL of 5 mg/mL MTT and incubated for 4 h. Formazan crystals were dissolved in 100 μL DMSO and absorbance at 570 nm was noted using Varioskan™ Flash Multimode Reader (Thermoscientific,USA).

2.6. Angiogenesis assays

2.6.1. Three dimensional spheroid models (In vitro angiogenesis assay)

Endothelial cell monolayer was trypsinized and the cells were resuspended in basal endothelial cell growth medium (ECGM) containing 20% of methylcellulose. The cell suspension was distributed as drops on the petri dish lid to obtain 5000 cells/drop and incubated for 24 h at 37 °C. The spheroids formed in each drop were embedded within the collagen matrix with 100 ng/mL of serine proteinase or 10 ng/mL of bFGF (positive control) and further cultured for 24 h. The length and number of sprouts per spheroid were measured morphologically by using ImageJ software (NIH, USA).

2.6.2. Tube forming assay (In vitro angiogenesis assay)

Tube forming assay was performed using HUVEC. A 50 μL of matrigel was coated on 96 well plates and incubated for 1 h at 37 °C. Cells were seeded at the density of 2×10^4 cells/well on matrigel cushion either in presence or absence of serine proteinase (100 ng/mL) or bFGF (10 ng/mL) and further incubated at 37 °C with 5% CO_2 for 6 h. Kinetics of tube formation in different conditions was observed.

2.6.3. Agarose plug transplantation assay

In order to demonstrate pro-angiogenic activity of serine proteinases *in vivo*, we employed agarose plug transplantation assay as described earlier (Brill et al., 2005). The study was approved by Institutional Animal Ethics Committee (IAEC), Kasturba medical college Manipal, Manipal University (IAEC/KMC/48/2015). Swiss albino mice (6–8 weeks) were used in four groups as control (n = 6), bFGF treated (n = 6), serine proteinase (*B.brazili)* (n = 6) and serine proteinase *(B.atrox)* (n = 6). Agarose gel (2%, 50 μL) was mixed with 1 μg/mL of serine proteinases/bFGF (100 ng/mL)/PBS and was allowed to solidify. The dorsal skin of mice was incised and the agarose pellets were introduced into subcutaneous space. After five days, tissue was excised and photographed with surrounding skin. Further, the skin was fixed with 4% formalin and 5 μm sections were prepared using microtome (Leica RM2125RT, Wetzlar, Germany) and sections were stained with Hematoxylin and Eosin.

2.7. Immunohistochemistry

Paraffin embedded histological sections were rehydrated using washes in xylene and series of decreasing concentrations of ethanol (100%–30%) followed by water and PBS for 5 min each. Antigen retrieval was performed in citrate buffer (10 mM Sodium Citrate, 0.05% Tween 20, pH 6.5) at 95 °C for 30 min. After a brief wash with PBS and 1.5% hydrogen peroxide, tissues were blocked with 5% BSA for one hour. Sections were treated with anti CD31 antibody (Cell Signaling, Danvers, MA, USA) overnight followed by incubation with anti-Rabbit IgG HRP-conjugate (Jacksons Laboratory, US) for one hour. After three washes with PBS, sections were treated with

chromogenic substrate 3,3′-Diaminobenzidine (Dako Agilent Pathology Solutions, CA, USA). Slides were dried, examined under Olympus BX51 (Olympus, Tokyo, Japan) upright microscope and captured using Olympus DP80 camera. Influence of various control and treatment groups on the blood vessel formation was quantified by counting number of CD31 stained vessels per field in each section.

2.8. Immunoblotting

HUVECs were serum starved (ECGM+0.5% BSA) for 3 h and further stimulated with serine proteinases (100 ng/mL) for 30 min. Cells were lysed with RIPA buffer (150 mM NaCl, 0.5% sodium deoxycholate, 50 mM Tris pH 8.0, 0.1% SDS, 1% Triton X 100, cocktail of protease inhibitors) and lysates were processed for immunoblotting and stained for total and p-AKT^{ser473}; p-eNOS^{ser1177} and β-actin. Imunoreactivity was determined using chemiluminescence (ECL) (Supersignal West Pico, Thermo Scientific, Pierce, USA) detection system using luminescent image analyzer (ImageQuant LAS4000, GE health care, UK).

2.9. Statistical analysis

All the experiments were performed in triplicates independently. Unpaired two-tailed Student's test and ANOVA were performed with or without *post-hoc* tests using Graphpad Prism. Data is represented along with standard deviations and p value < 0.05 was considered as significant change.

3. Results and discussion

3.1. Purification of serine proteinases from the venoms of B. brazili and B. atrox

The molecular size exclusion chromatography of *B. brazili* crude venom resulted in seven peaks numbered 1a to 7a, all of which were analyzed by SDS-PAGE gels (Fig. 1A). The fourth peak (4a) containing protein with a molecular weight of approximately 35 kDa was pooled and applied to a benzamidine sepharose 4 Fast flow affinity column which was separated into 3 sub-fractions as shown by a SDS-PAGE gel (Fig. 1B). The peak 3b from the affinity chromatography was pooled and subjected to cation exchange chromatography that resulted in further separation into five fractions. We obtained two proteins, a) 32 kDa in the first three peaks and b) 34 kDa in last two peaks (Fig. 1C). Based on earlier reports, we considered 32 kDa as serine proteinase and further characterized. The purity was confirmed by SDS-PAGE (Fig. 2C).

The molecular size exclusion chromatography of *B. atrox* crude venom resulted in four peaks numbered 1a to 4a, all of which were analyzed by SDS-PAGE (Fig. 2A). The proteins in the second peak (2a) with molecular weight of around 35 kDa were pooled and applied to benzamidine sepharose 4 Fast flow affinity column, which resulted in three peaks and were subsequently analyzed by SDS-PAGE (Fig. 2B). The pure serine proteinase was obtained in peak 3b. Purity of serine proteinase from *B. atrox* is shown in Fig. 2C.

Further, the serine proteinases from *B. brazili* and *B. atrox* were subjected to peptide mass fingerprinting. LC/MS/MS analysis of tryptic digested peptides of *B. atrox* serine proteinase led to the identification of a thrombin like enzyme, Bothrombin (*B. jararacas*; P81661). Five unique peptides matched with the thrombin like enzyme with a significant score of 2134. Analysis of serine proteinase from *B. brazili* showed four unique peptides which matched with venom serine proteinases HS114 (*B. jararaca*; Q5W959) with the score of 1084 (Table 1).

Protein homology of serine proteinases from different species of *Bothrops* genus was 65% and 30%–40% when compared to mammalian thrombin and trypsin (Fernandes de Oliveira et al., 2013). Serine proteinases from *B. brazili*, *B. atrox, B. jararaca, B. jararacussu* and *B. leucurus* exhibit sequence homology of approximately 79% with each other (Zaqueo et al., 2016). The relative masses of the serine proteinases from different snake venoms are presented in Table 2. The serine proteinases isolated from *B. brazili* and *B. atrox* venom in this study indicated a mass of 32 kDa which is in agreement with studies investigated in other *Bothrops* species (Fernandes de Oliveira et al., 2013; Stocker and Barlow, 1976; Zaqueo et al., 2016).

3.2. Serine proteinases from B. atrox and B. brazili possess amidolytic, coagulatory, fibrinogenolytic and gelatinolytic activity

We first characterized the serine proteinases based on

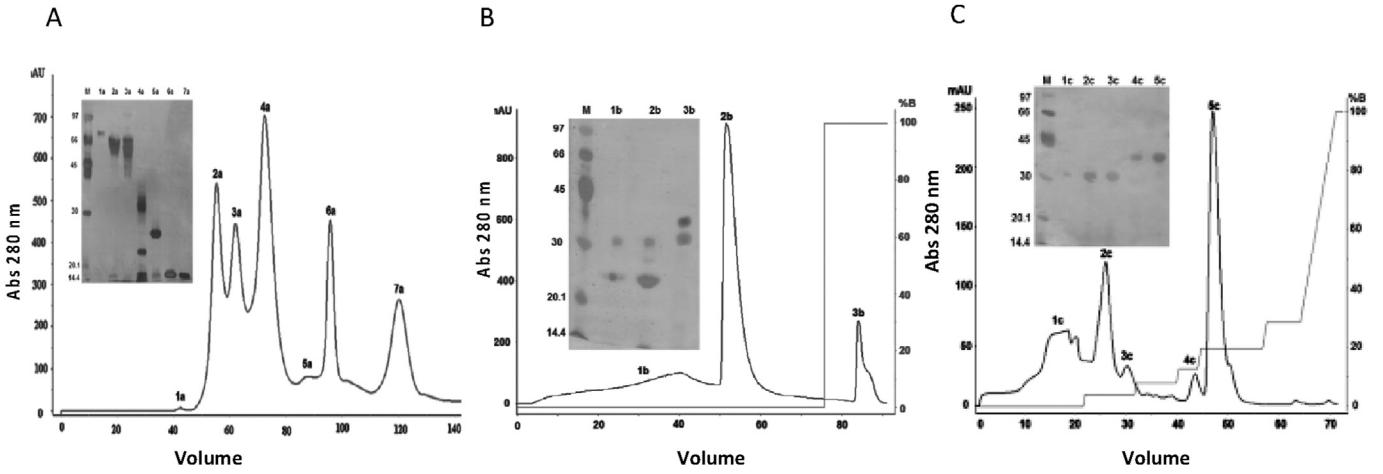

Fig. 1. Isolation and purification of serine proteinase from *B. brazili*. (A) Profile of molecular size exclusion chromatography on Sephacryl S-100 crude venom of *B. brazili* indicating the elution of seven peaks. Inset. SDS-PAGE analysis of peaks from molecular size exclusion chromatography. M; molecular weight markers (kDa), 1a to 7a corresponding peaks protein. (B) Affinity chromatography profile (peak 4a from MSEC of *B. brazili*) showing elution of three peaks numbered 1b through 3b. Inset. SDS-PAGE analysis of peaks fraction from affinity chromatography M; molecular weight markers (kDa), 1b–3b corresponding peaks proteins. (C) Ion exchange chromatography profile for (peak 3b of affinity chromatography) of *B. brazili* showing elution of five peaks numbered 1c through 5c. Inset. SDS-PAGE analysis of peaks from ion exchange chromatography M; molecular weight markers (kDa), 1c to 5c corresponding peaks proteins. Lane 1c, 2c and 3c represent 32 kDa serine proteinase and lane 4c and 5c 34 kDa serine proteinase.

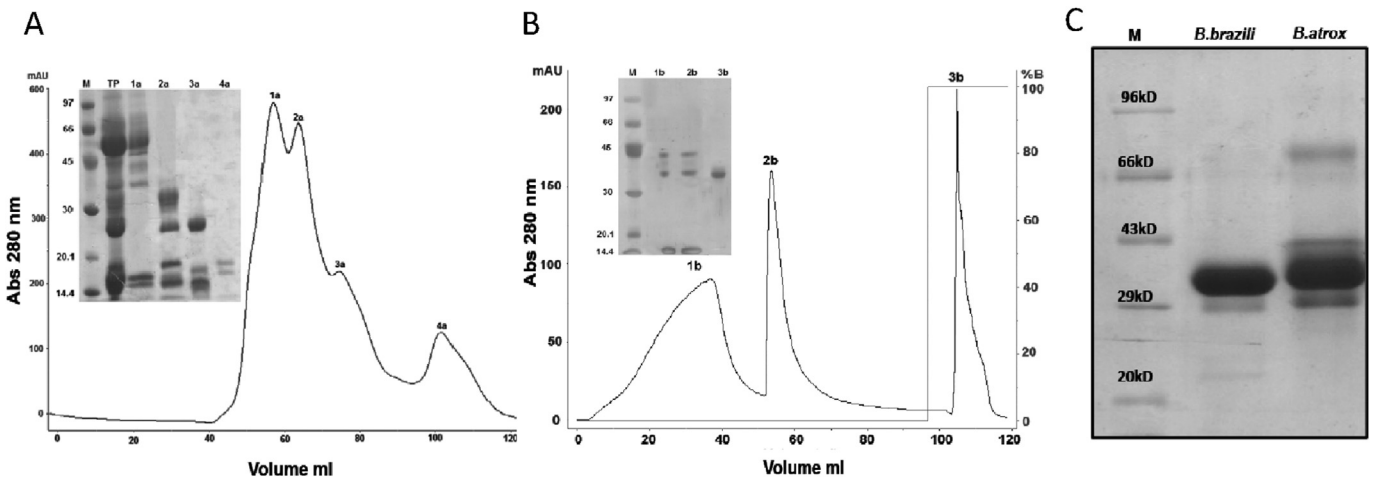

Fig. 2. Isolation and purification of serine proteinase from *B. atrox* (A) Profile of molecular size-exclusion chromatography on Sephacryl S-100 crude venom of *B. atrox* indicating the elution of 4 peaks. Inset. SDS-PAGE analysis of peaks from molecular size exclusion chromatography. M; molecular weight markers (kDa), TP: total proteins, 1a to 4a protein in corresponding peaks. (B) Affinity chromatography profile (peak 2a from MSEC *of B. atrox*) showing elution of three peaks numbered 1b through 3b Inset. SDS-PAGE analysis of peaks fractions from affinity chromatography M; molecular weight markers (kDa), 1b–3b corresponding peak proteins. Lane 3b represents pure serine proteinase. (C) SDS-PAGE analysis of serine proteinase from *B. brazili* and *B. atrox*.

Table 1
Peptide fingerprinting of serine proteinases.

Sample	Mascot score	Accession ID	Protein name	Observed peptide sequence	MW (da)
Serine proteinase *B. atrox*	2134	P81661	Thrombin like enzyme bothrombin	IYLGIHTRSVANDDEVIRYPKEKFICPNK KNVITDKDIMLIRLNRPVK EAYNGLPAK KPAFYTK TATCPP	26252
Serine proteinase *B. brazili*	1084	Q5W959	Venom serine proteinases HS114	TLNQDEQTR IFCPNK NDDALDKDLMLVRLDSPVSDSEHIAPLSLP VIDYNTWIESVIAGNTAATCPP	28509

Table 2
Comparison of functional characterization of serine proteinases from different species to the present study.

Protein	Species	Molecular mass (kDa)	Amidolytic	Coagulation	Fibrinogenolytic	Gelatinase	Reference
BbrzSP-32	*Bothrops brazili*	32	ND	+	+	ND	Zaqueo et al., 2016
BpirSP-39	*Bothrops pirajai*	39	+	+	+	+	Zaqueo et al., 2014
Russelobin	*Daboia russelii russelii*	51.3	+	+	+	ND	Mukherjee and Mackessy, 2013
Rhinocerase	*Bitis gabonica rhinoceros*	31	+	+	+	ND	Vaiyapuri et al., 2010
Thrombin-like enzyme	*Cryptelytrops purpureomaculatus*	35	+	+	+	ND	Tan, 2010
Bhalternin	*Bothrops alternatus*	31.5	ND	+	+	ND	Costa et al., 2010
AH143	*Agkistrodon halys*	30	+	+	ND	ND	Ghorbanpur et al., 2009
ABUSV-SPase	*Agkistrodon blomhoffii ussurensis*	26.7	+	ND	ND	ND	Liu et al., 2008
Thrombin-like enzyme	*Trimeresurus jerdonii*	38	+	+	+	ND	Lu et al., 2000
Serine proteinase	*Bothrops atrox*	32	+	+	+	+	Present study
Serine proteinase	*Bothrops brazili*	32	+	+	+	+	Present study

ND-Not determined.

amidolytic activity using BApNA, a known synthetic substrate for serine proteinase. Serine proteinases from both *B. atrox* and *B. brazili* exhibited amidolytic activity with minimum of 1 μg of protein. Time dependent analysis showed hydrolysis of BApNA within first 5 min and continued until 60 min. Amidolytic activities of enzymes from both the species were comparable (Fig. 3A). Further characterization of serine proteinase was performed using various metal cofactors and chelators. Both EGTA and EDTA significantly reduced the enzyme activity to 50% suggesting calcium as pre-requisite for enzyme activity. Amidolytic activity was inhibited by $ZnCl_2$ and $HgCl_2$; suggesting zinc and mercury divalent ions might act as competitive inhibitor of calcium in the enzyme buffer. However, magnesium did not show any effect on the enzyme activity. The proteinase inhibitor PMSF inhibited serine proteinase activity of *B. brazili* by 70% and *B. atrox* by 50% respectively. Interestingly, reducing agent β-mercaptoethanol did not show any effect on enzyme activity of *B. brazili*, however we observed a 20% inhibition of enzyme activity in *B. atrox* derived serine proteinase (Fig. 3B and 3C).

Several studies have characterized serine proteinases from

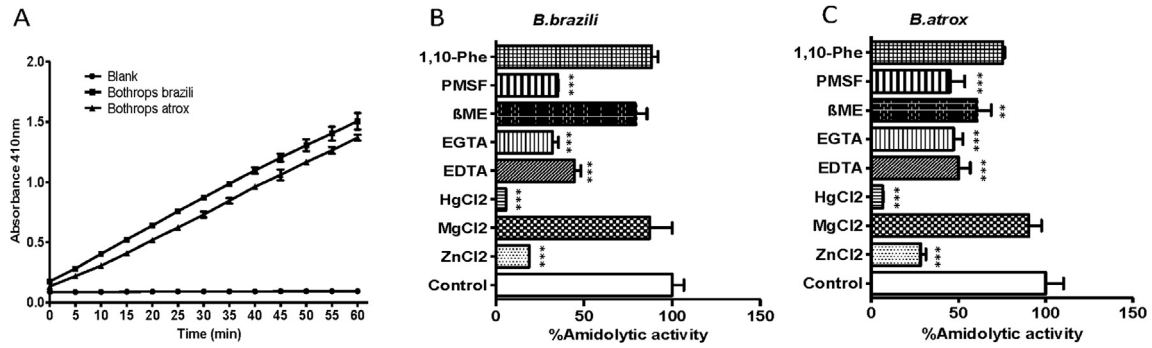

Fig. 3. Characterization of serine proteinase by amidolytic activity. (A) Serine proteinases from *B. brazili* and *B. atrox* were treated with BApNA substrate (1 mM) for 60 min and absorbance at 410 nm was measured along the time. Data is represented as arbitrary units. Amidolytic activity of serine proteinase from *B. atrox* (B) and *B. brazili* (C) was measured in presence or absence of metal chelators such as EDTA (10 mM) and EGTA (10 mM), divalent metals such as mercuric chloride ($HgCl_2$, 10 mM), magnesium chloride ($MgCl_2$, 10 mM) zinc chloride ($ZnCl_2$, 10 mM), and protease inhibitors 1, 10 phenanthroline (1,10, Phe, 10 mM), PMSF (10 mM) and beta-mercaptoethanol (βME, 10 mM). Release of 4-nitroanilide hydrochloride due to cleavage of BApNA substrate was measured at 410 nm. Enzyme activity is represented as percentage of control (Serine proteinase) and statistical significance is denoted by asterisk $^{**}p < 0.01$, $^{***}p < 0.001$.

various snake venoms based on their amidolytic activities. Serine proteinase (Ba III-4) isolated from venom of Peruvian *B. atrox* exhibited amidolytic activity with K_m 0.2 × 10^{-1} M and V_{max} 4.1 × 10^{-1} nmoles p-NA/lt/min (Ponce-Soto et al., 2007). Further, authors showed inhibition of amidolytic activity upon inclusion of the protease inhibitor PMSF. Thrombin like serine proteinase isolated from *B. pirajai* (BpirSP-39) exhibited amidolytic activity with 10 μg protein which was inhibited with PMSF (Zaqueo et al., 2014). Thrombin like enzyme BjussuSP-I isolated from *B. jararacussu* exhibited hydrolytic activity on BApNA substrate and possessed procoagulant and kallikrein-like activities (Sant' Ana et al., 2008). Two serine proteinases, Cdc SI (28.5 kDa) and Cdc SII (28.7 kDa), isolated from *Crotalus durissus cumanensis* showed amidolytic activity at higher concentration (10 μg) and was inhibited by PMSF but not by EDTA unlike serine proteinases from our preparation from *Bothrops* species (Patiño et al., 2013).

Next, we determined the ability of serine proteinases to induce coagulation of human plasma. Concentration dependent analysis of serine proteinases from both *B. atrox* and *B. brazili* indicated comparable and significant coagulation of plasma (Fig. 4A). Minimal coagulation dose (MCD) for coagulating plasma within 60 s for serine proteinases from both species was 2.4 μg/mL. We also observed an increase in the concentration of serine proteinase led to a decrease in the coagulation time. Similar to our observation, Zaqueo et al. (2016) have earlier demonstrated that the MCD of serine proteinase (BbrzSP-32) from *B. brazili* was 2.4 μg/mL. However, serine proteinases isolated from other *Bothrops* species exhibited diverse MCD. Two serine proteinases (MSP1-32 kDa and 34 kDa proteins) from *B. moojeni* did not show any coagulation activity (Serrano et al., 1993). Recent study by Fernandes de Oliveira et al. (2013) showed MSP1 (BM-IIB32 + BM-IIB34) from *B. moojeni* with high MCD (1 μg/mL). In the same study, Fernandes de Oliveira et al. (2013) demonstrated that the serine proteinases from *B. alternatus* showed weaker MCD at 6 μg/mL. Serine proteinases from *B. apser* and *B. pirajai* showed MCD of 4.1 μg/mL and 3.5 μg/mL respectively (Menaldo et al., 2012; Pérez et al., 2008). Interestingly, two serine proteinases (Cdc SI (28.5 kDa) and Cdc SII (28.7 kDa) isolated from *Crotalus durissus* differed significantly in inducing coagulation. The MCD for Cdc SI was 25 μg/mL as opposed to Cdc SII which showed high MCD value of 0.571 μg/mL (Patiño et al., 2013).

Serine proteinases have been shown to degrade fibrinogen which subsequently leads to coagulation. Based on the ability to cleave fibrinogen chains, serine proteinases have been classified as α, β, γ fibrinogenases. We found serine proteinases (1 μg/mL) from both *B. atrox* and *B. brazili* cleaved Aα and Bβ chains, but not γ chain

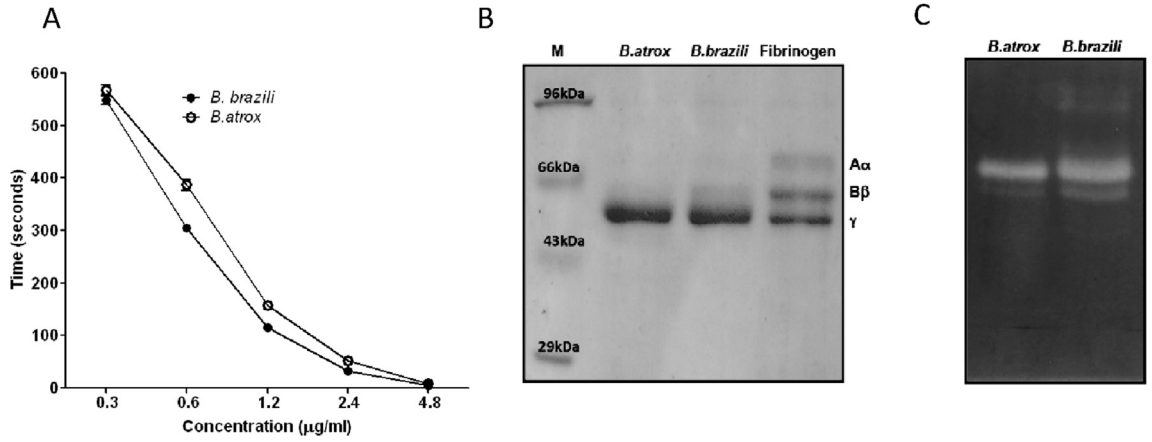

Fig. 4. Serine proteinase possesses coagulatory, fibrinogenolytic and gelatinase activity. (A) Human plasma was incubated with indicated concentrations of serine proteinase and coagulation was measured across the time. (B) Serine proteinase (1 μg/mL) was treated with fibrinogen (10 μg) for two hours at 37 °C. Fibrinogen degradation was analysed on a SDS PAGE. (C) Serine proteinase (2 μg) was loaded on zymography gel containing gelatin (0.3%). Zone of clearance on SDS-PAGE was considered as read out for gelatinase activity.

(Fig. 4B). The serine proteinase isolated from *B. pirajai* (Menaldo et al., 2012) and Jararassin-I isolated from *B. jararaca* (Vieira et al., 2004) showed specificity towards only Bβ chain. On the contrary, batroxobin from *B. atrox* (Stocker and Barlow, 1976) and Bhalternin from *B. alternatus* (Costa et al., 2010) cleaved Aα chain. Interestingly, Fernandes de Oliveira et al. (2013) observed that out of two serine proteinases from *B. moojeni*, BM-IIB32 cleaved both Aα and Bβ chain, whereas BM-IIB34 showed specificity only to Aα chain of fibrinogen.

We next investigated gelatinolytic activity of serine proteinases from *B. atrox* and *B. brazili*. We observed significant gelatinase activity of serine proteinases (2 μg) in gelatin zymogram (Fig. 4C). Inclusion of EDTA did not influence the gelatinase activity suggesting metal independent gelatin degradation by serine proteinases (data not shown). Similar gelatinase activity of serine proteinase was also observed in the venom of *B. pirajai* (Zaqueo et al., 2014).

3.3. Serine proteinases from B. atrox and B. brazili possesses angiogenic property

We next examined the ability of serine proteinases isolated from *Bothrops* venom to modulate angiogenesis. Prior to three dimensional assays, cytotoxicity of *Bothrops* venom and purified proteins were tested (Fig. S1). MTT based cytotoxicity assay was conducted for 48 h using endothelial cells, PBMC, MDCK, IMR32, fibroblasts, HepG2, HEK293 cells and HaCat cells. In all of these cell types, we observe 80–90% cell survival after 48 h treatment suggesting lack of significant cytotoxic effects of serine proteinases.

Next, we performed series of *in vitro* and *in vivo* assays to assess pro/anti-angiogenic property of serine proteinases. Collagen spheroid assays revealed that serine proteinases from *B. atrox* and *B. brazili* facilitated significant outgrowth of sprouts both in number and length. Statistical analysis suggested nearly 2.5–3 fold increase in sprout length upon induction by serine proteinase which was comparable with bFGF (Fig. 5A and 5B). We further, evaluated the effect of *Bothrops* serine proteinases on tubule formation in endothelial cells using a matrigel based assay. Similar to the effect on collagen spheroid as above on matrigel cushion, the endothelial cells reorganized into interconnecting tube like structures in response to serine proteinase. The interconnecting event of endothelial cells upon stimulation with serine proteinase was significantly higher (2 fold) than that of untreated control. Kinetics analysis showed serine proteinases and bFGF induced tube formation within two hours and further significantly increased at four hours and six hours (Fig. 5C and 5D).

Subsequently we employed agarose plug assay in mouse to validate the *in vitro* findings of pro-angiogenic property of serine proteinase in animal model (Fig. 6A). Analysis after 5 days of transplantation, we observed growth of new capillaries on the

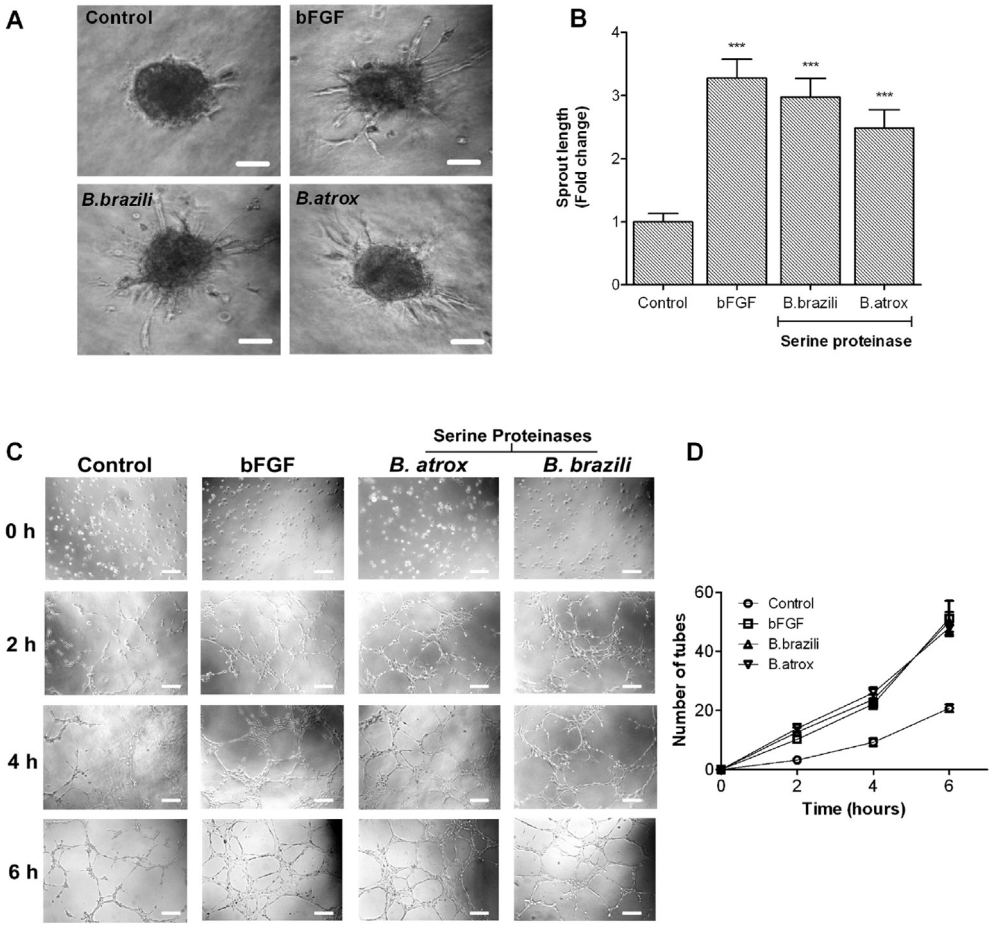

Fig. 5. Serine proteinase from *Bothrops* venom induces sprout and tubule formation. (A) and (B) Endothelial cell spheroids formulated in methylcellulose (20%) were further cultured on collagen gels (2 mg/mL) for the induction of sprouts in presence or absence of serine proteinase (100 ng/mL) and or bFGF (10 ng/mL). Number and length of sprouts were measured after 24 h. Data is represented as fold change in sprout length relative to untreated control. Statistical significance is represented as asterisk ***$p < 0.001$. (C) HUVEC were seeded on matrigel substrate and treated with serine proteinase or bFGF for 6 h. Images were captured every two hours. (Scale = 50 μm). (D) Data is represented as number of intact tubes formed at every 2 hours.

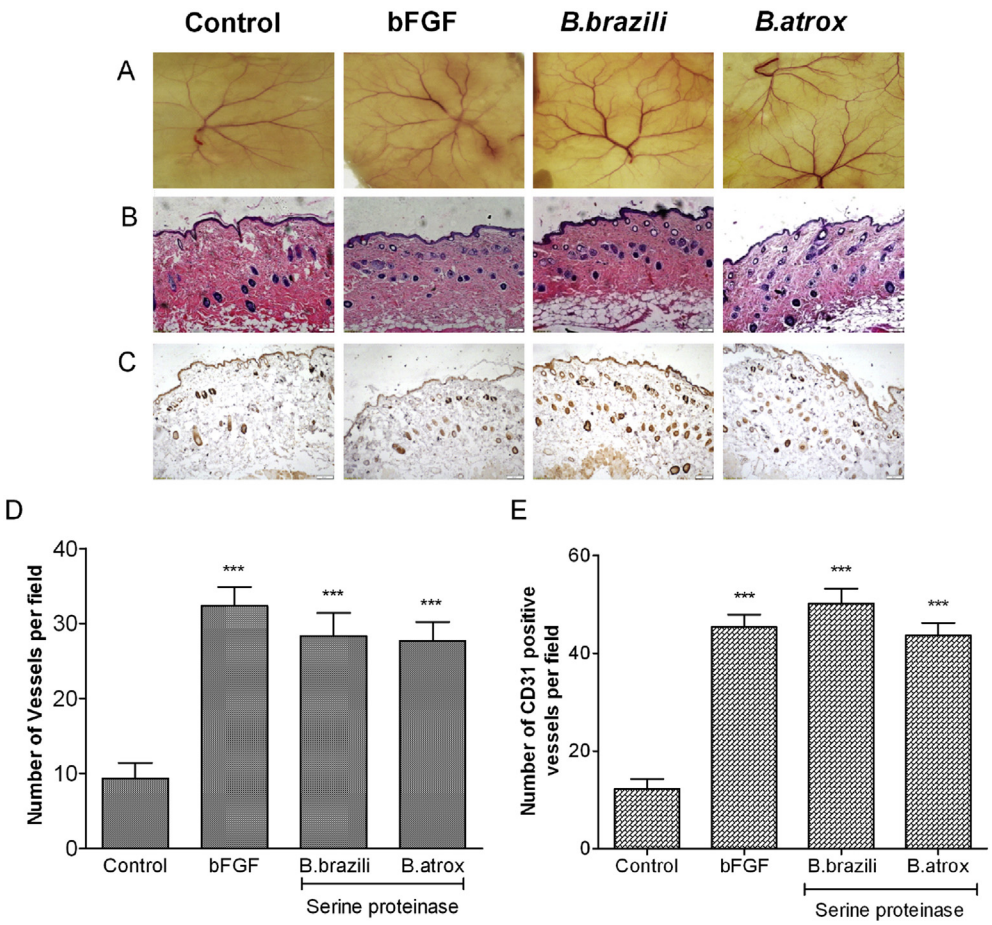

Fig. 6. Serine proteinases induce angiogenesis *in vivo*. (A) Agarose plug embedded with serine proteinase (1 μg/mL) or bFGF (100 ng/mL) or PBS was placed under subcutaneous space of mice and observed for growth of new capillaries after 5 days. (B) and (D) Tissues were sectioned and stained with hemotoxylin and eosin. Representative images of three independent experiments are given. Serine proteinase and bFGF induces blood capillaries. Graphical representation of influence of serine proteinases on capillary growth is provided. Significant formation of capillary is represented as asterisk ***$p < 0.001$ *vs* Control. (Scale = 20 μm) (C) and (E) Immunohistochemistry of the tissue stained with CD31 antibody. CD31 positive vessels were counted. Statistically significant changes are represented by asterisk ***$p < 0.001$ *vs* Control. (Scale = 20 μm).

tissue in proximity to the agarose bead loaded with either serine proteinase or bFGF. Further histology analysis confirmed significant induction of new blood vessel formation in response to serine proteinases (Fig. 6B and 6D). We next stained the sections for the presence of CD31, an endothelial specific marker to quantify growth of blood vessels. Treatment with serine proteinases from both *B. atrox* and *B. brazili* showed a 3–4 fold increase in number of CD31 positive vessels compared to control (Fig. 6C and 6E). Several studies have shown that snake venom harbor proteins modulating angiogenesis both inhibiting or stimulating neovascularization. VEGF like protein was found in venom of *Trimeresurus flavoviridis* Habu snake, which showed high specificity to VEGFR-1 similar to $VEGF_{165}$ and induced auto-phosphorylation of VEGFR-1 leading to angiogenesis (Minami et al., 2004). A disintegrin-like protein alternagin-C (ALT-C) isolated from *B. alternatus* exhibited potent *in vitro* and *in vivo* pro-angiogenic activity upon increased expression of VEGF and VEGF-R2 proteins and subsequently induced migration and proliferation in HUVEC (Ramos et al., 2007). Aggretin, a collagen like α2β1 agonist isolated from *Calloselasma rhodostoma* induced VEGF expression leading to endothelial proliferation and migration, and subsequently to angiogenesis in chick embryo model (Chung et al., 2004). On the other hand, MVL-PLA2, a snake venom phospholipase A2 has been demonstrated to suppress angiogenesis in *in vitro* and *in vivo* models (Bazaa et al., 2010). Triflavin, a disintegrin from *Trimeresurus flavovridis* reported to effectively inhibit TNF-α induced angiogenesis in chick chorioallantoic membrane models (Sheu et al., 1997).

3.4. Serine proteinases from B. atrox and B. brazili activates PI3K/Akt pathway

It has been reported that endothelial cells utilize PI3K/Akt pathway for the induction of angiogenesis (Kawasaki et al., 2003). Hence, we explored the influence of serine proteinases on the activation of Akt/eNOS signaling pathway. Stimulation of endothelial cells with serine proteinases from both *B. atrox* and *B. brazili* resulted in significant induction of phosphorylation of Akt^{ser473} and $eNOS^{ser1177}$, suggesting critical role of activation of Akt signaling pathway to facilitate angiogenesis. Both serine proteinases induced a 2–3 fold induction of phospho-Akt^{ser473} with a significant activation of phosho-$eNOS^{ser1177}$ (Fig. 7A and 7B). We observed that the inclusion of PI3K inhibitor LY294002 (10 μM) completely abrogated serine proteinase induced sprout formation, suggesting serine proteinase induced angiogenesis is PI3K/Akt dependent (Fig. 7C). Similar to our observation, Chung et al. (2004) have demonstrated that the aggretin induced angiogenesis utilizing PI3K/Akt signaling pathway.

Serine proteinases have been demonstrated as important regulators of angiogenesis in several *in vitro* and *in vivo* models

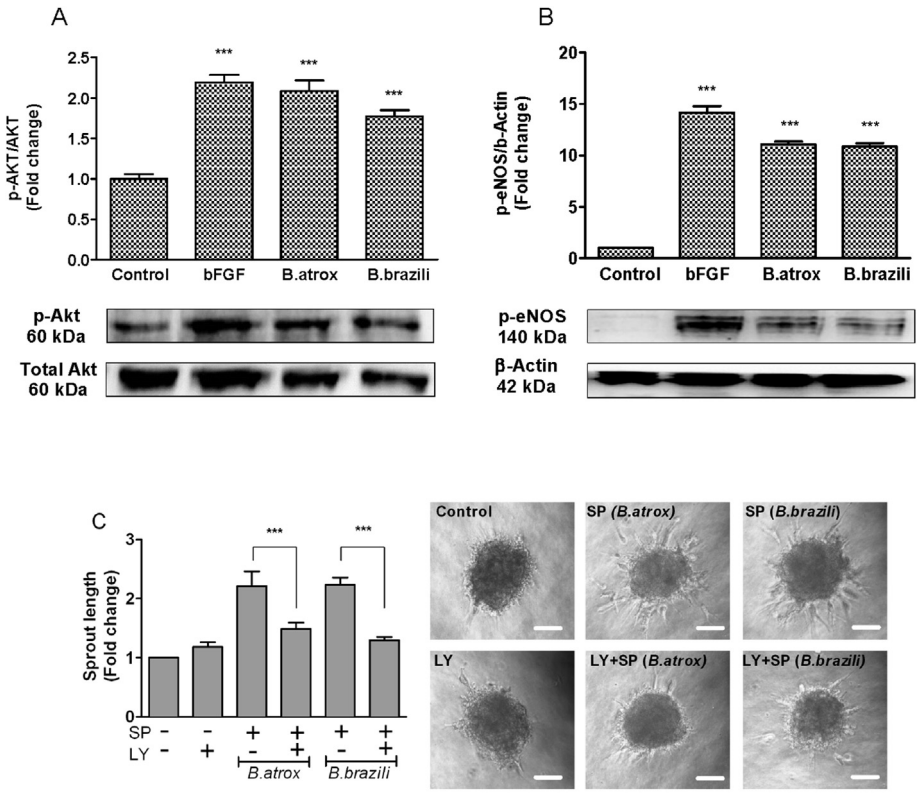

Fig. 7. Serine proteinase activates Akt/eNOS phosphorylation. (A) and (B) HUVECs were serum starved for 3 h and treated with serine proteinase from *B. atrox* and *B. brazili* for 30 min. Cell lysates were processed for immunoblotting and stained for phospho- and total Akt and eNOS. Data is represented as fold change of phospho-Akt and eNOS levels. Significant increase in phosphorylation status is represented by asterisk ***$p < 0.001$. (C) PI3K inhibitor (LY294002) inhibits serine proteinase induced angiogenesis. Spheroids were treated with serine proteinase (100 ng/mL) in the presence or absence of PI3K inhibitor (10 μM). Induction of sprouts were measured after 24 h. Data is represented as fold change in sprout length relative to untreated control. LY294002 and SP- serine proteinase. Statistical significance is represented as asterisk ***$p < 0.001$ vs Serine proteinase. (Scale = 50 μm).

(Weijers and van Hinsbergh, 2013). Serine proteinases such as chymotrypsin, trypsin, elastase and plasmin are activated upon cleavage of their N-terminal pro-peptide domain resulting in exposure of their catalytic triad and further induce degradation and remodeling of the extracellular matrix, cell migration and invasion, as well as the release and modification of growth factors leading to angiogenesis (van Hinsbergh et al., 2006). Tissue-type plasminogen activator (tPA) and urokinase plasminogen activator (uPA) degrades plasminogen to plasmin which is known to influence vascular remodeling such as endothelial migration and proliferation (Tarui et al., 2002). Studies have also shown that tPA increased mobilization of endothelial progenitor cells and induced angiogenesis in murine ischemia model (Yip et al., 2013).

Serine proteinase is one of the abundant protein present in the venom of *Bothrops* species that interfere with the haemostatic system (Cominetti et al., 2003). Serine proteinase from *Bothrops* genus was first characterized by Stocker and Barlow (1976) who isolated the coagulant enzyme Batroxobin from *B. atrox*. Since then, many *Bothrops* venom serine proteinases have been isolated which have found therapeutic use in the treatment of thrombosis, myocardial infarction, peripheral vascular diseases, acute ischemia and renal transplant rejection (Zaqueo et al., 2014). In the present study, we performed detailed functional characterization of serine proteinases from *B. atrox* and *B. brazili*. Both enzymes exhibited significant amidolytic, coagulatory, fibrinogenolytic and gelatinase activity. For the first time using *in vitro* and *in vivo* assays, we demonstrate that serine proteinases from snake venom possess pro-angiogenic property acting via PI3K/Akt signaling axis. Further studies using cellular, molecular, animal and clinical models are required to explore therapeutic applications of serine proteinases.

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Transparency document

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Appendix A. Supplementary data

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Conflict of interest

The authors declare that there is no conflict of interest.

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