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Research paper

Coagulant thrombin-like enzyme (barnettobin) from *Bothrops barnetti* venom: Molecular sequence analysis of its cDNA and biochemical properties

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ABSTRACT

The thrombin-like enzyme from *Bothrops barnetti* named barnettobin was purified. We report some biochemical features of barnettobin including the complete amino acid sequence that was deduced from the cDNA. Snake venom serine proteases affect several steps of human hemostasis ranging from the blood coagulation cascade to platelet function. Barnettobin is a monomeric glycoprotein of 52 kDa as shown by reducing SDS-PAGE, and contains approx. 52% carbohydrate by mass which could be removed by N-glycosidase. The complete amino acid sequence was deduced from the cDNA sequence. Its sequence contains a single chain of 233 amino acid including three N-glycosylation sites. The sequence exhibits significant homology with those of mammalian serine proteases e.g. thrombin and with homologous TLEs. Its specific coagulant activity was 251.7 NIH thrombin units/mg, releasing fibrinopeptide A from human fibrinogen and showed defibrinogenating effect in mouse. Both coagulant and amidolytic activities were inhibited by PMSF. N-deglycosylation impaired its temperature and pH stability. Its cDNA sequence with 750 bp encodes a protein of 233 residues. Indications that carbohydrate moieties may play a role in the interaction with substrates are presented. Barnettobin is a new defibrinogenating agent which may provide an opportunity for the development of new types of anti-thrombotic drugs.

1. Introduction

Venoms of several snake species contain abundant active proteases and peptides that cause changes in the hemostatic system of human victims or experimental animals by a variety of mechanisms. One group of pharmacologically useful venom components is the serine proteases (SVSPs) which are widely distributed in the venoms of the snake families Viperidae, Elapidae and Colubridae [1]. SVSPs belong to the clan PA, subclan S and the trypsin family S1 [2]. They display quite diverse substrate specificity. Due to their versatility of pharmacological action, these enzymes affect the blood coagulation cascade, the fibrinolytic, kallikrein-kinin system, endothelial cells and platelet function [1,3], and play key roles in hemorrhage and blood clotting disorders [4]. Since the fibrinogen clotting (TLE) enzymes bring about clotting in vitro but anticoagulation (defibrination) in vivo, they are intermediate between true coagulants and anticoagulant factors [5,6]. The SVSPs that have been sequenced show high homologies in the primary structure, particularly in the region surrounding the catalytic site, however, they are quite specific toward a given macromolecular substrate. A subgroup of SVSPs, the so-called TLEs, contain endopeptidases which possess primarily one of the essential functions of thrombin: the clotting of fibrinogen (Fg) [7]. The ability to clot blood is due to the direct action of the venom TLEs on the Fg components of the blood. Some of them can hydrolyze Fg specifically and release fibrinopeptide A or B or both [7]. In addition, the TLEs do not

Abbreviations: *Bb*-TLE, thrombin-like enzyme from *Bothrops barnetti*; DL-BAPNA, α-N-benzoyl-DL-arginine-*p*-nitroanilide; *p*NA, *p*-nitroanilide; TAME, Nα-tosyl-L-Arg-methyl ester; SBTI, soybean trypsin inhibitor; PMSF, phenylmethanesulfonyl fluoride; Fg, fibrinogen; TLCK, N-tosyl-lysine chloromethyl ketone; FPA, fibrinopeptide A.

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activate any other coagulation factor, therefore, the fibrin formed is not cross-linked and is readily degraded by the fibrinolytic system. These remarkable properties enable their clinical use as defibrinogenating agents [6,8]. These enzymes are defined by a common catalytic mechanism, that includes a highly reactive serine residue that plays a key role in the formation of a transient acyl-enzyme complex, which is stabilized by the histidine and aspartic residues within the reactive site [9]. The active site serine also reacts with amides, esters and peptides, and is sensitive to inhibition by serine-modifying reagents PMSF and DFP. Due to their therapeutic value, some of these enzymes have been used as anti-thrombotics to facilitate tissue oxygenation and prevent arterial embolism [3,4,10], and are widely used in laboratories in the detection of Fg in heparinized blood and as a reagent in coagulation studies [11,12]. Furthermore, thrombin inhibitors like anti-thrombin-III (AT-III), hirudin and heparin usually do not inhibit TLEs.

Bothrops barnetti (Barnett's Lancehead) is an endemic venomous pit viper native to the Pacific coast of northern Perú and the south east of Ecuador. Its habitats are low elevations in arid to semi-arid tropical scrub [13] where it is the main poisonous snake responsible for human accidents [14]. Although *B. barnetti* is recognized as a medically relevant species in the Pacific coast of northern Perú, there are only scattered reports on the identification and characterization of active venom components involved in local and systemic pathological effects. Recently, the protein composition as well as the main pharmacological properties of *B. barnetti*'s venom in comparison with the venoms of two other Peruvian pit vipers of medical importance *Bothrops atrox* and *Bothrops pictus* has been reported by our group [15]. The venom of *B. barnetti* contains large amounts of metalloproteases, SVMPs, (74.1%) including P-I class SVMPs comprises only a metalloproteinase domain (23 kDa) and by the multidomain P-III class comprises metalloproteinase domain, disintegrin-like domain and cysteine rich domain (55 kDa), respectively and 6.7% serine proteases among other venom components [15]. Thus, its venom presents a toxicological profile similar to those of other *Bothrops* sp. venoms in Latin America.

The present study aims to determine the structural and functional characterization of a novel TLE (*Bb*-TLE), termed barnettobin from the *B. barnetti* venom. Analysis of a cDNA, encoding barnettobin as well as the deduced amino acid sequences is discussed. We show that barnettobin is a fibrinogenolytic enzyme and that its carbohydrate moieties play an important role in its interaction with substrates and the protection of its catalytic activities.

2. Materials and methods

2.1. Materials

Bovine thrombin, PNGase F, and *o*-glycosidase, human fibrinogen essentially plasminogen free, fibrinopeptides A and B, DL-BAPNA, TAME, S-2251 were obtained from Sigma Chemical Co. (St. Louis MO, U.S.A.). All other chemicals were of analytical reagent grade.

2.2. Purification of barnettobin

Barnettobin was isolated from *B. barnetti* venom by size exclusion on Sephadex G-100, ion exchange on CM Sephadex C-50 and a second Sephadex G-100 chromatography. Fractions were analyzed by SDS-PAGE and for enzymatic activity by using DL-BAPNA and Fg as substrates. Approx. 319 mg of crude venom was dissolved in 3 ml of 50 mM ammonium acetate buffer, pH 5, and centrifuged at 3000 *g*. The supernatant solution (300 mg protein) was loaded onto a Sephadex G-100 column (1.4 × 64 cm) equilibrated and eluted with the above buffer at 14 ml/h. Fractions containing the *Bb*-TLE (~52 kDa protein revealed by SDS-PAGE) were collected and concentrated to1.2 ml with an Ultracell Centrifugal Filter Unit (Amicon), then this material (105.8 mg) was applied to a CM Sephadex C-50 column (1.2 × 45 cm) equilibrated with the above buffer and eluted with a linear gradient of 0.1-1 M NaCl in the same buffer at 14 ml/h. Active fractions containing *Bb*-TLE were collected, concentrated (1 ml, 6.4 mg protein) and re-chromatographed on a Sephadex G-100 column (1 × 30 cm) equilibrated and eluted with 50 mM ammonium acetate buffer, pH 5.

Protein concentration was determined by BCA protein assay (Pierce Chemical, USA). Anti-barnettobin antiserum was raised in a rabbit (New Zealand 2.5 kg) as described in Ref. [16]. The IgG fraction of immune rabbit serum was purified by affinity chromatography on protein A Sepharose.

2.3. Assay of fibrinopeptides release

Fibrinopeptides (FPs) were generated by incubation of 4 μg of *Bb*-TLE with 1.0 ml of human Fg (3.5 mg/ml) in 50 mM Tris-HCl buffer, pH 7.4, containing 70 mM NaCl at 37 °C for 1.0, 10, and 20 min. Insoluble proteins were removed with 2% TCA and the supernatants were subjected to reverse phase HPLC on an analytical column (4.6 mm × 250 mm) of Vydac C18 (218TP54) using a linear gradient (0–15%, v/v, during 15 min; then 15–30% during a further 60 min) of acetonitrile in 0.1% TFA. The times of elution of FPA and FPB were determined by passing controls samples of human FPs A and B (Sigma) through the column as described [17].

2.4. Gel electrophoresis

SDS-PAGE without or after reduction with 4% β-mercaptoethanol was carried out according to Laemmli [18], using 10 or 12% gels and stained with coomassie blue R-250. The relative molecular mass (Mr) of the isolated protease was estimated from reducing gels by comparison with a protein calibration mixture consisting of bovine serum albumin (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (29 kDa), and lysozyme (14.3 kDa).

2.5. N-terminal sequence determination

The amino terminal amino acid sequence of the S-pyridylethylated intact *Bb*-TLE (100 μg) was determined by Edman degradation using a Shimadzu PPSQ-21A automated protein sequencer as described [17].

2.6. MALDI-TOF mass spectrometry

Protein masses were determined by Matrix assisted laser desorption/ionization time-of- flight (MALDI-TOF-TOF) mass spectrometry. Spectra were recorded and analyzed using a Bruker Autoflex III Smartbeam instrument in a linear positive mode controlled by the proprietary COMPASS™ 1.2, as described in a previous report [16].

2.7. Deglycosylation of the native barnettobin

50 μg of barnettobin was dissolved in 50 μl of denaturing buffer (0.5% SDS, 1% β-mercaptoethanol). The sample solution was denatured by boiling for 5 min. After addition of 50 μl of reaction buffer (50 mM Tris-HCl, pH 8.0), 2.5 μl of detergent solution (IGEPAL 15%, Roche) and 2 units of recombinant PNGase F or *o*-glycosidase, the samples were incubated for 24 h at 37 °C. The reaction was terminated by boiling for 5 min and PAGE loading buffer was added to the reaction mixture. The native and the deglycosylated enzymes were analyzed by SDS-PAGE after reduction as described above. In

order to maintain the activity of barnettobin the enzyme was also deglycosylated under non denaturing conditions, by incubating the enzyme with PNGase F without the addition of denaturing and reducing agents for 48 h according to [19].

2.8. Enzymatic activity

The *Bb*-TLE was assayed for its clotting activity in vitro or by its amidolytic activity. Amidolytic activity of the *Bb*-TLE was routinely determined using the substrate DL-BAPNA at 37 °C in 50 mM Tris–HCl buffer, pH 8.0 following the increase in absorbance at 405 nm per unit of time at 37 °C. Clotting activity was measured by mixing appropriate concentrations of the samples (0.5–2.4 μg) with 0.98 ml of Fg (3.5 mg/ml) in 100 mM Tris-HCl buffer, pH 7.4, containing 1 mM $CaCl_2$ at 37 °C. One unit of coagulant activity was considered to be equivalent to one NIH thrombin unit. Specific activity was defined as the number of NIH thrombin units per 1 mg enzyme.

2.9. Determination of procoagulant and defibrinogenating activities

Procoagulant activity was measured by in vitro determination of the minimum coagulant dose (MCD) using either a solution of bovine Fg and named (MCD-F) or a standard citrated solution of human plasma (MCD-P), according to [20]. To estimate the minimum defibrinogenating dose (MDD), the whole blood clotting assay (MDD-WBC) was used. The MDD-WBC was defined as the minimum dose of *Bb*-TLE that produced non-clotting blood within 60 min of intravenous injection [20]. The defibrinogenating activity was evaluated in adult mice (CF strain, 20–22 g). Animals were randomly assigned to form six groups (four animals per group). Doses of barnettobin (50, 25, 12.5, 6.0, 3.0, 1.0 μg/22 g mouse) in 0.1 ml of saline solution (89% NaCl) were injected into the tail vein according to [20]. The experiments reported here were done within the guidelines established by the Brazilian College for Animal Experimentation and overseen by FUNED Animal Ethics Committee.

2.10. Digestion of fibrin(ogen)

Fibrino(geno)lytic activity was measured as described in the previous report [21] as follows: 0.1 ml of Fg (3 mg/ml) in 50 mM Tris-HCl (pH 7.4) buffer was incubated with 9.6 nM barnettobin at 37 °C for 15, 30, 60 and 120 min. The digestions were stopped by adding SDS-PAGE sample buffer and heated at 100 °C for 3 min. The reaction mixtures were analyzed by SDS-PAGE (12% gel).

2.11. Stability assays

pH and temperature effects on amidolytic and coagulant activities were conducted at pH values ranging from 2 to 10, and at different temperatures from 20 to 100 °C. The following buffers (200 mM) were used in the reactions: sodium acetate buffer (pH 2.0–4.0), sodium phosphate buffer (pH 6.0–7.0), Tris-HCl buffer (pH 8.0–10.0). All buffers contained 0.15 M NaCl to maintain constant ion strength. To assess the temperature stability, the *Bb*-TLE was incubated at temperatures between 20 and 100 °C. The enzyme was used with a final concentration of 0.2 μM throughout all the assays. Tris–HCl buffer (50 mM, pH 7.4) was used as the solvent for the estimation of the optimum temperature.

2.12. Inhibition studies

The effects of several protease inhibitors on the purified enzyme were examined by determining its activity on DL-BAPNA. Purified enzyme (1 μg) was pre-incubated with PMSF, EDTA, TLCK, and DTT (5 mM each), SBTI (200 μg/ml), chymostatin (50 μM) and heparin (5 units) in 50 mM Tris-HCl buffer, pH 7.5, for 15 min at 37 °C. The mixtures were subjected to amidolytic assay as described above.

2.13. Synthesis and sequencing of cDNA

For this study a juvenile (female) specimen of *B. barnetti* captured near the arid locality of Chiclayo in the department of Lambayeque, north of Perú was maintained in the serpentarium of the Instituto Nacional de Salud (INS, Lima-Perú). Venom was obtained by manual compression of the venom glands. 500 μl of fresh venom collected in a sterile vial free of DNAses and RNAses was frozen immediately. Two primers were designed on the basis of the highly conserved cDNA sequences encoding batroxobin (J02684.1), bothrombin (AB178321.1), BjussuSP-I (AY251282.1), *Bothrops insularis*-TLE, BITS01A (AF490536.1), *Bothrops asper*-TLE (DQ2447724.1) and *Gloydius ussuriensis*-TLE (AF336126.1) and synthesized (Invitrogen Custom Primers). For extraction and purification of mRNA the kit Total RNA Purification (Amresco), and for synthesis of the complement cDNA, the kit Transcriptor First Strand cDNA Synthesis (Sigma) were used in accordance with the manufacturer's instructions. To amplify the gene of barnettobin the kit Master Mix Platinum® Taq DNA Polymerase (Invitrogen) was used according to the manufacturer's instructions. Sequencing of the amplification products was performed on an ABI 3730 XL automated sequencer (Macrogen, Inc, South Korea). Both the cDNA and deduced protein sequences were compared with other sequences deposited in GenBank and SwissProt.

2.14. Immunoblot and enzyme-linked immunoabsorbent assay (ELISA)

For analysis of the immunological reactivity of several TLEs from pit viper venoms against the anti-*Bb*-TLE-IgG, immunoblotting and ELISA were performed. Samples for immunoblot analysis were subjected to reducing SDS-PAGE (12% gel), followed by transfer to nitrocellulose membrane in accordance to the manufacturer's (Bio Rad Laboratories) instructions. Blotting procedures, using rabbit IgG (4 μg) against *Bb*-TLE were conducted as described [16]. For ELISA, multiwell plates were coated overnight with 0.5 μg/well of each antigen (TLEs of *B. barnetti*, *B. leucurus*, *B. atrox*, *Lachesis muta muta* and *Crotalus durissus terrificus*) in 50 mM carbonate buffer, pH 9.6. After washing with 0.05% Tween-saline, a blocking solution (2% casein in phosphate buffered saline, PBS) was added (1 h at room temperature). After two additional washing steps with the same solution, anti-*Bb*-TLE IgG diluted in PBS containing 0.25% casein and 0.05% Tween 20 was incubated for 1 h at 37 °C. After six washes, peroxidase-coupled anti-rabbit IgG (Sigma, diluted 1:12,000) was added and incubated 1 h at room temperature. The wells were washed and 100 μl of peroxidase substrate *o*-phenylenediamine (0.33 mg/ml in citrate buffer, pH 5.2 in the presence of 0.03% hydrogen peroxide) was added and the color reaction was run for 15 min at 37 °C in the dark. The reaction was stopped by adding 20 μl of a 1:20 dilution of sulfuric acid. Absorbance was read at 492 nm.

2.15. Phylogenetic tree of serine proteases and 3D model of barnettobin

The three dimensional structure of barnettobin was obtained with the Pymol program, using the structure of TSV-PA (PDB 1BQY) as a templete [22]. In order to analyze the evolutionary relationship among thrombin, trypsin, and the SVSPs, a phylogenetic tree was

Table 1
Purification of *Bb*-TLE from *B. barnetti* venom.

Steps	Protein mg recovery (%)		Clotting activity[a]			Amidolytic activity[b]		
			Total NIH units	Specific units/mg	P.F.[c]	Total U	Specific U/mg	P.F.[c]
Crude venom	300	100	2700	9	1	8.078	0.027	1
Sephadex G-100	106	35	3150	30	3	8.019	0.076	2.8
CM-Sephadex C-50	6.4	2	838.4	131	14.5	4.847	0.755	28
Sephadex G-100	1.8	0.6	460.3	257	28.5	2.519	1.407	52

[a] One unit of coagulant activity on bovine Fg was considered to be equivalent to one NIH thrombin unit; specific coagulant activity was defined as the number of NIH units per mg.
[b] One unit of amidolytic activity was defined as the amount of enzyme hydrolyzing 1.0 μmol of DL-BAPNA per min (at 37 °C in Tris-HCl, buffer, pH 8.0).
[c] P.F. purification factor.

constructed based on the complete amino acid sequences of twenty SVSPs of viperidae snakes using the program MEGA 4.1.

2.16. Other assays

Hydrolysis of the synthetic peptides: thrombin substrate Glycyl-Prolyl-Arginyl-4-*p*NA acetate (Chromozyn TH) was assayed according to [23]. Esterase activity was tested on the TAME and Benzoyl Arginil ethyl-ester (BAEE) substrates [24]. Plaminogen activating activity was tested on plasmin substrate Val-Leu-Lys-*p*NA (S-2251) as described [25]. All assays were conducted in 50 mM Tris–HCl buffer, pH 8.0, containing 0.1 M NaCl at 37 °C for 15 min. After the addition of enzyme (1 μg), product formation was followed by measuring the change in absorbance at 247 nm (TAME), 253 nm (BAEE) and 405 nm (DL-BAPNA, chromozyn TH and S-2251). The determination of activity is based on the difference in absorbance between the *p*-nitroanilide formed and the original substrate. The increase in absorbance is proportional to the enzymatic activity.

GenBank accession code of Barnettobin: JX499027.

3. Results

3.1. Purification of barnettobin

The purification of the *Bb*-TLE (barnettobin) from *B. barnetti* venom was achieved as summarized in Table 1. In the initial Sephadex G-100 fractionation of the crude venom (300 mg protein) four peaks having absorbance at 280 nm were obtained (P1-P4) (not shown). The Fg clotting and amidolytic activities were found in peak 2 and were chosen for further purification. In the second step, purification was carried out by ion exchange chromatography on a column of CM Sephadex C-50. The active material from the preceding step (105.8 mg) was applied as described in Methods. Seven peaks of absorbance at 280 nm were obtained (P1-P7) (not shown). The amidolytic activity that corresponds to clotting activity was eluted in peak 6. Final purification of barnettobin was performed by rechromatography on a Sephadex G-100 column as described in Methods. From 6.4 mg protein in this step, 1.8 mg of protein was obtained with a purification factor of 25.7 and 52.2 based on clotting and amidolytic activities, respectively (Table 1). In all purification steps it was observed that clotting and amidolytic activities eluted in the same fractions. The homogeneity of the protease was assessed by SDS-PAGE and reverse phase HPLC (not shown). As shown in Fig. 1A, the purified enzyme gave a single protein band on SDS-PAGE under both non-reduced (NR) or reduced (R) conditions. The molecular mass of the protease was estimated to be about 52 kDa under reducing conditions. Barnettobin is a highly glycosylated protein containing approx. 52% carbohydrate by mass, and its apparent molecular mass was reduced to approx. 28 kDa after digestion with PNGase F (Figs. 1B and 2). Virtually all glycosylation of the protease was shown to be due to N-linked sugar chains since treatment with *o*-glycosidase did not affect the mobility of the protein (Figs. 1B and 3). The Mr of native enzyme detected by mass spectrometry was 39,077.948 (not shown). We could not obtain a clear value for MH^+ by MALDI TOF/TOF mass spectrometry analysis of the intact protein, probably due to heterogeneity of the sugar chains.

On the other hand, the N-terminal amino acid sequence of the S-pyridyl-ethylated native protein was determined up to the 20th residue as being VIGGDECDINEHPFLAFLYS (single-letter amino acid code), which is identical to the deduced from the cDNA sequence protein (Fig. 2).

3.2. Nucleotide sequencing of the protease cDNA

In this study, we have used an alternative strategy [26] to obtain cDNA from total RNA of snake venom. By using the RT-PCR 25 ng/ml cDNA was synthetized from 500 μl of fresh venom of one *B. barnetti* specimen obtained as described in Materials and methods. As a result, mRNA was obtained from fresh venom without sacrificing the snake. After cDNA amplification a value of approx. 750 bp (not shown) was found which codes for a primary structure of a protein with 233 amino acid residues (Fig. 2). The complete amino acid sequence of barnettobin predicted from the cDNA nucleotide sequence was aligned with those of other SVSPs and was found to be closely homologous with them (Fig. 3). All of the SVSPs conserved the 12 cysteine residues paired in six disulphide bridges. By comparison with other members of the SVSP family the amino acid residues that are essential for serine proteases such as the catalytic center triad His40, Asp85 and Ser179 (barnettobin numbering), their flanking sequences and the substrate-binding Asp178 were found to be conserved in barnettobin, indicating that the enzyme is a serine protease. In confirming that the protein is highly glycosylated, barnettobin was found to contain three potential glycosylation sites, N-X-S/T, located at amino acid residues 129-131, 145-147 and 226-228 (Fig. 3).

3.3. Fibrinogen clotting and release of fibrinopeptides

We have tested the fibrinogenolytic activity of barnettobin with human Fg (plasminogen free) as substrate. The time-course digestion of Fg and fibrin at 37 °C by the purified enzyme (molar ratio enzyme: Fg 1:976) at different time intervals of incubation was accompanied by SDS-PAGE. Under our experimental conditions, the enzyme digests the α chains of Fg and fibrin time-dependently (Fig. 4A and B), and does not require any other factors for this activity. No significant alterations in the electrophoretic mobility of Bβ and γ chains of Fg could be detected even at 120 min incubation period. The purified enzyme was able to clot human Fg with a specific activity of 251.7 NIH

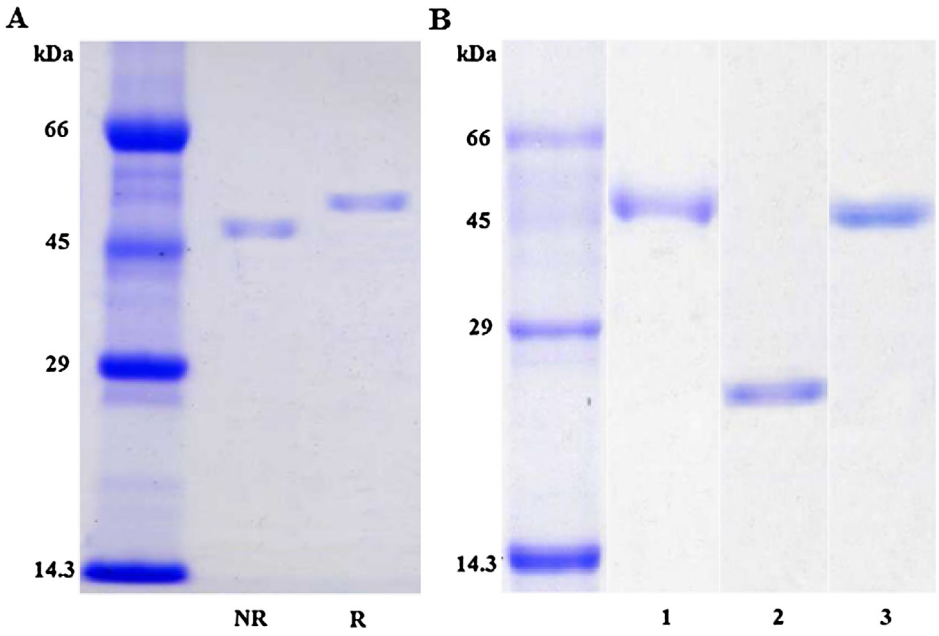

Fig. 1. SDS-PAGE (12% gels) analysis of native and deglycosylated barnettobin. A, native enzyme under non-reducing (NR) and reducing (R) conditions; B, native enzyme control (1) and after treatment with PNGase F (2) or with *o*-glycosidase (3). The gels were stained with coomassie blue. Marker proteins are indicated at left.

thrombin units/mg. This value is similar to that reported for batroxobin (210-230 NIH units/mg) from *B. a. moogeni* [17] and for other TLEs e.g. leucurobin from *B. leucurus* [17]. RP-HPLC analysis of *Bb*-TLE-treated human Fg supernatants after 0, 1, 10, and 20 min incubation at 37 °C are shown in Fig. 5, The retention times for standard FPA and FPB are presented in panel A. The only detected product being FPA and its phosphorylated derivative AP are shown in Fig. 5, panels C-D; essentially similar results to those after 10 min digestion was obtained by RP-HPLC analysis of incubation mixture for 20 min (not shown).

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  1 gccccaaaggaattacaggtttcatacgcacacaagtcatctgaa
    A  P  K  E  L  Q  V  S  Y  A  H  K  S  S  E
 46 ctggtcattggaggtgatgaatgtgacataaatgaacacccttc
    L  V  I  G  G  D  E  C  D  I  N  E  H  P  F
       V  I  G  G  D  E  C  D  I  N  E  H  P  F
 91 cttgcattcctgtactctcgcgggaatttctgtggtttgactttg
    L  A  F  L  Y  S  R  G  N  F  C  G  L  T  L
    L  A  F  L  Y  S
136 atcaaccaggaatgggtgctgaccgctgcacactgtgacaggaga
    I  N  Q  E  W  V  L  T  A  A  H  C  D  R  R
181 tttatgcccatataccttggtatacataccctaagtgtaccaaat
    F  M  P  I  Y  L  G  I  H  T  L  S  V  P  N
226 gatgatgaggtgataagatacccaaaggataatttcatttgtccc
    D  D  E  V  I  R  Y  P  K  D  N  F  I  C  P
271 aataatatatattgacgaaaaggacaaggacattatgttgatc
    N  N  N  I  I  D  E  K  D  K  D  I  M  L  I
316 aggctgaacagacctgtcaaaaacagtgaacacatcgcccctatc
    R  L  N  R  P  V  K  N  S  E  H  I  A  P  I
361 agtttgccttccaaccttcccagtgtgggctcagtttgccgtgtt
    S  L  P  S  N  L  P  S  V  G  S  V  C  R  V
406 atgggatggggctcaatcacagctcctaacgacacttttcccgat
    M  G  W  G  S  I  T  A  P  N  D  T  F  P  D
451 gtccctcattgtgctaacattaacctgttcaatgatacggtgtgt
    V  P  H  C  A  N  I  N  L  F  N  D  T  V  C
496 catggagcttacaaaaggtttccggtgaaaagcagaacattgtgt
    H  G  A  Y  K  R  F  P  V  K  S  R  T  L  C
541 gcaggtgtcctgcaaggaggcaaagataaatgtatgggagactct
    A  G  V  L  Q  G  G  K  D  K  C  M  G  D  S
586 gggggacccctcatctgcaatggaccatttcacggcattttattt
    G  G  P  L  I  C  N  G  P  F  H  G  I  L  F
631 tggggagatgatccctgtgccctgccgcgtaagcctgccctctac
    W  G  D  D  P  C  A  L  P  R  K  P  A  L  Y
676 accaagggctttgaatatcccccctggatccagagcattattgca
    T  K  G  F  E  Y  P  P  W  I  Q  S  I  I  A
721 aaaaatacaactgagacttgccccccgtga 750
    K  N  T  T  E  T  C  P  P  *
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Fig. 2. cDNA sequence and deduced amino acid sequence of barnettobin. The predicted amino acid sequence for barnettobin precursor protein is underlined and the pro-peptide region is indicated by dotted line. The predicted amino acid sequence is shown above; the numbering starts at the amino terminal amino acid of mature protein (valine). Direct N-terminal sequence of the mature protein determined by Edman degradation is in bold. The three potential N-glycosylation sites are in gray.

In connection with these results we have determined in vitro the MCD-F/P of *Bb*-TLE and in vivo the MDD-WBC for the native and deglycosylated enzyme. As can be seen in Table 4, the enzyme exerted a direct thrombin-like effect on Fg and therefore also clots human plasma. In addition, native *Bb*-TLE showed in vivo anticoagulant activity as evaluated by the MDD-WBC; 1.1 μg/mouse failed to clot blood within 60 min of i.v. injection. However, deglycosylated *Bb*-TLE loses its clotting and defibrinogenating effects.

3.4. Optimum temperature and pH determination

The relative hydrolyzing activity of *Bb*-TLE toward BAPNA as a function of temperature was measured between 20 and 100 °C. The optimal temperature for the hydrolysis of the BAPNA peptide substrate by *Bb*-TLE is reached at a temperature of approximately 40 °C with an optimal temperature between 35 and 50 °C (Fig. 6A). At temperatures below 35 and above 60 °C, its hydrolytic activity decreased rapidly, demonstrating that Bb-TLE is heat labile. Compared to these results, the enzyme exhibited optimal coagulating activity at approx. 40 °C, Fg coagulates in 60 min (Fig. 6B). On the other hand, we have assessed the effect of pH between pH 2.0 to 10.0 on the hydrolyzing activity of *Bb*-TLE toward Fg, BAPNA and coagulation of Fg in vitro. When human Fg was incubated with *Bb*-TLE, the Aα chain was preferentially degraded with optimal activity between pH 6.0 to 8.0 (Fig. 7A). No apparent alterations in Bβ and γ chains in Fg molecule were observed, as shown by SDS-PAGE (Fig. 7A). Furthermore, the relative amidolytic activity (compared to maximum hydrolytic activity of BAPNA at pH 7.5) of *Bb*-TLE was measured (Fig. 7B). Like the fibrinogenolytic activity, the *Bb*-TLE exhibits optimal hydrolysis of BAPNA between pH 6.0–8.0. In vitro, coagulation of Fg by *Bb*-TLE shows a narrowed optimal pH (~pH 7.0) as shown in Fig. 7C. Coagulation of Fg by the venom enzyme decreases dramatically at pH above 8 and below 5.

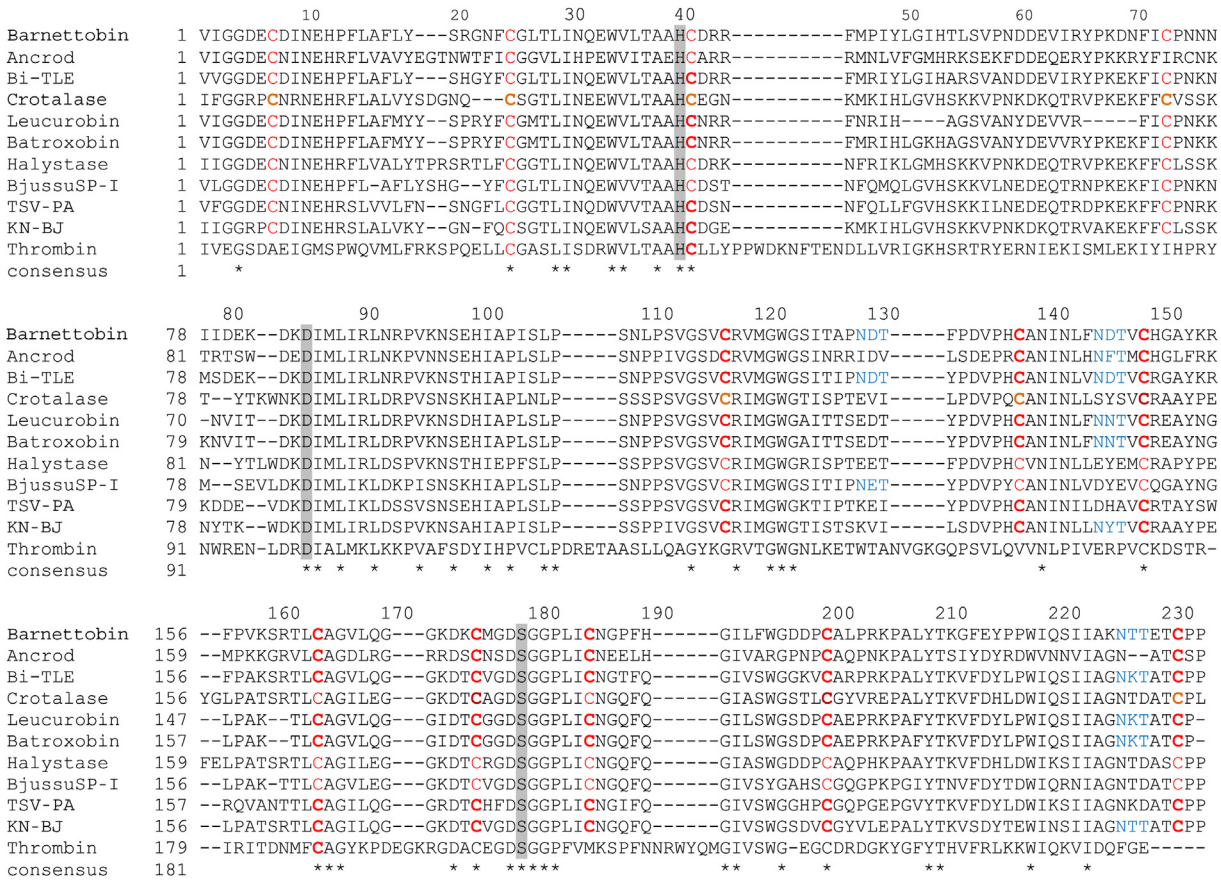

Fig. 3. Amino acid sequence comparison of barnettobin with snake venom serine proteinases and thrombin using CLUSTAL X Program. The numbering starts at the amino-terminal amino acid of barnettobin (this work). Sequences were from the following sources: ancrod from *A. rhodostoma* (P26324); Bi-TLE from *B. insularis* (Q9DG83); crotalase from *C. adamanteus* (F85114); leucurobin from *B. leucurus* (P0DJ86); batroxobin from *B. atrox* (P04971); TSV-PA from T. *stejnegeri* (Q91516); KN-BJ from *B. jararaca* (013069); halystase from *A. h. blomhoffii* (P81176); BjussuSP I from *B. jararacussu* (Q2PQJ3); and thrombin (45). The catalytic triad residues (H40, D85, S179) are in gray, and the N-glycosylation sites are in blue. Gaps have been introduced to optimize the sequence homology.

3.5. Effects of protease inhibitors and hydrolysis of chromogenic substrates

The effect of several enzyme inhibitors on the BAPNA hydrolyzing activity by *Bb*-TLE is summarized in Table 2. The enzyme activity was inhibited (71%) by PMSF, a reagent for active-site serine residues of serine proteases, functionally proving that the purified enzyme acts as a serine-type protease. Its amidolytic activity was also inhibited by chymostatin, an inhibitor of serine and cysteine proteases (43%). Other inhibitors of trypsin-like serine proteases SBTI and TLCK were able to decrease the enzymatic activity (26 and 10%, respectively), however, the enzyme was not affected by EDTA and heparin. The rapid loss of activity (45%) promoted by the disulfide-reducing agent DTT indicate the importance of disulfide bonds to the structural integrity of the protease. Table 3, shows the hydrolyzing activity of *Bb*-TLE toward various chromogenic substrates. The enzyme most effectively hydrolyzed the amidase substrate BAPNA (55 times higher than the crude venom). Also, the purified enzyme showed relatively high activity toward TAME and chromozym TH (27 and 13 times higher than the crude venom). In contrast, the plasmin substrate S2251 (D-Val-Leu-Lys-*p*NA) with basic amino acid residue Lys in place of Arg at P1 was not hydrolyzed by *Bb*-TLE.

3.6. Enzymatic assays by deglycosylated Bb-TLE

With the aim of studying the role of the extensive glycosylation of *Bb*-TLE a non-denaturing process was used to remove the N-linked glycans. The effects of amidolytic activity by deglycosylated *Bb*-TLE as a function of time, pH and temperature are shown in Fig. 8, in comparison with the native enzyme. Hydrolysis of BAPNA by deglycosylated enzyme decreased gradually after several hours incubation to about 55% at 72 h (Fig. 8A). Like these, deglycosylated enzyme retains only 52% activity at optimal pH (pH 7) (Fig. 8B). Finally, 38% amidolytic activity of PNGase F-treated *Bb*-TLE was lost at optimal temperature (40 °C) as shown in Fig. 8C. These results indicate that N-linked carbohydrate chains are important for in vitro activity of *Bb*-TLE and may play an important role in the in vivo activity.

3.7. Immunological assays

Next we investigated how purified rabbit anti-*Bb*-TLE IgG would cross- react with related TLEs isolated from the pit viper venoms, *B. barnetti*, *B. atrox*, *B. leucurus*, *L. m. muta* and *C. d. terrificus*, to experience its phylogenetic limits. Immunological cross-reactivity was assessed by ELISA and western blotting. The antibody against *Bb*-TLE reacted with highest intensity with *Bb*-TLE and with the homologous enzymes from *B. atrox* and *B. leucurus* and with less intensity with the enzymes of *C. d. terrificus* and *L. m. muta* (Fig. 9). Similar results were obtained from western blotting (not shown).

3.8. 3D model of Bb-TLE and phylogenetic tree of serine proteases

The theoretical *Bb*-TLE 3D model was generated by knowledge-based homology modeling (program PyMOL) using the

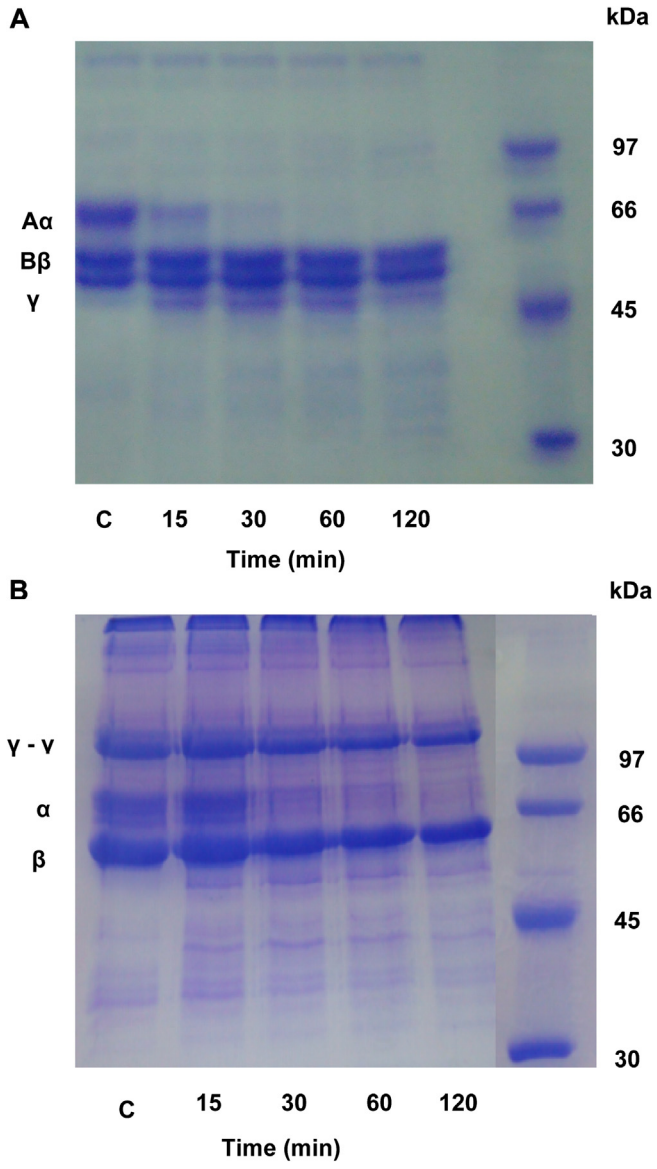

Fig. 4. Time course analysis of fibrin(ogen)olytic activity of purified *Bb*-TLE on SDS-PAGE (12% gel). A, Fg digestion; B, Fibrin digestion. Fg or fibrin incubated with the purified enzyme at 37 °C for 15, 30, 60 and 120 min. C, Fg/fibrin controls in the absence of enzyme. Note that *Bb*-TLE shows specific cleavage on the α chains of both macromolecular substrates.

crystallographic structure of the plasminogen activator from *Trimeresurus stejnegeri* (TSV-PA) as a template [22; PDB code 1BQY]. The overall structure of theoretical *Bb*-TLE shows the typical fold of a trypsin-like serine proteases (Fig. 10) and thus belongs to the trypsin/kallikrein gene family. For instance, the spatial organization of the catalytic triad residues His40, Asp85, and Ser179 at the active site cleft between the two subdomains of the β-barrel structure is preserved as in the other SVSPs. Amino acid sequences of 20 SVSPs, trypsin and thrombin were aligned and a tree was constructed by using the program MEGA 4.1 (Fig. 11). The distance matrix was used to reconstruct the tree by the neighbor-joining method [27] and boot-strap estimates of the confidence levels were conducted in 1000 replicates. Phylogenetic trees have been used to represent the evolutionary past and the probability of common ancestry to SVTLEs and other serine proteases [28]. Data in the literature indicate that the amino acid sequences of venom gland serine proteases have diversified in an accelerating manner [29]. In

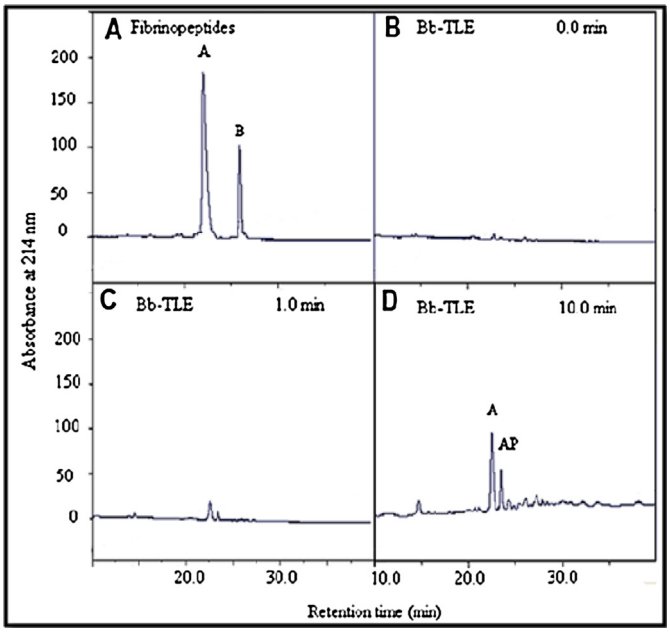

Fig. 5. Time-dependent elution profiles on RP-HPLC of peptides released from human Fg by the action of *Bb*-TLE. A, standard peptides A and B as controls, and from B–D: time dependent liberation of FPs. Details of conditions are given in the text.

accordance with Wang et al. [28] and others, Fig. 11 shows the independent and parallel evolution of three major protease subtypes, the coagulating enzymes (CLs), the kininogenases (KNs), and the plasminogen activators (PAs). In spite of their great variety of physiological properties, the SVSPs are composed of approx. 234 amino acids and are all highly homologous in sequence.

4. Discussion

Venoms of South American pit vipers contain serine proteases which exhibit fibrin(ogeno)lytic activity and lead to spectacular changes in hemostatic mechanism [3,6,30,31]. The present report details the structural and biochemical characterization of the TLE termed barnettobin to reflect its origin from *B. barnetti* venom. *Bb*-TLE is a highly glycosylated protease that contains the catalytic triad His40, Asp85 and Ser179 (barnettobin numbering) which is the main criterion for classification as a serine protease. The Mr estimated by reduced SDS-PAGE was approx. 52 kDa which reduces to 28 kDa after treatment with N-Glycosidase. This difference in Mr was expected because glycoproteins have an impaired capacity to

Table 2
Effect of some inhibitors on amidolytic activity of barnettobin.

Inhibitor	Concentration (mM)	Residual activity (%)
None	–	100
PMSF	5	29.4 ± 1.5
DTT	5	55.7 ± 3.0
SBTI	1 μg/ml	74.4 ± 3.5
Chymostatin	50 μM	57.0 ± 1.8
EDTA	5	100 ± 5.0
TLCK	5	90 ± 3.0
Heparin	5 U	100 ± 5.0

The purified enzyme (1 μg) was pre-incubated with each compound at the indicated concentrations in 1 ml of 50 mM Tris-HCl buffer, pH 8.0 at 37 °C for 5 min, after which 50 μl aliquots were taken to determine amidolytic activity on DL-BAPNA as described under Materials and Methods. Controls were performed for each compound (no inhibitor). Values are expressed as percentages relative to control and are given as mean ± S.D., $n = 4$.

Table 3
Comparison of the hydrolyzing activities of barnettobin toward synthetic substrates.

Substrate	Concentration (mM)	Specific activity U[a]/mg	
		Crude venom	Barnettobin
TAME	2	4.64 ± 0.9	118.8 ± 3.5
BAEE	2	1.96 ± 0.7	5.7 ± 1.8
BAPNA	0.9	0.02 ± 0.003	1.1 ± 0.02
Chromozym TH	2	0.81 ± 0.04	10.6 ± 0.9
S-2251	0.8	0.00	0.01

[a] One unit was defined as Δ*A* nm/min. Specific activity was expressed in U/mg. These values are presented as mean S.D. ($n = 3$).

bind sodium dodecyl sulfate and consequently have lower electrophoretic mobility [32]. Thus, the Mr of *Bb*-TLE (52 kDa), compared with the Mrs of purified TLEs from, *B. atrox* (31.4 kDa), *B. leucurus* (35 kDa), *L. m. muta* (41–47 kDa) and *C. d. terrificus* (32 kDa) measured by SDS-PAGE were obtained due to different glycosylation levels (not shown). The carbohydrate content in SVSPs varies considerably depending on the snake species and the class of SVSPs, TLEs/coagulating (CLs), kininogenases (KNs) and plasminogen activators (PAs) [28,33]. The Mr of native *Bb*-TLE detected by mass spectrometry was 39,077.948. As mentioned earlier mass spectrometric characterization of intact glycoproteins is more difficult than other simple spectrometric analysis of other simple proteins due to the extensive microheterogeneity of the carbohydrate moieties. Although MALDI-TOF/TOF mass spectrometry is in principle capable of recording spectra of intact glycoproteins, resolution of individual glycoforms can only be achieved for small proteins (~40 kDa). In addition, glycoproteins are less efficiently ionized [32,34].

In addition to its clotting activity *Bb*-TLE catalyzed the hydrolysis of the thrombin substrate chromozyn TH and was shown to have esterase activity toward basic amino acid esters: TAME and BAEE. However, it was inactive against the plasmin substrate S-2251 (Table 3) suggesting that arginine but not lysine is required at the P1 site for substrate recognition. The fact that PMSF was able to inhibit its esterase, amidolytic and clotting activities also identified it as a serine protease. Furthermore, it appears that the same active site is utilized by these functions. In accordance with studies on the mechanism by which the TLEs Ancrod from *Calloselasma rhodostoma* (formerly *Agkistrodon rhodostoma*) [35] and batroxobin from *B. atrox* [36] produce nonclotting blood suggest that these enzymes produced an aberrant Fg which either (i) cannot be clotted by thrombin or (ii) form microclots that are dispersed in the circulation. It is assumed that *Bb*-TLE acts in the same manner. In addition, when the enzyme from *B. barnetti* was incubated with Fg or plasma (not shown) it immediately formed a fibrin clot even in the presence of the anticoagulant heparin (Table 2).

The determined structure of the cDNA allowed the elucidation of the complete amino acid sequence of *Bb*-TLE. Is N-terminal sequence was confirmed by automated amino acid sequencing that also confirmed the presence of a single amino-terminal residue (Fig. 2). Analysis of the sequence alignment of *Bb*-TLE with other SVSPs (Fig. 3), indicates remarkable conservation of the structural features of the serine proteases including the twelve cysteine

Table 4
Coagulant and defibrinogenating effects of *Bb*-TLE.

Bb-TLE	MCD-F[a] (μg)	MCD-P[a] (μg)	MDD-WBC[b] (μg/mouse)
Native	0.35	1.42	1.1
Deglycosylated	NA	NA	NA

NA, no activity. These values are the average of two determinations in duplicate.
[a] MCD-F/P, minimum coagulant dose over fibrinogen and human plasma.
[b] MDD-WBC, minimum defibrinogenating dose-whole blood clotting.

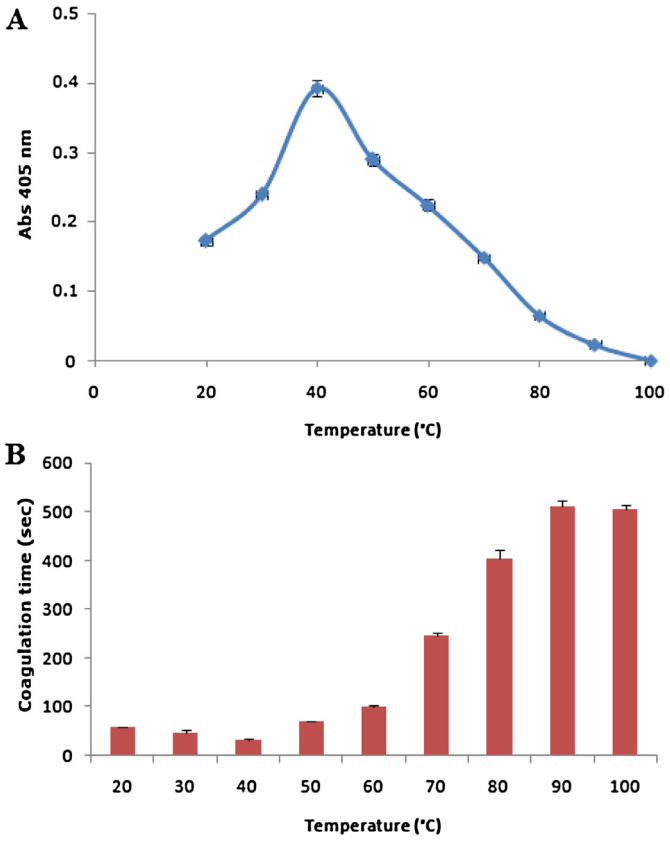

Fig. 6. Effect of temperature on the amidolytic and coagulating activities of *Bb*-TLE. A, amidolytic, and B, coagulating activities. *Bb*-TLE (1 μg) was assayed with BAPNA and Fg at 20–100 °C, respectively. Note that optimal activities were approx. at 40 °C (at this temperature, Fg coagulates in 60 s).

residues, and significant elements of secondary structure (α-helices, β-strands and loops). Based on the homology with batroxobin [36] (Fig. 3), and on the crystallographic structure of TSV-PA [37] the six disulphide bridges of *Bb*-TLE appear to be Cys7-Cys138, Cys25-Cys41, Cys73-Cys231, Cys117-Cys185, Cys149-Cys164, and Cys175-Cys200. Residues Asp85, Asp173 and Asp179, which are involved in the catalytic triad or in the formation of buried salt bridges are highly conserved in *Bb*-TLE. Furthermore, residues involved in the catalytic center are crucial for the specificity of these proteases since their secondary structure, through their β-sheets and α-helices, play an important role in their specificity, as described for TSV-PA and batroxobin [37,38]. It has been reported that phenylalanine 177 (*Bb*-TLE numbering) of TSV-PA plays a crucial role in substrate and inhibition recognition and represents one mechanism by which TSV-PA escapes from serpins [38], this equivalent position is occupied by glycine in the majority of TLEs (Fig. 3). In addition, experimental observations and theoretical data indicated that the TLEs are not allosterically regulated by sodium ions such as trypsin. This result was expected due to the conservative proline at position 206 (this work) in that coagulant SVTLEs group, instead of tyrosine presented in thrombin [39]. In the theoretical *Bb*-TLE model, the potential N-glycosylation sites are near the catalytic site and on three opposite loops (Fig. 10). This prediction agrees with its biochemical properties, since it shows that the carbohydrate in the structure may affect the activity of the enzyme, interacting with the residues of the catalytic center and/or with its surroundings [39,40]. Structural evidence for the function of the carbohydrate in the SVSPs, AaV-SP-I and AaV-Sp-II from *Agkistrodon acutus* has been reported earlier [41].

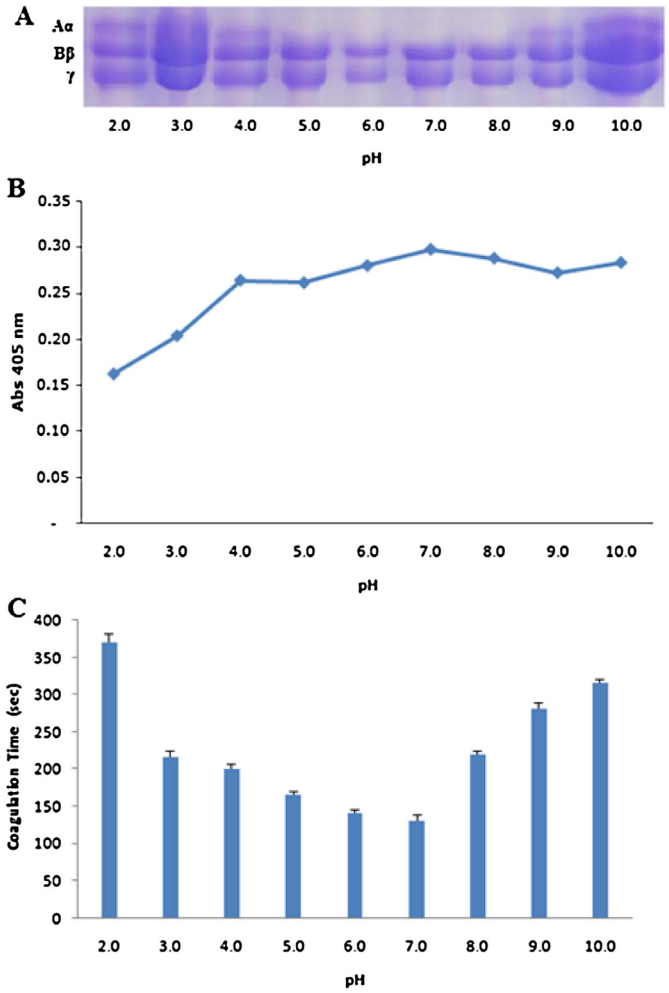

Fig. 7. Effect of pH on the activity of purified venom enzyme. A, fibrinogenolytic; B, amidolytic on DL-BAPNA; C, coagulant activity on citrated human plasma. The reaction mixture was adjusted to the desired pH for 30 min at room temperature prior to addition of enzyme (1 μg).

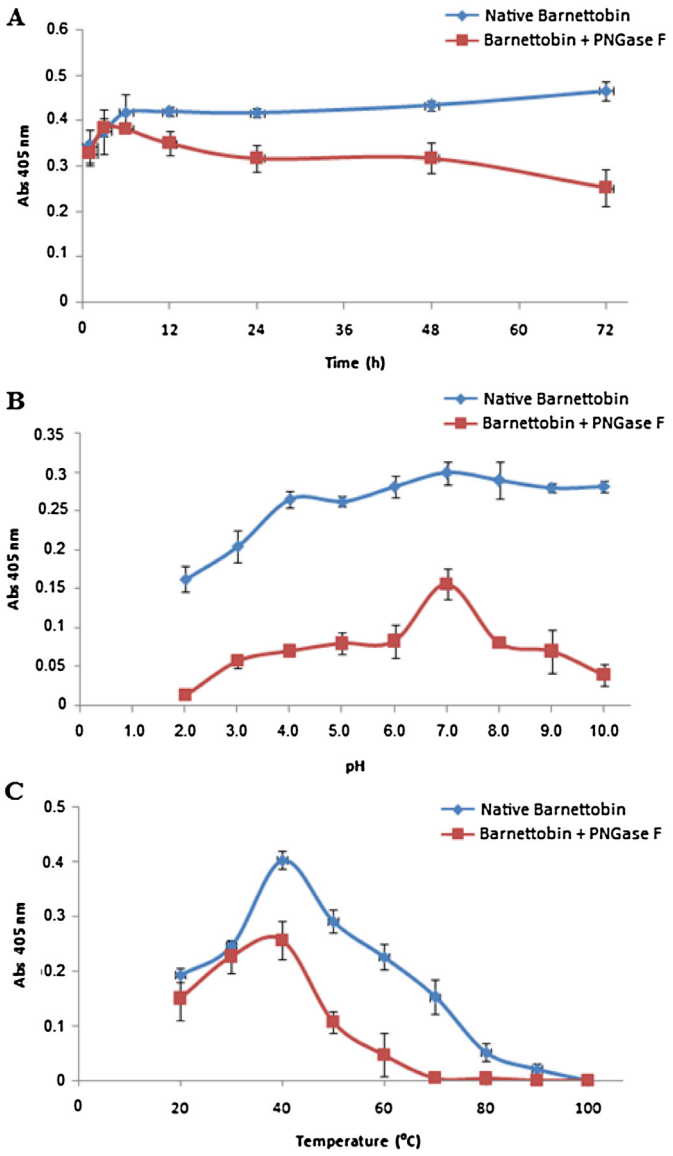

Fig. 8. Effect of *Bb*-TLE carbohydrate content on the susceptibility of the amidolytic activity as a function of time (A), pH (B) and temperature (C). Results are presented as S.D. ($n = 3$), considering the amidolytic activity at 37 °C, pH 7 of native or deglycosylated enzymes as 100% activity. Native enzyme (50 μg) was deglycosylated by incubation with 2 units of PNGase F as described in Materials and methods after which the amidolytic activity on DL-BAPNA was determined. Note that loss of N-glycosylation affected the venom enzyme activity.

Digestion experiments conducted at different intervals showed that *Bb*-TLE has both fibrinogenolytic and fibrinolytic activities as well as a defibrinogenating effect in vivo, but specifically cleaved Fg releasing FPA and its derivative (venombins A [7],) promoting coagulation. Other venombin A enzymes capable of preferentially releasing FPA from Fg include Arvin, batroxobin, leucurobin, ancrod, the TLE from bushmaster and gyroxin [3]. It is known that clots formed by TLEs are, therefore, soluble and not cross-linked and are readily degraded by the fibrinolytic system [11]. These properties enable their clinical use as defibrinogenating agents [8,11,12,31]. In addition, like thrombin and other analogous SVTLEs (bushmaster TLE, gyroxin, leucurobin), barnettobin produced axial gyrations upon intravenous injection of mice at levels of 0.133 μg/g mouse, perhaps due to the liberation of neuroactive peptides. The gyroxin effect has been related to the clotting activity of a number of SVTLEs and was elicited in mice by tail vein injection of purified enzyme containing 0.25–0.75 NIH thrombin units of activity [42]. Because of its fibrinogenolytic activity, barnettobin may be directly involved in decreasing the levels of Fg in the plasma through defibrinogenation, and also reduces blood viscosity, improving blood circulation as reported for other SVTLEs [5,10–12,43–45]. It is known that defibrinogenation is part of thrombolytic therapy. In this regard, few TLEs have been successfully approved for clinical use [3,11,12]. Ancrod (Viprinex™) a venombin A from *C. rhodostoma* is currently used for treating heparin-induced thrombocytopenia and thrombosis [10,43–46]. Batroxobin (Defibrase) another enzyme from this group found in *B. a. moogeni* venom has been indicated for treating acute cerebral infarction, angina pectoris and sudden deafness [45]. Recent studies on the outcomes of clinical trials assessing Ancrod in the treatment of acute ischemic stroke [44] and other SVTLEs which are at different stages of clinical trials, highlight the re-emerging interest in snake venom fibrinogenases as anti-thrombotics [45–47]. Desirable properties of some SVTLEs include the increased selectivity for Fg in plasma, resistance to inhibition by endogenous thrombin inhibitors (serpins), and minimal collateral effects e.g. bleeding. In this concept, a serine protease termed *Bothrops* protease A (BPA) found in *B. jararaca* venom, is a highly glycosylated protease (~62% carbohydrate content) showed selective fibrinogenolytic effect on human and rat plasma Fg [31]. Like BPA, barnettobin (52 kDa) is a glycoprotein (approx. 52%

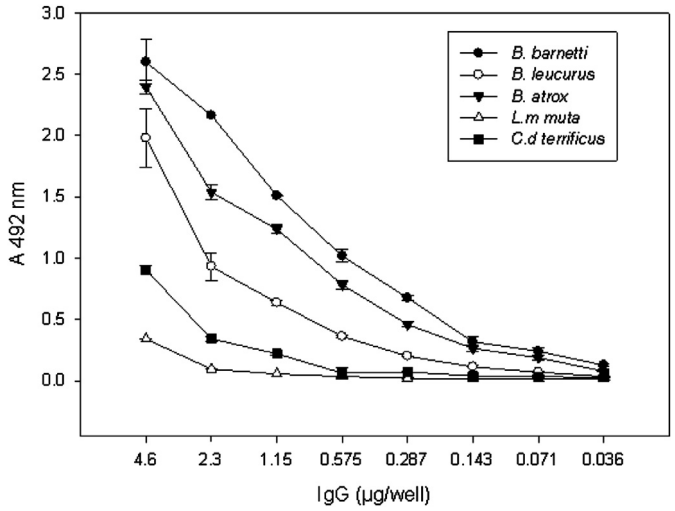

Fig. 9. Reactivity of anti-*Bb*-TLE IgG against SVTLEs analyzed by indirect ELISA. 96-well microtitration plates were precoated with 0.5 µg/well of purified: *Bb*-TLE (●), batroxobin from *B. atrox* (▼), leucurobin from *B. leucurus* (○), *L. muta*-TLE (△), *C. d. terrificus*-TLE (■). Anti- *Bb*-TLE IgG was added at different dilutions. Binding was visualized by incubation with peroxidase-coupled anti-rabbit IgG (diluted 1:12,000) and by further addition of *o*-phenylenediamine. The absorbance of preimmune serum (control) was subtracted. Results are presented as S.D. ($n = 3$).

carbohydrate content) which digests the α-chains of Fg and fibrin in vitro and produced defibrinogenating effects in mice. As demonstrated in this study, the enzyme is insensitive to inhibition by thrombin inhibitors e.g. heparin and serpins and only partially inhibited by the serine protease inhibitors (PMSF, TLCK, SBTI) probably due to its high carbohydrate content.

Conclusions. In the present study we have clarified the structure of the major coagulant TLE (barnettobin) of *B. barnetti*. Its

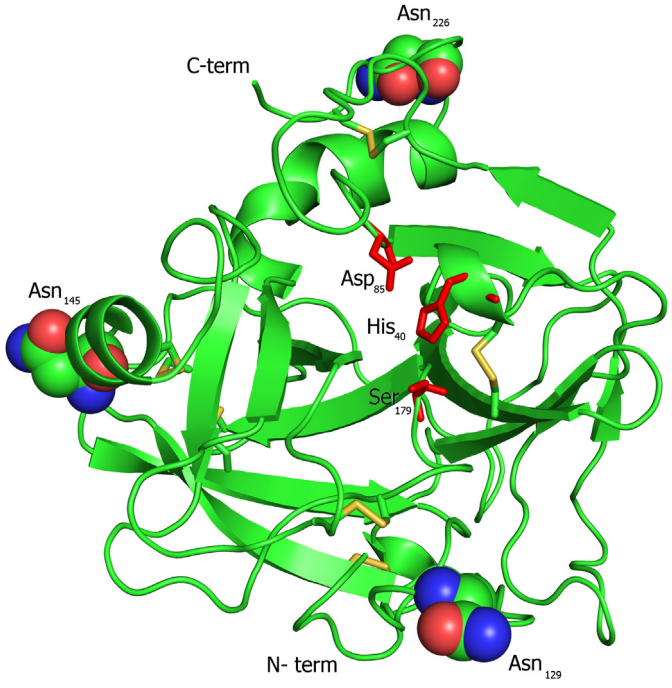

Fig. 10. Theoretical *Bb*-TLE 3D model. The representation shows the secondary structural features, with disulfide bridges in *yellow*, the catalytic triad His40, Asp85, and Ser179 in *red*, the predicted N-glycosylation sites (Asn129, Asn145, and Asn226) in *blue* spheres. The β/β hydrolase fold typical of serine proteases from the chymotrypsin family and the C-terminal extension characteristic of SVSPs is indicated. Model was generated by the Pymol program [48].

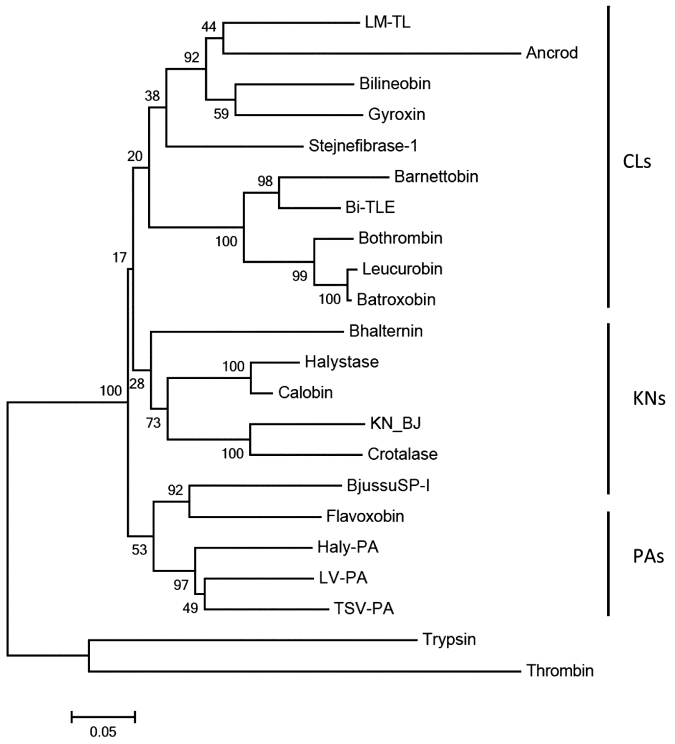

Fig. 11. Phylogenetic tree of snake venom serine proteases. The computer program Mega 4.1 and the amino acid sequences of 20 SVSPs, trypsin and thrombin so far available were used to construct the tree. The enzyme abbreviations and references are TLEs from *L. muta muta* (LM-TL), *C. rhodostoma* (ancrod, P26324), *A. bilineatus* (bilineobin, Q9PSN3), *C. d. terrificus* (gyroxin, Q58G94), *Trimeresurus stejnegeri* (stejnefibrase-1, Q8AY80), *B. barnetti* (barnettobin, JX499027), *B. insularis* (Bi-TLE, Q9DG83), *B. leucurus* (leucurobin, P0DJ86), *B. atrox* (batroxobin, P04971), *B. alternatus* (Bhalternin, P0CG03), *Gloydius blomhoffii* (halystase, P81176), *A. caliginosus* (calobin, Q91053), *B. jararaca* (KN-BJ, O13069), *C. adamanteus* (crotalase, F85114), *L. m. muta* plasminogen activator (LV-PA, Q27J47), *T. stejnegeri* (TSV-PA, Q91516), *A. halys* (Haly-PA, AF017736), *Protobothrops flavoviridis* (flavoxobin, P05620), *B. jararacussu* (BjussuSP-I, ABC24687), *B. jararaca* (bothrombin, P81661), trypsin and thrombin [49].

carbohydrate content ~52% plays an important role in the structure and activity of this glycosylated enzyme. Since the protease selectively targets fibrinogen and fibrin in vitro and showed defibrinogenating effect in in vivo models, it indeed suggests that there may be great potential in exploiting this novel fibrinogenolytic enzyme as anti-thrombotic agent.

Conflict of interest

The authors declared there is not conflict of interest.

Acknowledgments

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