

Research Article

Biochemical and Pharmacological Characterization of TLBbar, a New Serine Protease with Coagulant Activity from *Bothrops barnetti* Snake Venom

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A thrombin-like enzyme named TLBbar was isolated from *Bothrops barnetti* snake venom and its biochemical and pharmacological characteristics were determined. TLBbar was purified using size exclusion chromatography and reverse phase HPLC, showing molecular mass of 28750.7 Da determined by mass spectrometry. TLBbar serine protease is basic (*pI* 7.4) and its structure shows similarity with other serine proteases of snake venom. Optimal proteolytic activity was at 37°C and pH 8; this activity was strongly inhibited by PMSF and Leupeptin, however; heparin, and soybean trypsin inhibitor (SBT-I) were ineffective. Kinetic studies on BApNA chromogenic substrate have revealed that TLBbar presents a Michaelis-Menten kinetics, with values of K_m and V_{max} of 0.433 mM and 0.42 nmol/min, respectively. TLBbar showed high clotting activity upon bovine and human plasma, presenting IC₅₀ of 125 and minimum dose coagulant (MDC) of 2.23 $\mu\text{g}/\mu\text{L}$. TLBbar cleavages the A α chain of bovine fibrinogen, with maximal efficiency at 30–40°C in the presence of calcium after two hours incubation; this fibrinolytic activity was inhibited by PMSF and Leupeptin, confirming its classification in the group of serine proteases. In addition, TLBbar is capable of aggregating platelets in the same way that thrombin in concentrations of 2.5 $\mu\text{g}/\mu\text{L}$.

1. Introduction

The snake venom contains a variety of proteins that are studied in the world for biological and pharmacological importance; within their complex composition has proteolytic enzymes which belong to two groups: serine proteases and metalloproteases. Both groups affect the hemostatic system through several mechanisms [1, 2].

Over 20 serine proteases families (named S1 to S66) have been identified so far and they are grouped according to their functional and structural similarity. Snake venom serine proteases belong to trypsin S1 family clan SA. To date, a large number of studies, including molecular cloning, have led to the isolation and identification of TLEs mainly from the venom of subfamily Viperinae and Crotalinae [3–9].

These enzymes have a common catalytic mechanism, which includes high reactivity of the serine residue that has an

important role in the formation of the transient acyl-enzyme complex, which is stabilized by the presence of histidine residues and aspartic acid within the active site. The amino acids involved in this mechanism correspond to the catalytic triad (Ser 195, His 57, and Asp 102) that is highly conserved [10]. Serine proteases from snake venom (SVSPs) generally have 12 cysteine residues, 10 of which form five disulfide bonds based on homology to the trypsin, with the other two forming a single disulfide bridge conserved among SVSPs found in length C-terminus [11, 12].

SVSPs exert their effects through the ability to disrupt the blood clotting system of the prey [13]. Studies of these toxins have allowed the elucidation of several pharmacological mechanisms and substantially helped the pharmaceutical industry in search of new drugs [14, 15] based on their biological functions, they were classified as fibrinolytic activators, procoagulant, anticoagulant, and platelet aggregation

activate. The SVSPs procoagulant activate FVII, FX, and prothrombin, and to shorten the clotting times. SVSPs coagulants act directly or indirectly activating prothrombin in the presence of factor V, calcium, and phospholipids. SVSPs anti-coagulants act by consumption of functional fibrinogen of the victim and inactivation of the fibrin stabilizing factor (XIII). SVSPs also act on platelet activation, binding to the thrombin receptor to initiate the process of platelet activation and adhesion resulting in the formation of a platelet thrombus [16].

Some of these thrombin-like enzymes from snake venom have been used as anticoagulants in the prevention and treatment of a wide variety of thrombotic conditions, vascular surgery and others. Likewise they are widely used in laboratories and on detection of fibrinogen as a reagent for studies coagulation [15]. Although the list of biochemical and biological actions attributed to thrombin continues to grow, the term gender as applied to the thrombin enzyme poison often implies the ability to induce clotting of fibrinogen. The thrombin-like enzymes have been extensively studied over the past decade for potential therapeutic uses. For example, Ancrod, Batroxobin, and Reptilase are commercially available for the treatment of cardiovascular diseases [17].

SVSPs are found mainly in the venoms of snakes belonging to the Viperidae family with a few occurring in members of the Elapidae, Colubridae, and Hydrophiidae families [12]. *Bothrops barnetti* of the family Viperidae is an endemic species from Peru that is distributed in the coast between 0 and 1000 m [18]. The vast majority of snakebites in Peru are inflicted by species of the genus *Bothrops* [19]. The biology and the biochemistry of a lot of toxins from bothropic venoms have been documented; however, there is scantiness of information about the *B. barnetti* snake venom, recently been reported the proteomic characterization of the venom and some biochemical characteristics of barnettobin, a thrombin-like enzyme from *B. barnetti* [20, 21]. In this paper we report the purification and the biochemical (proteolytic, kinetic, and structural) and pharmacological characterization (fibrinogenolytic and platelet aggregation) of a new serine protease named TLBbar from *B. barnetti* snake venom.

2. Material and Methods

2.1. Purification of TLBbar. Samples of 400 mg of desiccated crude venom from *B. barnetti* were suspended in 8.0 mL of 0.05 M ammonium bicarbonate (pH 8.1) and centrifuged at 12.000 × g for 10 min at 0°C. The clear supernatant was applied on a molecular exclusion chromatographic column of Sephadex G-75 (110 × 4 cm), previously equilibrated and then eluted with the same buffer. Fractions of 2.5 mL/tube were collected at a flow rate of eluting with a flow rate of 10 mL/h at room temperature. The fraction showing clotting and proteolytic activity upon BApNA substrate (N-benzoyl-L-arginine-p-nitroanilide) was lyophilized and then applied on C8 column in reverse phase HPLC. Briefly, 5 mg of fraction was dissolved in 250 μL of solution A (0.1% trifluoroacetic acid—TFA) and centrifuged at 4500 × g and the supernatant was then applied on the analytical reverse phase HPLC Supelco C8, previously equilibrated with solution A for 15 min. The elution of the protein was conducted using a

linear gradient of solution B (66.5% acetonitrile in solution A) and the chromatographic run was monitored at 280 nm. After elution, the fractions were lyophilized and stored at -20°C.

2.2. Enzyme Assays and Determination of Kinetic Parameters. Thrombin-like enzyme activity was measured using the BA_pNA synthetic substrate in 96-well plates. The standard assay mixture contained 40 μL of buffer (10 mM Tris-HCl, pH 8.0, 10 mM CaCl₂, 100 mM NaCl), 200 μL of substrate (1 mM), 5 μL of water, and 5 μL of sample (thrombin-like enzyme) in a final volume of 250 μL. After the addition of the sample (5 μg), the mixture was incubated for up to 10 min at 37°C, with the absorbance being read at 10 min intervals. The enzymatic activity, expressed as the initial velocity of the reaction (V_0), under the described conditions was calculated based on the amount of p-nitroaniline released [22]. One unit of enzymatic activity was defined as the amount of enzyme able to release 1 μmol of p-nitroanilide/min. Under the same conditions the proteolytic activity was evaluated at different temperature and pH.

The inhibition assays were determined by incubating 5 mM of the inhibitors PMSF (phenylmethyl sulfonyl fluoride), Leupeptin (acetyl-leucyl-leucyl-argial) acid glycol-bis (2 aminoetileter)-N,N,N,N-tetraacetic (EGTA), Soybean trypsin inhibitor SBT-I (5 μg/μL), Leupeptin, and heparin (5000 UI/mL) with BA_pNA (1 mM) in Tris-HCl buffer, pH 8.0, (1:1, v/v) for 30 min at 37°C. The remaining proteolytic activity was calculated as the percent activity in relation to the control preparation incubated with no inhibitors, which was considered to be 100% activity. The Michaelis constant (K_m) and maximum velocity (V_{max}) of sample were determined using a Lineweaver-Burk plot. All assays were done three times and the absorbance at 410 nm was measured using a VERSAMAX 190 microplate reader (Molecular Devices Corporation, Sunnyvale, CA).

2.3. Electrophoresis. Electrophoresis was carried out following the Laemmli method [23]. The degree of purity of fractions was assessed by discontinuous electrophoresis using a final acrylamide concentration of 12% in the resolving gels (1.0 M Tris-HCl, pH 8.8) and 5% in the stacking gel (0.5 M Tris-HCl, pH 6.8). Electrophoretic separation was carried out in a 250 Mighty Small (Hoefer Scientific Instruments) for SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis). Samples were heated at 100°C for 5 min and then ran under both reducing (SDS + β-mercaptoethanol) and no reducing conditions. The gel was stained with 0.1% Coomassie Brilliant Blue G-250. Molecular weight markers (Sigma) were phosphorylase b (97.000 Da), bovine serum albumin (62.000 Da), ovalbumin (45.000 Da), glyceraldehyde-3-phosphate dehydrogenase (30.000 Da), carbonic anhydrase (21.000 Da), and α-lactalbumin (14.200 Da).

2.4. Amino Acid Analysis. Amino acid analysis was done on a Pico-Tag amino acid analyzer (Waters Corporation, Massachusetts, USA) as described by Heinrikson and Meredith [24]. The purified protein (30 μg) was hydrolyzed at 105°C for 24 h in 6 M HCl acid (Pierce sequencing grade)

containing 1% phenol (w/v). The hydrolyzates were reacted with 20 μ L of derivatization solution (ethanol:triethylamine:water:phenylisothiocyanate, 7:1:1:1, v/v) for 1 h at room temperature after the phenylthiohydantoin (PTC) amino acids were identified and quantified by HPLC by the comparison of their retention times and peak areas with those of a standard amino acid mixture.

2.5. Determination of the Molecular Mass of the Purified Protein by Mass Spectrometry. An aliquot (4.5 μ L) of the purified protein was injected by C18 (100 μ m \times 100 mm) RP-UPLC (nanoAcuity UPLC, Waters) coupled with nano-electrospray tandem mass spectrometry on a Q-ToF Ultima API mass spectrometer (MicroMass/Waters) at a flow rate of 600 nL/min. The gradient was 0–50% acetonitrile in 0.1% formic acid over 45 min. The instrument was operated in MS continuum mode and the data acquisition was from m/z 100–3.000 at a scan rate of 1 s and an interscan delay of 0.1 s. The spectra were accumulated over about 300 scans and the multiple charged data by the mass spectrometer on the m/z scale were converted to the mass (molecular weight) scale using Maximum Entropy-based software supplied with Masslynx 4.1 software package. The processing parameters were output mass range 6.000–20.000 Da at a “resolution” of 0.1 Da/channel; the simulated isotope pattern model was used with the spectrum blur width parameter set to 0.2 Da; the minimum intensity ratios between successive peaks were 20% (left and right). The deconvoluted spectrum was then smoothed (2 \times 3 channels, Savitzky Golay smooth) and the mass centroid values obtained using 80% of the peak top and a minimum peak width at half height of 4 channels.

2.6. Enzymatic Hydrolysis. The purified protein was hydrolysed with sequencing grade bovine pancreatic trypsin in 0.4% ammonium bicarbonate, pH 8.5, for 4 h at 37°C, at an enzyme : substrate ratio of 1:100 (w/w). The reaction was ceased by lyophilization.

2.7. Analysis of Tryptic Digests. The protein was reduced with 5 mM DTT (Dithiothreitol) for 25 min at 56°C and alkylated with 14 mM Iodoacetamide for 30 min prior to the addition of trypsin (Promega-Sequence Grade Modified). After the trypsin addition (20 ng/ μ L in ambic 0.05 M), the sample was incubated for 16 hr at 37°C. To stop the reaction, formic acid 0.4% was added and the sample centrifuged at 2500 \times g for 10 min. The pellet was discarded and the supernatant dried. The resulting peptides were separated by C18 (100 μ m \times 100 mm) RP-UPLC (nanoAcuity UPLC, Waters) coupled with nano-electrospray tandem mass spectrometry on a Q-ToF Ultima API mass spectrometer (MicroMass/Waters) at a flow rate of 600 nL/min. The gradient used was 0–90% acetonitrile in 0.1% formic acid over 20 min. Before performing a tandem mass spectrum, an ESI/MS mass spectrum (TOF MS mode) was acquired for each HPLC fraction over the mass range of 100–2000 m/z , in order to select the ion of interest, where these ions were subsequently fragmented in the collision cell (TOF MS/MS mode).

Raw data files from LC-MS/MS runs were processed using MassLynx 4.1 SCN662 software package (Waters) and analyzed using the Mascot Distiller v.2.3.2.0, 2009 (Matrix Science, Boston, MA) with SNAKES database (snakes_jun2011 was downloaded from NCBI Taxonomy) release from June 2011, using the following parameters: peptide mass tolerance of \pm 0.1 Da, fragment mass tolerance of \pm 0.1 Da, oxidation as variable modifications in methionine, and trypsin as enzyme.

2.8. Clotting Activity upon Plasma. This activity was assessed using 200 μ L of citrated human plasma at 37°C. The activity was characterized for the immediate appearance of fibrin network in the presence of 0.1 M calcium chloride. The minimum dose coagulant (MDC) is defined as the amount of sample able to coagulate plasma (200 μ L) in 60 seconds [25]. The clotting time was characterized by the first sudden appearance of the fibrin net. This activity was performed with 200 μ L of human plasma obtained by centrifuging the blood samples (800 \times g for 10 min). Different concentrations (1.0, 2.5, 5.0, 10, 15, and 20 μ g) of sample were tested. The coagulation index (CI) was determined according to Cavinato et al. [26], using the formula $CI = t^{-1} \times 1000$, where t^{-1} = reverse time.

2.9. Platelet Aggregation. Platelet aggregation activities were assayed as described by Lee et al. [25]. Briefly, venous blood was collected with informed consent from healthy volunteers who denied taking any medication in the previous 14 days. Collected blood was immediately transferred into polypropylene tubes containing one-tenth of final volume of acid citrate dextrose (ACD-C; citric acid 3%, trisodium citrate 4%, glucose 2%; 1:9 v/v). Platelet-rich plasma (PRP) was obtained by centrifuging whole blood at 200 \times g for 15 min. PRP was washed in a wash buffer solution (NaCl 140 mM, KCl 5 mM, sodium citrate 12 mM, glucose 10 mM, and saccharide 12 mM; pH 6; 5:7 v/v) and centrifuged at 800 \times g for 12 min at 20°C. The platelet pellet was gently resuspended in Krebs-Ringer solution and counts were performed on a Neubauer chamber. The final platelet suspension was adjusted to 1.2×10^8 platelets/mL. The supernatant was discarded. Platelet aggregation was carried out using 400 μ L of the washed platelet solution in a cuvette and incubated at 37°C with constant stirring. The desired concentration of protein was added 3 min prior to the addition of a platelet aggregation inducer (thrombin). Aggregation was subsequently recorded for 5–10 min with an aggregometer (Chrono-log Lumi-Aggregometer model 560-Ca, Havertown, PA, USA). Assays were performed at 37°C with 500 μ L and aggregation of washed platelets was analyzed after 2 minutes of incubation with different concentrations of the sample (0.03125, 0.0625, 1.25, and 2.5 μ g) dissolved in 0.15 M NaCl. Control experiments were performed using thrombin as aggregate platelet.

2.10. Fibrinogenolytic Assay. The fibrinogenolytic activity was determined using methods described by Edgar and Prentice [27] with some modifications according to Rodrigues et al. [28] and Vaiyapuri et al. [29]. The experiment

was performed with bovine fibrinogen solution of 1.5 mg/mL resuspended in Tris-HCl 10 mM pH 7.4 containing 10% of 0.01 M CaCl₂. The sample was incubated in said solution at different concentrations: 0.625, 1.25, 2.5, 5.0, 10, and 20 µg at 37°C for two hours in a water bath. The reaction was stopped by adding 50 µL of denaturing solution (10 M urea, 0.2% SDS 0.55%, 50 mM Tris-HCl, β-mercaptoethanol, and 0.05% bromophenol blue) followed by boiling at 100°C for 5 minutes. The samples were analyzed by SDS-PAGE 15%. The evaluation of the stability of the proteolytic activity of sample was analyzed in the presence of inhibitors (PMSF, Leupeptin, EDTA, EGTA, SBT-I, and heparin) and different divalent ions (Ca⁺⁺, Mg⁺⁺, Cd⁺⁺, Ba⁺⁺, Cu⁺⁺, and Zn⁺⁺).

2.11. Statistical Analyses. They were reported as mean ± SEM. The significance of differences among means was assessed by analysis of variance followed by Dunnett's test, when several experimental groups were compared with the control group. Differences were considered statistically significant if $P > 0.05$.

3. Results

The chromatographic profile of *Bothrops barnetti* snake venom on Sephadex G-75 revealed four fractions (Bbt I–Bbt IV) (Figure 1(a)). Each fraction was screened for coagulant and proteolytic activities. Only the fraction Bbt Ia showed coagulant activity on bovine fibrinogen at concentrations ranging from 20 µg/mL and proteolytic activity upon BApNA substrate (0.166 nmol/min). This fraction was collected for purification on reversed phase HPLC chromatography, resulting in 13 peaks (1–13) (Figure 1(b)). The fraction 11, named TLBbar (thrombin-like *B. barnetti*), showed high serine protease enzymatic activity upon BApNA substrate (0.35 ± 0.017 nmol/min) with properties thrombin-like and was selected for biochemical and pharmacological characterization. The purity of this peak was confirmed by rechromatography on an analytical RP-HPLC Supelco C8 column, showing the presence of only one peak (Figure 1(c)).

SDS-PAGE revealed the presence of an electrophoretic band with M_r around 28.5 and 37.5 kDa in the absence and presence of DTT, respectively (Figure 1(b), insert). Mass spectrometry analysis confirmed the homogeneity of the peak TLBbar and determined the molecular mass of 28750.7 Da (Figure 2). Amino acid analysis revealed the following composition: B/31, Z/2, S/18, G/26, H/16, R/112, T/14, A/13, P/23, Y/7, V/15, M/5, C/8, I/16, L/19, F/9, K/15. Two-dimensional electrophoresis determined isoelectric point of 7.4 (data not shown).

The primary structure was determined by sequence tryptic digested (three peptides via MS/MS mass spectrometry) and deduction of the SwissProt database <http://br.expasy.org/>.

The study conducted of sequence homology in all three peptides in alignment with other serine proteases from snake venom, as determined and the sequences registered in the database, has demonstrated a high degree of homology with the thrombin-like Calobin, Crotalase, and Flavoxobin isolated from *Gloydius ussuriensis*, *Crotalus adamanteus*,

and *Trimeresurus flavoviridis*, respectively (Figure 3). The closed regions represent the consensus sequence between the different thrombin-like serine proteases, indicating both highly conserved and variable regions. The conserved amino acid His at position 57 shows the presence of unchanged catalytic site of serine proteases.

In order to estimate the serine protease activity of TLBbar, DL-BApNA was used and the kinetic parameters were calculated. The proteolytic activity of the purified protein (0.37 ± 0.017 moles/min) was higher than that of the whole venom and sephadex fraction (0.02 ± 0.0018 and 0.16 ± 0.01 nmol/min, resp.), showing the efficiency of purification procedure herein described (Figure 4(a)). This activity was inhibited by PMSF, and leupeptin (8.82% and 4.01% of residual activity, resp.). Chelating agents such as EDTA and EGTA inhibit the proteolytic activity of TLBbar in approximately 50% the heparin does not produce significant inhibition (Figure 4(b)). TLBbar showed a Michaelis-Menten kinetic behavior (Figure 4(c)) K_m and V_{max} were 0.433 mM and 0.42 nmoles/min, respectively (Figure 4(d)). The maximum enzyme activity occurred at 37°C (Figure 4(e)) and pH 8.0 (Figure 4(f)).

TLBbar was able to induce platelet aggregation on washed platelet preparations (Figure 5). This activity was similar to thrombin (control) at the concentration of 2.5 µg/mL (95% of aggregation) and was strongly inhibited by preincubation of this protein with PMSF. Lower concentration (0.625 and 1.25 µg/mL) also induced aggregation reaching 70% of the activity of thrombin in all cases the aggregation begins after three minutes as compared to control. The TLBbar thrombin-like showed coagulant activity upon human plasma, at different concentrations, was able to induce clot formation with minimum dose coagulant (MDC) of 2.23 µg/µL (Figure 6) and clotting activity on bovine fibrinogen with coagulant index (CI) of 125, corresponding to 8 seconds.

The TLBbar thrombin-like was found to degrade the Aα chain of fibrinogen but not the Bβ chain protein fragments appeared as degradation products. The degradation of the Aα chain was detectable after 15 minutes of incubation and this chain was degraded completely at two hours (Figure 7(a)). This activity was dose dependent, observing degradation from 1.25 µg of TLBbar (Figure 7(b)), with optimal activity between 30°C and 40°C (Figure 7(c)) and the presence of calcium, magnesium, and barium ions (Figure 7(d)). Fibrinogenolytic activity was reduced by PMSF and leupeptin (Figure 7(e)).

4. Discussion

The new serine protease, TLBbar, was purified from the venom *B. barnetti* by combination of two chromatographic steps: molecular exclusion chromatography Sephadex G-75 followed by reverse phase HPLC (Figures 1(a), and 1(b)). The purification procedure herein described was efficient for the obtainment of the TLBbar toxin, showing high yield with high purity levels (Figure 1(c)) and maintaining their functionality, which could be used in biological studies.

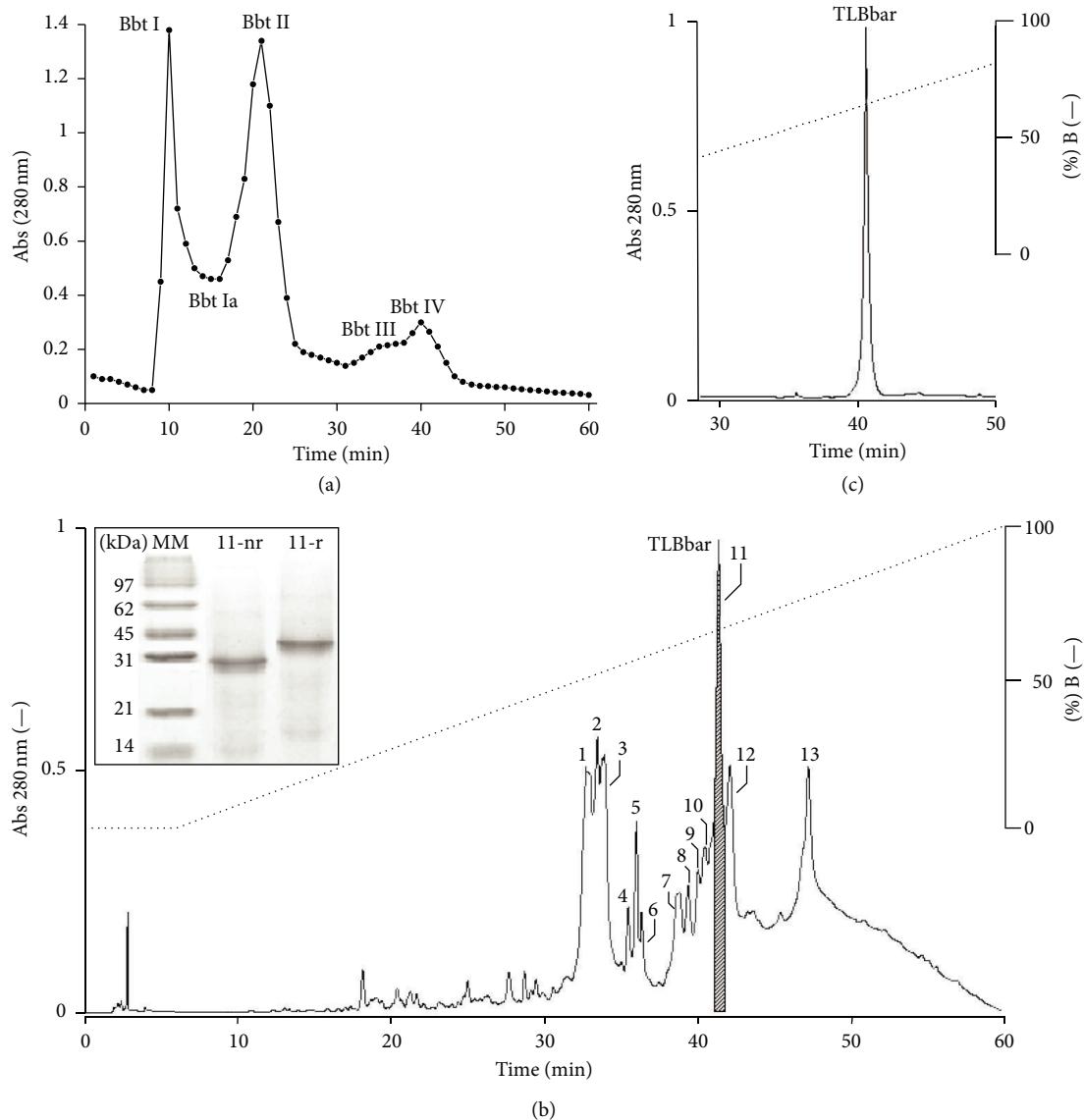


FIGURE 1: Chromatographic profile of the purification procedure and purity assay of TLBbar. (a) Molecular exclusion chromatography on Sephadex G-75. (110 × 4.0 cm) from *B. barnetti* snake venom. Fraction Bbt Ia (–) showed coagulant and proteolytic activities upon bovine fibrinogen and BApNA substrate, respectively. (b) Elution profile of Bbt Ia fraction by RP-HPLC on an analytical Supelco C8 column. Fraction 11 (TLBbar) showed high serine protease enzymatic activity with properties thrombin-like. Insert: electrophoretic profile in SDS-PAGE: (MM) molecular mass markers, (11-nr) TLBbar not reduced, (11-r) TLBbar reduced with DTT (1 M). (c) Rechromatography on RP-HPLC of TLBbar.

Analysis by SDS-PAGE showed that TLBbar, has one band with $M_r \sim 28.5$ kDa, being a protein constituted of a single polypeptide chain (Figure 1(b) insert). TLBm of *Bothrops marajoensis* [9], Ba III-4 of *Bothrops atrox* [7], and PA-BJ of *Bothrops jararaca* [30] are serine proteases from snake venom that also have structure monomeric.

Figure 2 shows the mass of TLBbar determined by mass spectrometry (ESI positive ionisation). Each peak shown in the spectrum is the protein carrying a different number of charges (protons) [31]. Figure insert shows the deconvolution of the spectrum obtained with the help of software Maximum Entropy. The molecular masses obtained of 28750.7 Da appeared to be similar to those of other snake venom serine proteases as PA-BJ of *Bothrops jararaca* 30 kDa [30],

Mucrosobin of *Trimeresurus mucrosquamatus* 28 kDa [32], and RVV-V of *Vipera lebetina* 24 kDa [33].

Sequence comparison of the peptide containing N-terminal region of TLBbar with several other venom thrombin-like enzymes shows high degree of homology (Figure 3), in particular with Calobin thrombin-like isolated from *Agkistrodon caliginosus* [34], Crotalase from *Crotalus adamanteus* [35], and Flavoxobin from *Trimeresurus flavoviridis* [36]. The other two tryptic peptides have Cys170 and His57 residues highly conserved in the sequence of serine proteases [16]. The whole pattern of Cys residues at the molecular level is forming disulfide bridge and is identical to the mammalian trypsin [3]. Likewise, TLBbar retains the histidine residue at position 57 of the catalytic triad of serine proteases, giving the basic

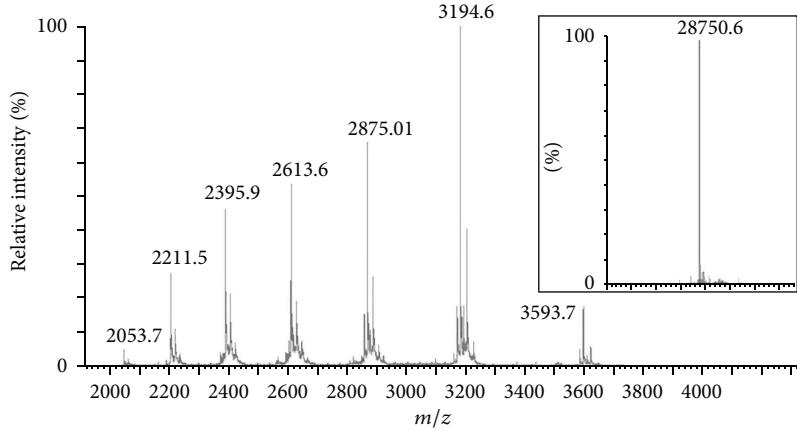


FIGURE 2: Electrospray positive mass spectrum of TLBbar determination by ESI mass spectrometry. The corresponding deconvoluted mass spectrum is shown in the insert.

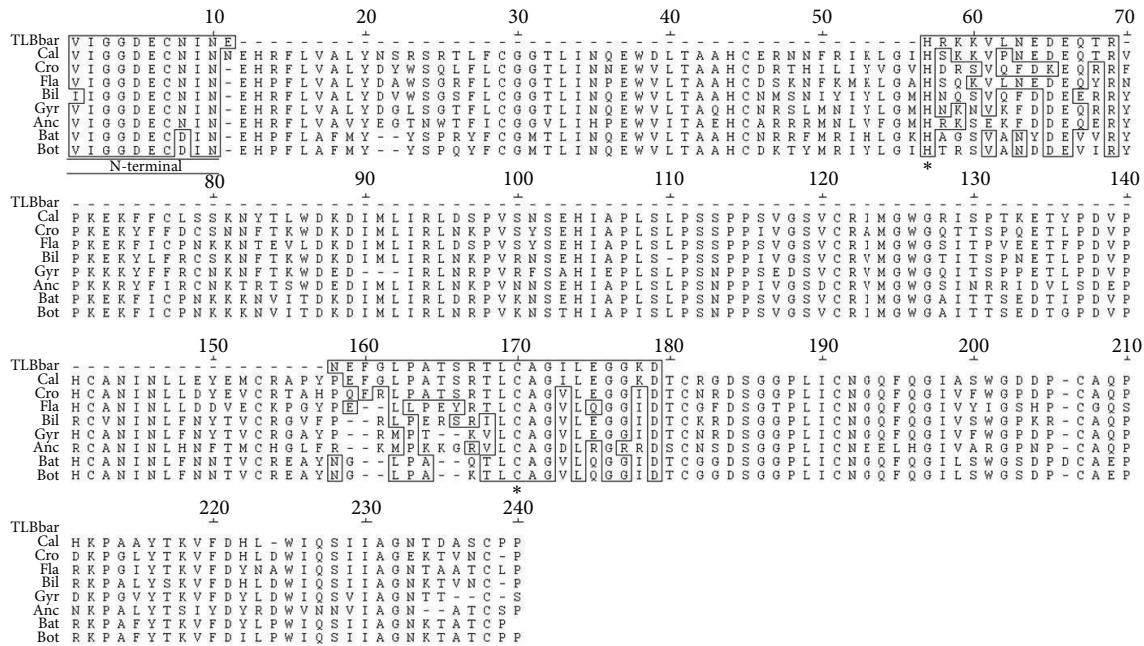


FIGURE 3: Sequence alignment of TLBbar with other SVSPs with thrombin-like activity. Calobin of *Agkistrodon caliginosus* GI: 1389589 [34]; Crotalase (Cro) of *Crotalus adamanteus* GI: 384951520 [35]; Flavoxobin (Fla) of *Trimeresurus flavoviridis* GI: 3915685 [36]; Bilineobin (Bil) of *Agkistrodon bilineatus* GI: 998903 [50]; Gyroxin (Gyr) of *Lachesis muta muta* GI: 998903 [51]; Ancrod (Anc) of *Calloselasma rhodostoma* GI: 247212 [52]; Batroxobin (Bat) of *Bothrops atrox* GI: 114837 [37], and Bot TL (Bot) of *Bothrops jararaca* GI: 999161 [53]. The cysteine and histidine residues (*) are highly conserved in all sequences.

polarity in the catalytic triad for hydrolysis of the reaction of the peptide bond [16]. This analysis of homology confirms that TLBbar belongs to the serine proteases family of snake venom.

TLBbar showed V_o of 0.35 nmoles/min on BApeNA substrate (Figure 4(a)), with Michaelis-Menten kinetic behavior (Figure 4(c)) and V_{max} and K_m of 0.42 nmol/min and 0.433 mM, respectively (Figure 4(d)); these values are similar to other thrombin-like snake venom, as TLE-1 and TLE-2 isolated from *B. atrox* (K_m 0.33 mM and 0.46 mM, resp.) [37] and thrombin-like from *Lachesis muta muta* (K_m of 0.075 mM and V_{max} of 0.36 nmol/min) [38]. These results suggested that

the catalytic mechanism of this enzyme is probably involved in an active site organization similar to that of trypsin. Proteolytic activity occurs at 37°C and pH 8 (Figures 4(e) and 4(f)), but activity is still in the range of 40°C to 45°C which indicates the stability of the molecule before the heat due to the presence of disulfide bridges and proper folding to give molecular stability; this stability; is also explained by the nature pectiothermic of snakes, which is considered the adequacy of body temperature against climate change, affecting physiological processes, and development [39].

The proteolytic activity TLBbar was inhibited by Leupeptin and PMSF which inhibit the proteolytic activity of

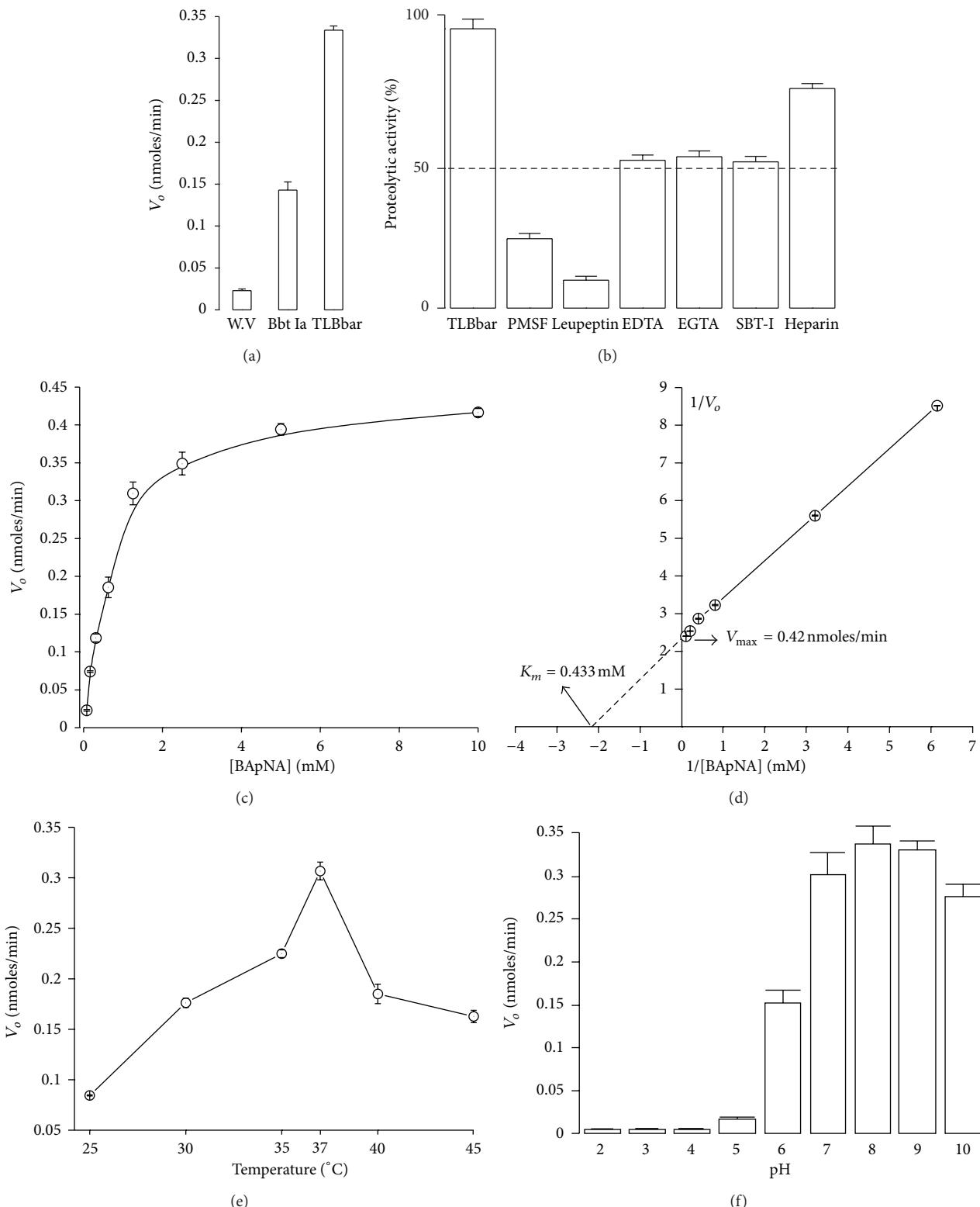


FIGURE 4: Kinetic properties and inhibition of the thrombin-like enzyme TLBbar from *B. barnetti* venom upon BApeNA substrate. (a) Proteolytic activity of the whole venom *B. barnetti* (WV) Bbt Ia and TLBbar fractions. (b) Inhibition of the proteolytic activity of TLBbar by PMSF (phenylmethyl sulfonyl fluoride), Leupeptin (acetyl-leucyl-leucyl-argial) EGTA (acid glycol-bis (2 aminoeteleter)-N,N,N,N-tetracetic), EDTA (Ethylenediamine tetraacetic acid), SBT-I (Soybean trypsin inhibitor), and heparin. (c) Effect of substrate concentration. (d) Lineweaver-Burk (double-reciprocal) plot. (e) Effect of temperature. (f) Effect of pH. The results of all experiments are the mean \pm S.E. of three determinations ($P < 0.05$).

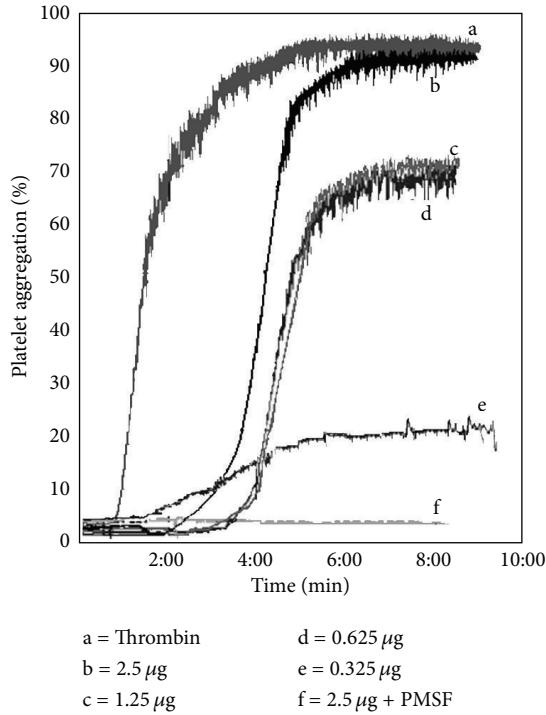


FIGURE 5: Effect of TLBbar on platelets. Different concentrations of TLBbar (0.03125, 0.0625, 1.25, and 2.5 μg) were evaluated on washed platelets at 37°C. The control was aggregating thrombin. Significant difference ($P < 0.05$).

SVSPs (Figure 4(b)). Leupeptin is a competitive inhibitor which competes with the substrate for the enzyme active site, being displaced when the substrate is in excess. The PMSF has the ability to bind to the amino acid residue serine of the catalytic site, leading to irreversible binding and inactivating it [40]. TLBbar had residual activity of 4.01% to 8.82% for leupeptin and PMSF. These results are compared with other serine proteases that have been inhibited their proteolytic activities with PMSF and Leupeptin, such as halystase from *Agirostrodon halys blomhoffii* venom [41] and MOO3 from *Bothrops moojeni* venom [42]. These data confirmed that TLBbar exhibits typical characteristics of snake venom serine protease thrombin-like enzymes.

EDTA and EGTA produce 50% inhibition; they are chelating agents that sequester the metal ions present in the environment, destabilizing the structure of the molecule and shortening its proteolytic activity. The trypsin inhibitor SBT-I inhibited in some cases at high concentrations [12], in TLBbar, SBT-I, and heparin, had no significant inhibition (Figure 4(b)); they are macromolecules found in the aqueous medium, increase their size as a result of hydration, and probably are unable to get near the catalytic site of serine proteases and prevent their activity. Therefore, the inability of heparin to inhibit the proteolytic activity of TLBbar indicates the existence of structural differences between this enzyme and thrombin, studies crystal structures of serine proteases mammal, both native states as linked to inhibitors should be studied to gain a better understanding this process.

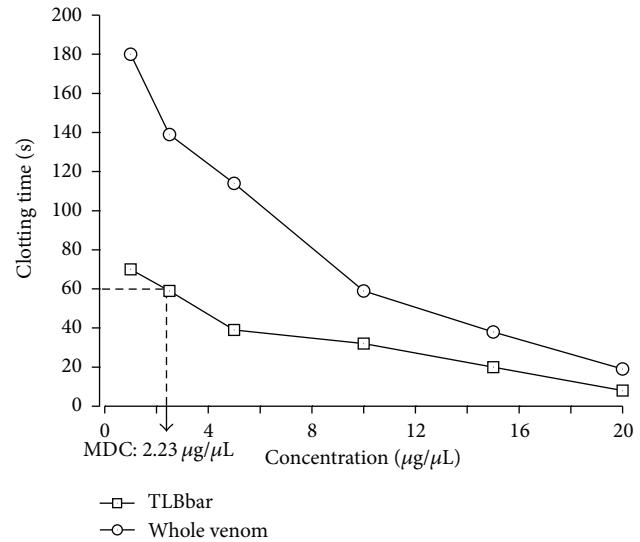


FIGURE 6: Coagulant activity of TLBbar. Minimum dose coagulant (MDC) on TLBbar human plasma at various concentrations (1, 3, 5, 10, 15 and 20 μg).

The spot appeared in two-dimensional electrophoresis (data not shown) to confirm this basic nature, showing an isoelectric point of 7.4. Acidic and basic serine proteases were isolated from snake venom. The basic serine proteases usually have direct activity on the platelet aggregation; however, the acid proteases exhibit proteolytic activity on protein substrates [12]. TLBbar shows activity on platelet aggregation, confirming the main effect of serine proteases basic.

The results show that TLBbar is capable of aggregating platelets and the concentration of 2.5 μg exhibits a behavior similar to the thrombin, reaching 95% of aggregation (Figure 5). Serine proteases like-thrombin with platelet aggregation activity as Bothrombin from *B. jararaca* [43], Cerastocitin from *Cerastes cerastes* [44], and BJ-PA from *B. jararaca* [30], among others have been described. Probably TLBbar activates the thrombin receptor platelet membrane (fibrinogen, factor V, and receptors 1 and 4) coupled to G protein to initiate intracellular signals that activate the phospholipases, which cleavage membrane phospholipids releasing arachidonic acid, thromboxane A₂, calcium, and ADP responsible for the activation of platelets [29, 45]. The aggregant activity caused by TLBbar was confirmed by inhibition PMSF, causing inhibitory effect as expected. Interestingly TLBbar stimulated aggregation but start time is different compared with thrombin (delayed by approximately 2-3 minutes). The interpretation of the graph has been assessed following the traces of the curve [46] evaluating these studies may suggest that TLBbar activates platelets in a longer time than thrombin which would indicate that the structural variation in membrane surface by releasing agents such as ADP and thromboxane A₂ is processes that develop more slowly to reach the platelet adhesion.

TLBbar showed clotting activity with IC of 125, corresponding to 8 seconds. The enzyme induces clotting of plasma with a minimum dose coagulant (MDC) of 2.23 $\mu\text{g}/\mu\text{L}$ (Figure 6). These values represent high coagulant activity the

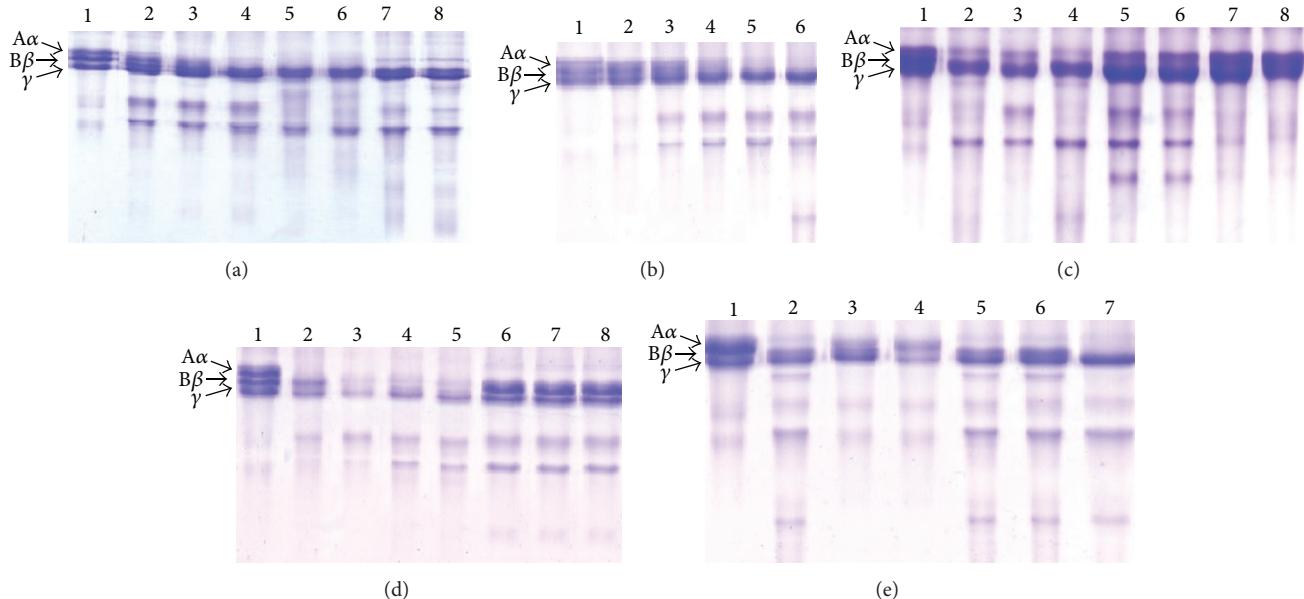


FIGURE 7: Fibrinogenolytic activity of TLBbar. (a) Time of incubation. (1) Control bovine fibrinogen 1.5 mg/mL; (2) 15 min; (3) 30 min; (4) 60 min; (5) 2 hours; (6) 4 hours; (7) 6 hours; (8) 24 hours. (b) Dose dependent. (1) Control bovine fibrinogen 1.5 mg/mL; (2) 1.25 μ g TLBbar; (3) 2.5 μ g TLBbar; (4) 5 μ g TLBbar; (5) 10 μ g TLBbar; (6) 20 μ g TLBbar. (c) Temperature. (1) Control bovine fibrinogen 1.5 mg/mL; (2) 20°C; (3) 30°C; (4) 40°C; (5) 50°C; (6) 60°C; (7) 70°C; (8) 80°C. (d) Ions. (1) Control of bovine fibrinogen 1.5 mg/mL; (2) TLBbar; (3) TLBbar with Ca²⁺; (4) TLBbar with Mg²⁺; (5) TLBbar with Ba²⁺; (6) TLBbar with Zn²⁺; (7) TLBbar with Cu²⁺; (8) TLBbar with Cd²⁺. (e) Inhibition. (1) Control bovine fibrinogen 1.5 mg/mL; (2) TLBbar; (3) Leupeptin; (4) PMSF; (5) EDTA; (6) EGTA; (7) Heparin.

difference of *Bothrops asper* serine protease which exhibit MDC of 4.1 μ g/ μ L [47]. The clot formed is semi rigid, probably TLBbar did not activate the factor XIII and consequently produces a soft clot, different of that produced by thrombin. An exception to this rule is *Bitis gabonica*, isolated by Pirkle et al. [35] which activates factor XIII [15, 48]. TLBbar coagulant activity leads to the consumption of the content of fibrinogen functional victim or attached without being able to form a stable clot, leading thereby to the blood incoagulability, therefore contributing to hemorrhage. TLBbar confirms its thrombin-like activity acting on fibrinogen to generate fibrin networks.

Snake venom serine proteases (SVSPs) possess three main functions that are responsible for their systemic effects in envenomed victims: fibrinogenolysis reduces the functional fibrinogen content; fibrinolysis dissolves the blood clots, and kininogenolysis generates kinin and bradykinin which alter blood pressure [29]. TLBbar showed fibrinogenolytic activity and degrades fibrinogen A α chain; its activity is time and dose dependent (Figures 7(a), and 7(b)), with similarity to Bothrokinin of *B. jararaca* [43] and Flavoxobin of *Trimeresurus avoviridis* [49]. The degradation was observed by cleavage A α chain with 15 μ g of the enzyme, which starts in 15 minutes, and at two hours there was complete degradation with maximum efficiency. TLBbar is a fibrinogenolytic enzyme that would be classified in the class A (Venombin A). Ratings temperature drop shows that the degradation of A α chain of fibrinogen is greater at temperatures of 30°C to 40°C (Figure 7(c)), and this activity was increased in the presence of calcium (Figure 7(d)) confirming that this ion is important and has a role in the coagulation cascade.

Leupeptin and PMSF inhibited the fibrinogenolytic activity of TLBbar (Figure 7(e)) confirming that its catalytic site is responsible for its pharmacological activity and that this activity is the responsibility of TLBbar.

The functions induced for TLBbar are consistent with the effects of systemic poisoning caused by *Bothrops* snakes such as clotting disorders (fibrinogenolytic activity) and bleeding (fibrinolytic activity) that alter the hemostatic system of the victim.

5. Conclusion

We have purified and characterized biochemical and pharmacologically a new serine protease thrombin-like from *B. barnetti* snake venom, named TLBbar. Experimental results have indicated that TLBbar is a new serine protease with fibrinogenolytic and coagulant activities and is capable of aggregating platelets. This enzyme showed structural homology with other thrombin-like serine proteases isolated from snake venom. The complete sequence of this protein must be determined. The complete understanding of the sequence, structure, and functional relationships of TLBbar could lead to clinical trials to investigate the potential of this enzyme and can be used to treat hemostatic disorders.

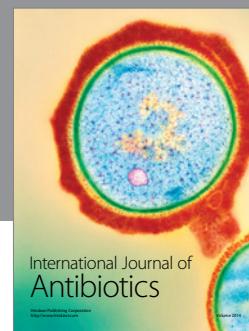
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