CHARACTERIZATION OF THROMBIN LIKE ENZYME FROM *Bothrops pictus* VENOM

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ABSTRACT

Objectives. To perform a biochemical and molecular characterization of the coagulant principle from *Bothrops pictus* venom. ***Materials and methods.*** We amplified the genetic sequence of this enzyme from cDNA and analyzed the homology of its nucleotide sequence and its deduced protein. This enzyme was also purified for N-terminal sequencing of first 20 amino acids and for coagulation assays using human plasma and human fibrinogen. Furthermore, cleavage pattern on fibrinogen was evaluated using SDS-PAGE and defibrinogenant activity on white mice (18-22 g). Finally, associated carbohydrate content, effect of protease inhibitors and chloride ions on its enzymatic activity were analyzed. ***Results.*** The Thrombin-like Enzyme from *Bothrops pictus* showed homology at primary level of structure with other previously reported TLEs from Viperidae family. Minimum Coagulant Dosis (MCD) on plasma and human fibrinogen were 18 and 6 µg, respectively, and its coagulant potency was 131.1 NHI Thrombin units. This TLE was stable under physiological conditions and chloride ions are not necessary for its activity. Detected associated carbohydrates were hexoses (25.76%), hexosamines (13.12%) and sialic acid (0.76%). Phenyl methyl sulphonyl fluoride (PMSF) and dithiothreitol (DTT) were the main inhibitors of its enzymatic activity, but heparin had no inhibitor effect. ***Conclusions.*** The coagulant principle of *Bothrops pictus* venom is a Thrombin-like enzyme.

Key words: Bothrops; Thrombin; Blood coagulation; Fibrinogen (source: MeSH NLM).

INTRODUCTION

Disorders of the hemostatic system are the hallmarks of ophidism caused by serpents of the Viperidae family (lanceheads). This is due to the range of toxic components (enzymes and nonenzymatic peptides) that constitute their poison and that have functions similar to those of some of the serum components of the prey or that mimic their structure leading to activation or atypical inhibition of the blood-clotting processes, respectively [1,2].

In particular, there is a group of enzymes that act on the circulating fibrinogen, whether by completely degrading it (fibrinogenolytic enzymes) or by producing specific cleavages similar to those of thrombin, resulting in the formation of fibrin clots (clotting enzymes). The latter are called thrombinlike enzymes (TLEs) [3]. Nevertheless, most TLEs release only one type of fibrinopeptide (A or B) or both in very few cases; this process produces a mesh that is completely unstable and susceptible to plasmin degradation [4].

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This paper forms part of the dissertation to obtain the PhD of Dan Vivas Ruiz.
Received:1/15/2015 Approved: 6/17/2015

Citation: Vivas-Ruiz D, Sandoval GA, Lazo F, Rodriguez E, Yarleque A, Flores-Sanchez E. Characterization of thrombin like enzyme from *Bothrops pictus* venom. Rev Peru Med Exp Salud Publica. 2015;32(4):652-8.

Among the serpents that inhabit Peru, TLE has been identified in the poisons of *Lachesis muta* [5], *Bothrops bilineatus* [6], *B. barnetti* [7], *B. atrox* [8], and *B. andianus* [9]. As for *B. pictus*, there are reports about a thrombinlike activity [10, 11] suggesting that it is one of the weakest in the genus *Bothrops* [12-14].

Bothrops pictus, the "desert lancehead," causes ophidism in the central coast of Peru. Its poisoning pattern is characterized by phlogosis, erythema, ecchymosis, and loss of function. Among laboratory findings, hypoprothrombinemia and prolongation of partial thromboplastin time occur in most cases [15]. Recently, our group reported partial characterization of the clotting protein of *B. pictus*; this protein has unusual features in the TLE group, such as a higher pH range for its activity and enhancement of the enzymatic activity by the Mn^{2+} ion [16].

The interest in the studies on TLEs lies in their potential application to the fields of clinical diagnosis and treatment of cardiovascular diseases such as deep vein thrombosis, acute myocardial infarction, peripheral arterial thrombosis, and sudden sensorineural hearing loss as well as the use for prevention of thrombus formation after a surgical procedure [17]. Some of these enzymes are widely used in diagnostic laboratories to detect fibrinogen in heparinized blood samples [17]. A vivid example is the drugs Ancrod® and Reptilasa® TLE produced from the poisons of *Agkistrodon rhodostoma* and *Bothrops atrox*, respectively [18].

In this study, we assessed biochemical features of the clotting enzyme of *B. pictus* poison and characterized it at the molecular level to determine its functions.

MATERIALS AND METHODS

POISON

The poison was obtained from *Bothrops pictus* specimens from the city of Pachacamac (Lima region) that are kept in the Oswaldo Meneses Serpentarium (MHN-UNMSM). A part of the extracted venom was lyophilized for purification purposes, while a smaller amount was immediately processed to obtain RNA.

CLONING AND SEQUENCING OF THE GENE

The cDNA was synthesized, and the gene was later amplified in accordance with the method of Vivas-Ruiz *et al.* [7], using the F primer: 5′-ATGGTGCTGATCAGAGTG-3′ and R primer: 5′-CTGCAATAATGCTCTGGA-3′ designed manually based on the multiple alignment of the batroxobin (J02684.1), bothrombin (AB178321.1), BjussuSP-I (AY251282.1), BITS01A (AF490536.1), *Bothrops asper*-TLE (DQ2447724.1), and barnettobin (JX499027) sequences. The amplified product was examined on a 1% agarose gel after electrophoresis. The gene sequencing was conducted on an ABI 3730 XL automated sequencer (Macrogen, Inc., South Korea). The multiple alignment was performed by means of the Clustal W algorithm of the BioEdit software, version 7.2.5. The protein sequence was deduced in the Translate Tool software, and the biochemical properties were predicted in the Protparam software. Both programs were obtained from http://web.expasy.org/. Both the cDNA sequence and the deduced protein sequence were deposited in the databases GenBank and UniProt.

ENZYME PURIFICATION

We resuspended 100 mg of lyophilized venom in 0.05 M ammonium acetate buffer pH 5.0 and centrifuged the mixture at 2000 rpm. The supernatant was loaded onto a CM-Sephadex-C50 (1.2 × 47.5 cm) ion exchange column equilibrated with the aforementioned buffer, and the enzyme was eluted with a 0.1 to 1 M NaCl linear gradient in the same buffer at a flow rate of 14 mL/h. The enzymatic activity was monitored by means of fibrinogen and the BApNA chromogenic substrate. The active fractions were concentrated using Microcom tubes. The resulting concentrate (33.9 mg) was applied to a Sephadex G-100 (1.4 × 64 cm) filtration column, equilibrated with the buffer from the first step at a flow rate of 14 mL/h (elution involved the same buffer). The active fractions from the previous step were concentrated again in a volume of 0.6 mL (6.4 mg). This sample was applied to a Sephadex G-75 column (1 × 30 cm) equilibrated with the same buffer at a flow rate of 15 mL/h (elution involved the same buffer).

N-TERMINAL SEQUENCING

The N-terminal sequencing of the enzyme was conducted on a Shimadzu PPSQ-21A automated sequencer based on Edman degradation, using a ~1 mg/mL solution of the purified enzyme. The procedure was performed in accordance with the report of Magalhaes *et al.* [19].

DETERMINATION OF THE LINKED CARBOHYDRATES

The presence of carbohydrates linked to the protein was detected by means of hexoses and hexosamines

according to the method of Winzler R. [20], and the presence of sialic acid was identified by the method of Warren L. [21] by means of an initial protein solution at the concentration of 0.5 mg/mL.

ENZYMATIC ACTIVITY

The clotting activity was measured by quantifying human fibrinogen (5 mg/mL) in a Tris-HCl buffer (0.05 M, pH 7.4) or citrated human plasma (0.2 mL) with appropriate amounts of the enzyme (0.5 to 12 µg). One unit of clotting activity was considered equivalent to one NHI unit of thrombin. The specific activity was defined as the amount of NHI units of thrombin per mg of protein. Likewise, the minimum clotting dose (MCD) was determined [22]. The amidolytic activity was determined by means of BApNA at 37ºC in 50 mM Tris-HCl pH 8.1 after an increase in absorbance at 405 nm and 37ºC.

FIBRINOGENOLYTIC ACTIVITY

This activity was determined by incubating the purified enzyme with 0.1 mL of 0.2% fibrinogen in 0.05 M Tris-HCl pH 7.5 at 37°C for 10, 20, 30, 60, or 120 minutes. The incubation was stopped by addition of electrophoresis sample buffer and subsequent heating at 100ºC for 3 minutes. The results were examined by means of SDS-PAGE [23].

DEFIBRINOGENATING ACTIVITY

This activity was tested in albino mice (BALB/c strain, weight 18–22 g) grouped randomly into six groups (four mice per group), which were injected via the tail vein with decreasing doses of the purified enzyme starting with 50 µg diluted in 0.1 mL of saline. The minimum defibrinogenating dose (MDD) was defined as the minimum dose of the enzyme that does not cause total blood clotting within 60 minutes of intravenous injection [22].

EFFECTS OF pH AND TEMPERATURE

Both parameters were evaluated by means of BApNA using 20 µL of the enzyme. The effect of pH was determined by means of the following buffers: 50 mM ammonium acetate (pH: 4.0–6.0), 50 mM sodium phosphate (pH: 6.0–8.0), and 50 mM Tris-HCl (pH: 8.0–10.0). The effect of the temperature was evaluated by preincubating the enzyme for 15 minutes in the range 4°C to 95ºC.

EFFECTS OF IONS AND INHIBITORS

The effects of various ions in the form of chlorides (cf.: 25 mM) on the amidolytic activity were tested by preincubating a solution of the relevant salt with 20 µL of the enzyme at 37ºC for 20 minutes. The inhibitors that were tested were soybean trypsin inhibitor (STI) 1 mg/mL, ethylenediamine tetraacetic acid (EDTA), tosyl-lysyl chloromethyl ketone (TLCK), phenylmethyl sulfonylfluoride (PMSF), and dithiothreitol (DTT) at the final concentration of 10 mM. In the case of heparin, three final concentrations were tested: 250, 175, and 87.5 IU (referential values in the literature). These results were compared to those obtained with bovine thrombin (100 IU) as the reference protein.

STATISTICAL ANALYSIS

The data on the enzymatic activity were expressed as mean ± standard deviation (SD). Evaluation of the differences was done by analysis of variance (ANOVA) and Student's t test. Differences with p values below 0.05 ($p < 0.05$) were considered significant.

RESULTS

We obtained a nucleotide sequence of 754 base pairs (GenBank: KF410948) encoding a protein with 250 amino acids (Figure 1). The mature protein, named TLE-Bp (GenBank: AGZ87932), corresponds to 233 aa with the N-terminal sequence V-I-G-G-D-E typical of serine proteases. The molecular weight and isoelectric point were found to be 25.9 kDa and 8.2, respectively. In addition, five sites for N-glycosylation were identified.

During the homology analysis (Figure 2), TLE-Bp showed regions conserved in other serine proteases and thrombinlike enzymes. According to these results, the positions of the catalytic triad (His^{41}, Asp^{85}, and Ser^{179}) stand out as well as the disposal of cysteine residues. TLE-Bp showed a 30% homology with thrombin. TLE-Bp turned out to be a 49-kDa monomeric protein (Figure 3a) with a clotting activity of 131.1 NHI units of thrombin toward human fibrinogen (Fg) and amidolytic activity on BApNA. The plasma MCD was 18 µg, while for fibrinogen it was 6 µg; in both cases, the clot produced by the enzyme was loose and unstable when compared

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gcaaaccttctgattctacaggtttcttacgcacaaaagtcttctgaactggtcattgga
 A  N  L  L  I  L  Q  V  S  Y  A  Q  K  S  S  E  L  V  I  G
ggtgatgaatgtaacataaatgaacatcgtttccttgcatttacgtactctcgcgggttt
 G  D  E  C  N  I  N  E  H  R  F  L  A  F  T  Y  S  R  G  F
ttctgtggtgggactttgatcaaccaggaatgggtgctgaccgctacacactgcgacagg
 F  C  G  G  T  L  I  N  Q  E  W  V  L  T  A  T  H  C  D  R
atatttatgcgcatataccttggtttgcataaccaaagtgtacgatatgatgatcagcag
 I  F  M  R  I  Y  L  G  L  H  N  Q  S  V  R  Y  D  D  Q  Q
ataagatacccaaaggagaagtacttttttccctgtagcaaaaacttaccaaatgggac
 I  R  Y  P  K  E  K  Y  F  F  P  C  S  K  N  F  T  K  W  D
aaggacatcatgttgatcaggctggacagacctgttaagaacagtgaacacatcgcgcct
 K  D  I  M  L  I  R  L  D  R  P  V  K  N  S  E  H  I  A  P
ctcagcttgccttccaaccctcccagtgtgggctcagtttgccgtgttatgggatggggc
 L  S  L  P  S  N  P  P  S  V  G  S  V  C  R  V  M  G  W  G
acaatcacagctcctaacgacacttatcccgatgtccctcattgtgctaacattaacctg
 T  I  T  A  P  N  D  T  Y  P  D  V  P  H  C  A  N  I  N  L
ttcaattatacggtgtgtcgtggagcttacaaagggttgccagcgacaagcagaacattg
 F  N  Y  T  V  C  R  G  A  Y  K  G  L  P  A  T  S  R  T  L
tgtgcaggtgtcctgcaaggaggcatagatacatgtgtgggtgactctgggggacccctc
 C  A  G  V  L  Q  G  G  I  D  T  C  V  G  D  S  G  G  P  L
atctgtaatggacaattccagggcattgtattttggggaggtgatccctgtgcccaaccg
 I  C  N  G  Q  F  Q  G  I  V  F  W  G  G  D  P  C  A  Q  P
cgtaagcctgccctctacaccaaggtctttgatcatcttcactggatcctgagcattatt
 R  K  P  A  L  Y  T  K  V  F  D  H  L  H  W  I  L  S  I  I
gcaggaaatacaactgcgacttgccccccgtgaa
 A  G  N  T  T  A  T  C  P  P  -
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Figure 1. Deduced sequence of TLE-Bp (GenBank accession # KF410948; UniProt: U5YCR8). Lowercase letters correspond to the nucleotide sequence. The dotted line denotes the signal peptide. The solid line indicates the activation peptide. The mature protein is highlighted in boldface. The first 20 amino acids in the mature protein were confirmed by N-terminal sequencing. The boxes indicate the motifs for N-glycosylation (according to the NetGlyc software).

to those produced by thrombin. Fibrinogenolytic tests showed degradation of the fibrinogen Aα chain (Figure 3b), whereas thrombin degraded both chains, and MDD was 1.2 µg/mouse.

In other experiments, the N-terminal amino acid sequence of the native protein was determined up to 20 amino acids: VIGGDECNINEHRFLAFTYS (single-letter code), which is consistent with the deduced protein sequence. The analysis of the linked carbohydrates revealed that most sugars are hexoses (25.76%), followed by hexosamines and sialic acid (13.1% and 0.76%, respectively).

The enzyme showed activity between 30°C and 50°C. Peak activity was observed at 35°C and from pH 5.0 to pH 11.0, pH 8.0 being optimal. PMSF reduced the enzymatic activity to 9% of the norm and completely inhibited the fibrinogenolytic activity, whereas the reducing agent DTT and STI inhibited the enzyme to 65% and 90% of normal activity, respectively (Figure 4a). Heparin at a concentration of up to 100 IU/mL did not inhibit the activity significantly. Furthermore, Mg^{2+} and Mn^{2+} ions caused a slight increase (by 5%

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TLE-Bp          1 VIGGDECNINEHRFLAFTY-SRG--FFCGGTLINQEWVLTATHCD----------RIFMRIYLGLHNQS-VRYDDQQIRYPKEKYFFPCSKNFT--KWDK
Barnettobina    1 VIGGDECDINEKTFLAFLY-SRG--NFCGLTLINQEWVLTAAHCD----------RRFMPIYLGIHTLS-VPNDDEVIRYPKDN--FICPNNNIIDEKDK
BPA             1 VIGGDECNITEHRFLVEIFNSSG--LFCGGTLIDQEWVLSAAHCD----------MRNMRIYLGVHNEG-VQHADQQRRFAREK--FFCLSSRNYTKWDK
CL4             1 VIGGDECNINEHRFLALVYTDR---FQCGGTLINPEWVLTAAHCD----------RRYMHIYLGVHNES-VQYDDEQRRFPKKK--YFCLSSKNYTRWDK
LM_TL           1 VIGGDECNINEHRFLVALYDGLSGTFLCGGTLINQEWVLTAQHCN----------RSLMNIYLGMHNKN-VKFDDEQRRYPKKKYFFRCNKNFT--KWDE
Crotalasa       1 IFGGRPCNRNEHRFLALVYSDGN---QCSGTLINEEWVLTAAHCE----------GNKMKIHLGVHSKK-VPNKDKQTRVPKEK--FFCVSSKTYTKWNK
Ancrod          1 VIGGDECNINEHRFLVAVYEGTNWTFICGGVLIHPEWVITAEHCA----------RRRMNLVFGMHRKS-EKFDDEQERYPKKRYFIRC-NKTRT-SWDE
Batroxobina     1 VIGGDECDINEHPFLAFMYYSPR--YFCGMTLINQEWVLTAAHCN----------RRFMRIHLGKHAGS-VANYDEVVRYPKEK--FICPNKKKNVITDK
Tripsina        1 IVGGYTCGANTVPYQVSLNSGYH---FCGGSLINSQWVVSAAHCY----------KSGIQVRLGEDNIN-VVEGNEQFISASKS---IVHPSYNSNTLNN
Trombina        1 IVEGSDAEIGMSPWQVMLFRKSPQELLCGASLISDRWVLTAAHCLLYPPWDKNFTENDLLVRIGKHSRT--RYERNIEKISMLEKIYIHPRYNWRENLDR

TLE-Bp         85 DIMLIRLDRPVKNSEHIAPLSLP-----SNPPSVGSVCRVMGWGTITAP-----NDTYPDVPHCANINLFNYTVCRGAYKGLP--ATSRTLCAGVLQG--
Barnettobina   85 DIMLIRLNRPVKNSEHIAPISLP-----SNLPSVGSVCRVMGWGSITAP-----NDTFPDVPHCANINLFNDTVCHGAYKRFP--VKSRTLCAGVLQG--
BPA            86 DIMLIRLNRPVNNSEHIAPLSLP-----SNPPSVGSVCRIMGWGTITSP-----NATFPDVPHCANINLFNYTVCRGAHAGLP--ATSRTLCAGVLQG--
CL4            85 DIMLIRLNRPVRNSAHIAHLSLP-----SKPPSVGSVCRVMGWGTITSP-----NETLPDVPRCANINLFNYTVCRGVFPWLP--ARSRILCAGVLQG--
LM_TL          88 D---IRLNRPVRFSAHIEPLSLP-----SNPPSEDSVCRVMGWGQITSP-----PETLPDVPHCANINLFNYTVCRGAYPRMP--TK--VLCAGVLEG--
Crotalasa      85 DIMLIRLDRPVSNSKHIAPLNLP-----SSSPSVGSVCRIMGWGTISPT-----EVILPDVPQCANINLLSYSVCRAAYPEYGLPATSRTLCAGILEG--
Ancrod         88 DIMLIRLNKPVNNSEHIAPLSLP-----SNPPIVGSDCRVMGWGSINRR-----IDVLSDEPRCANINLHNFTMCHGLFRKMP--KKGRVLCAGDLRG--
Batroxobina    86 DIMLIRLDRPVKNSEHIAPLSLP-----SNPPSVGSVCRIMGWGAITTS-----EDTYPDVPHCANINLFNNTVCREAYNGLP----AKTLCAGVLQG--
Tripsina       84 DIMLIKLKSAASLNSRVASISLP-----TSCASAGTQCLISGWGNTKSS-----GTSYPDVLKCLKAPILSDSSCKSAYPGQI---TSNMFCAGYLEG--
Trombina       99 DIALMKLKKPVAFSDYIHPVCLPDRETAASLLQAGYKGRVTGWGNLKETWTANVGKGQPSVLQVVNLPIVERPVCKDSTRIRI---TDNMFCAGYKPDEG

TLE-Bp        171 -GIDTCVGDSGGPLICNGQFQ------GIVFWGDPCAQPRKPALYTKVFDH-LHWILSIIAGNT-TATCPP
Barnettobina  171 -GKDKCMGDSGGPLICNGPFH------GILFWGDDPCALPRKPALYTKGFEY-PPWIQSIIAKNT-TETCPP
BPA           172 -GIDTCGGDSGGPLICNGTFQ------GIVSWGGHPCAQPGEPALYTKVFDY-LPWIQSIIAGNT-TATCPP
CL4           171 -GIDTCKRDSGGPLICNGQFQ------GIVSWGGDPCAQPREPGVYTKVFDH-LDWIQNIIAGNT-TATCPL
LM_TL         169 -GIDTCNRDSGGPLICNGQFQ------GIVFWGPDPCAQEDKPGVYTKVFDY-LDWIQSVIAGNT---TCS-
Crotalasa     173 -GKDTCAGDSGGPLICNGQFQ------GIASWGSTLCGYVQEPALYTKVFDH-LDWIQSIIAGNT-DATCPL
Ancrod        174 -RRDSCNSDSGGPLICNEELH------GIVARGPNPCAQPNKPALYTSIYDY-RDWVNNVIAGNA---TCSP
Batroxobina   170 -GIDTCGGDSGGPLICNGQFQ------GILSWGSDPCAEPRKPAFYTKVFDY-LPWIQSIIAGNK-TATCP-
Tripsina      169 -GKDSCQGDSGGPVVCSGKLQ------GIVSWGSG-CAQKNKPGVYTKVCNY-VSWIKQTIASN--------
Trombina      196 KRGDACEGDSGGPFVMKSPFNNRWYQMGIVSWGEG-CDRDGKYGFYTHVFRL-KKWIQKVIDQFGE------
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Figure 2. Multiple alignment of TLE-Bp with other serine proteases. The conserved region of the His-Asp-Ser catalytic triad is highlighted in gray. The cysteine residues are shown in boldface. The asterisk shows the aspartate residue in a conserved position in all the enzymes of the trypsin/kallikrein family. The variation of the Na+-binding region 2 positions downstream relative to thrombin is highlighted in black. The sequences were obtained from the following GenBank resources: barnettobin from *Bothrops barnetti* (JX499027), BPA: *Bothrops jararaca* (BAA89310) protease A, CL4: *Trimeresurus stejnegeri* serine protease (AAQ02909), LM-TL: *Lachesis muta* TLE (1919267A); Ancrod, *Calloselasma rhodostoma* (P26324); *Bothrops atrox batroxobin* (P04971), bovine trypsin (P00760), and human thrombin. The alignment was performed in the CLUSTAL W software and edited in BOXSHADE 3.21 (ExPASy: SIB bioinformatics resource).

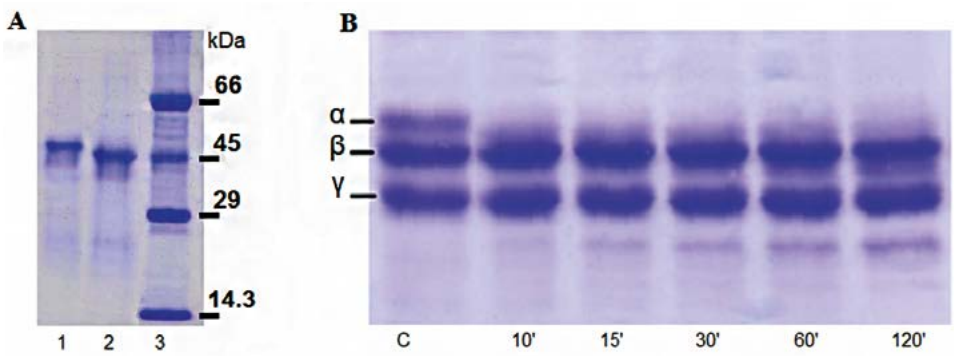

Figura 3. A) Evaluation of the purified enzyme by SDS-PAGE under reducing conditions (49 kDa; lane 1); the molecular weight patterns (lane 3) in descending order were bovine serum albumin, ovalbumin, carbonic anhydrase, and lysozyme. **B)** Fibrinogenolytic activity that shows degradation of the Aα fibrinogen chain in a time-dependent manner as compared to the control (c).

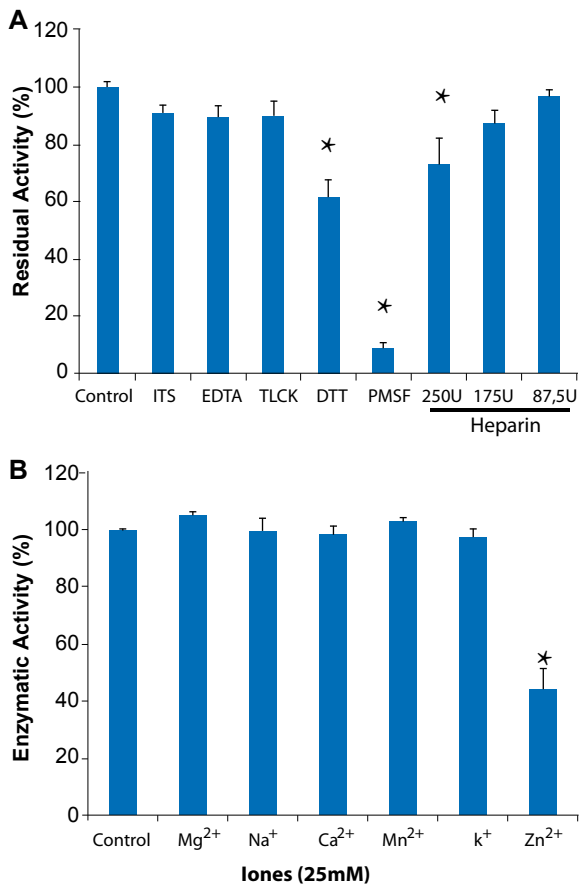

Figura 4. A) Effects of inhibitors on the amidolytic activity of TLE-Bp. The final concentrations were: EDTA, TLCK, DTT, and PMSF: 10 mM; ITS 1 mg/mL. Heparin was tested at three final concentrations. The data are expressed as percentages relative to the control and are presented as mean plus standard deviation. B) Analysis of the effects of various ions on the amidolytic activity; the ions (final concentration 25 mM) were tested in the form of chlorides. The data are expressed as percentages relative to the control and are presented as mean plus standard deviation (n = 4). *Significant differences from the control ($p < 0.05$).

and 2%, respectively), whereas the Zn^{2+} ion caused a reduction by 52% (Figure 4b).

DISCUSSION

The main functional difference of the TLEs from thrombin is the type of cut of the Fg molecule. Most TLEs cut only the Aα or Bβ chain and do not activate factor FXIII or clot the blood plasma, but inhibit *in vivo* clotting and are not inhibited by heparin. Structurally, TLEs are monomeric, with six disulfide bonds, and share only a 30% maximal similarity with thrombin [4]. Clotting alterations during *B. pictus* poisoning have been reported [14]. Previous studies have shown the presence of clotting activities in *in vitro* and *in vivo* [13] models. On the other hand, nobody performed molecular characterization of the active ingredient involved.

The primary structure of TLE-Bp, established by direct sequencing and deductive analysis of cDNA, shows 100% identity with the TLE of *Lachesis muta* and the Ancrod enzyme in the first 15 aa, as well as a strong homology with other TLEs (Figure 2) and with protease A of *Bothrops jararaca* and one CL4 serine protease of *Trimeresurus stejnegeri* (both fibrinolytic and nonfibrinogenolytic enzymes). A high homology between clotting enzymes was confirmed by the presence of the valine as the first N-terminal residue, a common feature of most TLEs [24].

Disposal of the amino acids belonging to active site (H-D-S) indicates that the enzyme belongs to the serine protease S1 family of the "PA Clan" of endopeptidases [18]. This observation is corroborated by the powerful inhibition by PMSF, which binds irreversibly to the serine residue in the active site (Figure 4a).

Likewise, TLE-Bp shares cysteine residues (all conserved) with other TLEs [4,20], and these residues participate in the formation of disulfide bonds important for the stability of the tertiary structure, as demonstrated by the inhibitory effect of the reducing agent DDT [4,20]. STI had no significant inhibitory effect; this result supports a structural difference between TLE-Bp and this protease.

Furthermore, TLE-Bp clotted human plasma and human fibrinogen; however, its injection into rodents produced an effect of blood incoagulability *in vivo* judging by the defibrinogenating activity. This result indicates that clots induced *in vivo* do not have optimal stability and could be removed by endogenous fibrinolytic processes, which cause significant depletion of the circulating fibrinogen. The instability of the clots produced by TLE-Bp may be explained by the single hydrolysis of the fibrinogen Aα chain (Figure 3b).

Unlike thrombin, TLE-Bp was not inhibited by heparin (up to 100 U/mL). This finding could be related to the absence of TLEs in certain amino acid regions present in the thrombin enzyme involved in the binding of this mucopolysaccharide [2,4,5,7]. Likewise, TLE-Bp activity was not enhanced by Ca^{2+} or any of the other ions tested, nor was it affected by the chelating agent EDTA. This finding indicates that the enzyme does not require divalent ions for its activity. On the other hand, the Zn^{2+} ion could be an endogenous serine protease inhibitor that prevents autoproteolysis in the venom-producing gland [2].

The role of the carbohydrate moieties of TLE-Bp has yet to be determined; they represent approximately 40% of the total protein weight. Additionally, participation of this enzyme in alteration of other physiological processes during poisoning is also unclear at present.

Our results enable us to conclude that the clotting enzyme of the *B. pictus* venom has a thrombinlike activity and can be called as such (GenBank name: pictobin) because it shares similar features with other TLEs present in other species.

Acknowledgments: This research was financially supported by the National Innovation Program for Competitiveness and Productivity (Contract 131 FINCyT-IB-2013) and the CONCYTEC (Peru)–CNPq (Brazil) agreement. This study is also a part of the doctoral thesis of Dan Vivas-Ruiz, carried out in the School of Biological Sciences - UNMSM.

Author Contributions: DVR, EFS, and AY participated in the conception and design of the study. DVR, GAS, and ER collected the materials and performed the experiments, and DVR and FL participated in writing of the manuscript. DVR and AY participated in the analysis and interpretation of data and in the approval of the final draft. AY, EFS, and GAS obtained the funding.

Funding Source: This work was possible thanks to the funds provided by the CONCYTEC (Peru)–CNPq (Brazil) Bilateral Cooperation Agreement, as well as the National Innovation Program for Competitiveness and Productivity – Innovate Peru (Contract 131 FINCyT-IB-2013).

Conflicts of Interest: The authors declare they have no conflicts of interest related to the execution and publication of this study.

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