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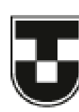

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Purificação de uma α-fibrinogenase com atividade thrombin-like e kallikrein-like da peçonha de Bothrops leucurus

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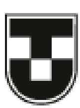
ABSTRACT

A thrombin-like enzyme from *Bothrops leucurus* snake venom, named TL-Leuc, was purified in two chromatography steps on CM-Sepharose fast flow and HiPrep Sephacryl S-300. This protease was homogeneous on SDS-PAGE and when analyzed in MS/MS (MALDI TOF\TOF) showed mass of 30710.25 and 32109.69Da after alkylation and reduction. The peptides with significant score were compared with those of other proteins used NCBI-BLAST2 alignment display, showing similarity with other serine proteases. TL-Leuc exhibited proteolytic activity against fibrinogen, being able to degrade only the Aα-chain. This enzyme caused defibrinogenation when administered intraperitoneally in mice, making the plasma incoagulable. The enzyme induced systemic defibrinogenation when administered intraperitoneally in mice, resulting in incoagulable plasma. Conversely, TL-Leuc also exhibited high coagulant activity against bovine plasma and fibrinogen solution. The hydrolytic activity of Tl-Leuc upon S-2238, S-2266 and S-2302 substrates suggests that this enzyme has thrombin and kallikrein-like activities, respectively. TL-Leuc not show hemorrhagic, myotoxic and fibrinolytic activities. Taken together, our data showed that TL-Leuc is in fact, a thrombin-like enzyme isoform isolated from *B. leucurus* snake venom.

***Keywords**: Bothrops leucurus*, snake venom, thrombin-like, kallikrein-like, anticoagulante, antithrombotic.



RESUMO

Uma enzima com atividade thrombin-like proveniente da peçonha bruta de *Bothrops leucurus*, denominada TL-Leuc, foi purificada em duas etapas cromatograficas, utilizando as resinas de troca iônica CM-Sepharose e gel filtração Sephacryl S-300. Esta protease mostrou-se homogênea em SDS-PAGE e quando analisada em MS/MS (MALDI TOF\TOF) apresentou massa de 30710,25 e 32109,69Da após alquilação e redução. Os peptídeos com pontuação significativa foram comparados com os de outras proteínas utilizadas no display de alinhamento NCBI-BLAST2, mostrando semelhança com outras serina proteases. O TL-Leuc apresentou atividade proteolítica sobre o fibrinogênio, sendo capaz de degradar apenas a cadeia αA. A enzima causou desfibrinogenação quando administrada por via intraperitoneal em camundongos, tornando o plasma incoagulável. Tl-Leuc mostrou alta capacidade de coagular plasma bovino e alta atividade de coagulação em plasma bovino e solução de fibrinogênio. A atividade hidrolítica do Tl-Leuc sobre os substratos S-2238, S-2266 e S-2302 sugere que esta enzima possui atividades semelhantes à trombina e à calicreína, respectivamente. A enzima TL-Leuc não apresentou atividades hemorrágicas, miotóxicas e fibrinolíticas. Tomados em conjunto, nossos dados mostraram que o TL-Leuc é, de fato, uma isoforma enzimática semelhante à trombina isolada do veneno da cobra *B. leucurus*.

Palavras-chave: *Bothrops leucurus*, peçonha de serpente, thrombin-like, kallikrein-like, anticoagulante.

INTRODUCTION

Snake venoms are produced in specialized glands capable of synthesizing and secreting a large amount of biologically active substances. The venoms, therefore, constitute true chemical arsenals with a potential to attract, paralyze and kill other organisms (JUNQUEIRA-de-AZEVEDO, HO, 2002). They may have several functions, such as attack, capture, digestion of food, or contribute to protect the animal against predators or attackers. The snake venoms have a wide composition and physiological action. The major systemic effects after envenomation by *Bothrops* species are the imbalance of hemostasis. Toxins that disrupt the mammalian hemostatic system exhibit either procoagulant or anticoagulant activity (MARKLAND, 1998; MARSH, WILLIAMS, 2005; SANCHEZ, SWENSON, 2007; GAY et al., 2009). By activating blood clotting factors, *Bothrops* venom induces fibrinogen depletion, frequently resulting in incoagulable blood (COSTA et al., 2009, COSTA et al., 2010; GOMES et al., 2009, GOMES at al., 2011).

Over the last decades the incidences of cardiovascular disease and stroke have been increased. According to the World Health Organization this type of pathology is already among the most serious and tends to grow, mainly on lifestyle related to a diet high in fat, lack of physical

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activity, stress, among others (CHAVES et al., 2010). Biochemical and pharmacological studies of snake venoms have shown a variety of components and physiological activities. Consequently, numerous attempts have been made to utilize these compounds as valuable tools in medical research (KOH et al., 2006; SANCHEZ, SWENSON, 2007; SAJEVIC et al., 2011). These findings support the inclusion of batroxobin (Defibrase ®; Pentapharm), isolated from Bothrops moojeni, as a potent agent utilized for the therapeutic and diagnostic management of hemostatic disorders. By cleaving only fibrinopeptide A from fibrinogen, Batroxobin rapidly promotes its removal from circulation, leading to its use in defibrination therapy. Other drugs used for the same application, such as Reptilase®, are also isolated from the venom of *Bothrops atrox*.

As components of snake venom, the serine proteases (SVSPs) maintain the highly conserved catalytic triad (Ser, His, and Asp) characteristic of the S1 family of peptidases (PIRKLE, 1998; CASTRO et al., 2004; MARSH, WILLIAMS, 2005; COSTA et al., 2010). The pharmacological importance of these enzymes stems from their direct action on components of the haemostatic system. Acting as thrombin-like enzymes (T-LEs), they target fibrinogen, making the blood incoagulable. Despite this potent effect, SVSPs are generally considered to have low in vivo toxicity, primarily because they do not activate Factor XIII (MARKLAND, 1998; CASTRO et al., 2004; HENRIQUES et al., 2004; MARSH, WILLIAMS, 2005; SANCHEZ and SWENSON, 2007; SAJEVIC, et al., 2011).

Enzymes Thrombin-like (TLEs) act on blood coagulation, primarily by degrading fibrinogen to fibrinopeptides A or B and may act on both (FPA, FPB or FPAB). The most common ones in snake venoms are those that degrade the Aα chain of fibrinogen producing fibrinopeptide A and consequently a weak fibrin clot which can be removed by endogenous components of the haemostatic system, and that these enzymes are generally incapable of the active Factor XIII (MAGALHÃES et al., 2007; PÉREZ et al., 2008; COSTA et al., 2010).

Moreover, these enzymes can activate the clotting cascade factors, especially factors FX, FVII, FV, FII and PC (protein C), usually leading to platelet aggregation, and can activate plasminogen and fibrin degrader, among other features (SAJEVIC et al., 2011). Consequently, the unique properties of these enzymes make them a subject of intense scientific investigation regarding their therapeutic potential in clinical haemostatic disorders (thrombosis, cardiovascular disorders among others), the thrombin-like enzymes are fundamentally characterized by their function as fibrinogenases (MARSH 1994; MARSH, WILLIAMS, 2005; SANCHEZ, SWENSON, 2007; SAJEVIC et al., 2011).

The species is endemic to Brazil and occurs in the Atlantic Forest coastal area, extending from the north of Espírito Santo State up to Ceará State. Consequently, its venom has been extensively

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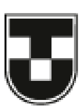

purified and characterized by researchers: two PLA2 (HIGUCHI et al., 2007), two metalloproteinases P-I class (BELLO et al., 2006, GOMES et al., 2011), a metalloproteinase P-III class (SANCHEZ et al., 2007), a serine proteinase (MAGALHÃES et al., 2007), a L-amino acid oxidase (SILVA et al., 2007), a lectin (NUNES et al., 2011).

OBJECTIVES

In this work, we purify and characterize a novel serine protease from Bothrops leucurus snake venom, with direct activity on the coagulation cascade and low toxicity, thus aiming at a possible clinical application in diseases related to blood coagulation.

MATERIAL AND METHODS

Venom and animals

Bothrops leucurus venom was purchased from CEPLAC (The south region of Bahia state, Brazil). This was lyophilized and maintained at low temperatures in the Chemistry Laboratory of Proteins from the Universidade Estadual do Sudoeste da Bahia (UESB). Male Swiss mice were kindly provided by the Institute Vallé (Uberlândia-MG, Brazil) and the experiments were approved by the ethics committee of the Federal University of Uberlandia (UFU); RESEARCH PROTOCOL number CEUA/UFU 046/09.

Reagents

CM-Sepharose fast flow was purchased from Amersham Biociences. Acrylamide, HiTrep Sephacryl S-300 RP, C2C18 column were purchased from GE Healthcare Bio-Sciences, Ammonium persulfate, Aprotinin, PMSF, Benzamidine, Bromopherol Blue, ethylenediaminetetraacetic acid (EDTA), Bovine fibrinogen, Human Thrombin, β-mercaptoethanol, N, N´-methylene-bis-acrylamide, N, N, N´, N´,-tetramethylethylenediamine (TEMED), 1,10-phenanthroline, Sodium dodecyl sulfate (SDS) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). The p-Nitroanilide substrates H-D-Phe-pipecolyl-Arg-pNA.2HCl (S-2238), N-Bz-Ile-Glu-(OR)-Gly-Arg-pNa.HCl (S-2222); H-D-Val-Leu-Arg-pNA.2HCl (S-2266), H-D-Val-Leu-Lys-pNA.2HCl (S2251) and and H-D-Pro-Phe-Arg-pNA.2HCl (S-2302) all came from Chromogenix. All other chemicals were of analytical reagents grade.

Isolation of TL-Leuc

TL-Leuc was purified from *B. leucurus* venom by a combination of ion- exchange chromatography on CM-Sepharore fast flow and HiPrep Sephacryl S-300 (26/60). Crude venom of *B. leucurus* (175 mg) was dissolved in 50 mM ammonium bicarbonate buffer (pH 7.8) and clarified by centrifugation at 5000xg for 10 min. The supernatant solution was chromatographer on a column CM-Sepharore fast flow (1.4x 26cm), previously equilibrated with 50mM, pH 7.8, ammonium bicarbonate buffer (AMBIC) and eluted with a concentration gradient

Revista Biociências - Universidade de Taubaté - v.32 - n.1 - p. 18-36, 2026 - ISSN: 14157411
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(50-500mM) of the same buffer at a flow rate of 2.0 ml/min (the gradient was established in fraction 50). Absorbance of the effluent solution was recorded at a wavelength of 280 nm. The active fraction CM6 was collected, lyophilized, dissolved in 50mM ammonium bicarbonate pH 7.8, and applied on a HiPrep Sephacryl S-300 column (26/60) previously equilibrated and eluted with the same buffer at a flow rate of 0.2 ml/min Using an AKTA prime plus equipment (Amersham Biosciences). The fraction showing clotting activity (now named TL-leuc) was collected, concentrated and stored at 4 °C for subsequent biochemical analysis.

Analysis of TL-Leuc in RP-HPLC

TL-Leuc sample of 300 µg was diluted in 500 µL of solvent A (0.1% trifluoroacetic acid and 5% acetonitrile) and analyzed on RP-HPLC using a C4 column of 4.6x150mm (Pharmacia Biotech), equilibrated with solvent A (0.1% trifluoroacetic acid and 5% acetonitrile) and eluted with a concentration gradient of solvent B (80% acetonitrile, 0.1% trifluoroacetic acid) from 0 to 100% at a flow rate of 0.5 mL/min at room temperature. Protein concentration was determined by the method of BRADFORD (1976).

Determination of Mr in SDS-gel and MALDI TOF

Determination of Mr: 12% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to LAEMMLI (1970). The molecular mass of TL-Leuc was also determinate by Matrix-assisted laser desorption ionization time of flight mass spectrometry (MALDI-TOF-MS) (Autoflex II, Bruker Daltonics, Germany).

Peptide mass fingerprinting on MALDI-TOF/TOF and bioinformatics

Sample TL-Leuc was incubated with 2% (w/w) trypsin (Promega, Madison, USA) for three hours at 37 oC. The digest was concentrated and mixed into 1µL of α-cyano-4-hydroxycinnamic acid matrix (1µg/µL) and left to air dry. Protein digest was subjected to peptide mass fingerprinting on a MALDI-TOF/TOF mass spectrometer (Autoflex II, Bruker Daltonics, Germany). Selected peaks were used for searches and identification, and one of the identified peptide was subjected to MS/MS fragmentation. Mass spectra were analyzed using FlexAnalysis and Biotools software (Bruker Daltonics) and Mascot software (PERKINS et al., 1999). The searches were performed against the NCBI database (JOHNSON et al., 2008) with 100 ppm mass tolerance and fragmentation mass tolerance of 0.5 Da.

The peptide fragments generated which showed significant score were aligned using the program BLAST bioinformatics (www.ebi.ac.uk/Tools/blastall/) and EMBOSS Pairwise Alignment Algorithms (http://www.ebi.ac.uk/Tools/emboss/align/)

The sequences of TL-Leuc was determined by Edman degradation (EDMAN, 1967). About 1

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mg/mL of the purified enzyme were sequenced by an automated protein sequencer model PPSQ-33A (Shimadzu Co., Japan) according to the manufacturer's instructions. The obtained sequences were compared with non-redundant protein sequences (nr) from Viperidae (taxid: 8689) available at BLAST database using the online blastp suite (https://blast.ncbi.nlm.nih.gov/Blast.cgi) (protein-protein blast) (https://www.ncbi.nlm.nih.gov/tools/cobalt/cobalt.cgi) .

Enzymatic activity

Coagulant activity

The coagulant activity of the crude venom, fraction CM6 and TL-Leuc, was assayed on citrated bovine plasma and bovine fibrinogen solution (2.0 mg/mL). Since samples of 5µg of crude venom of Bothrops leucurus, CM6 fraction and TL-Leuc all diluted in 10µL of PBS and applied to 150µL of citrated bovine plasma and 150µL of a solution of 2.0 mg/ml of bovine fibrinogen diluted in Tris-HCl 50mM pH 7.4. The tests were performed in a micro-processor Quick-Timer analyzer (DRAKE LTDA).

Proteolytic activity upon fibrinogen

Fibrinogenolytic activity was assayed as described by GOMES et al., (2009). Samples de 50µL of bovine fibrinogen (1.5 mg/mL was dissolved in saline) was incubated with 5µg of the enzyme at 37°C and different time intervals (5, 10, 15, 30, 60 and 120 minutes). The enzyme action on fibrinogen was also tested using the same methodology with doses of 1-10 µg at a time of 1 hour. The reaction was stopped with the addition of 50% of denaturing buffer containing 2% SDS; 5% β-mercaptoethanol; 10% glycerol and 0,005% bromophenol blue. Reaction products were analyzed by 12% SDS-PAGE.

The effect of inhibitors on the fibrinogenolytic activity was assayed after preincubation of the enzyme (5µg) with 10µL of EDTA, 1,10-phenanthroline, PMSF, β-mercaptoethanol, aprotinin and benzamidine (all 20mM).

Proteolytic activity upon fibrin

Proteolytic activity upon fibrin clots was assessed as described by GREMSKI et al., (2007) with modifications. Briefly, 5NIH units/mL human thrombin (Sigma) were added to 2.5 mg/mL bovine fibrinogen (Sigma) in 500µL of 0.2M Tris/HCl, pH 8.0, with 20mM $CaCl_2$ at room temperature. Immediately after mixing, 100µL of the polymerizing solution were transferred to tubes (0.5mL). After 2h, each solution was treated with 20µL (10µg) of the enzyme TL-Leuc in same buffer and incubated for different time intervals (30, 45, 60, 120 and 180 min.) at 37°C, followed by the addition of 60µL of denaturing solution containing 2% SDS; 5% β-mercaptoethanol; 10% glycerol and 0,005% bromophenol blue, for SDS-PAGE (12%) analysis.

Defibrinating activity

Revista Biociências - Universidade de Taubaté - v.32 - n.1 - p. 18-36, 2026 - ISSN: 14157411
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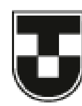

Defibrinating activity was tested by the method of GENE et al., (1989), with slight modifications. Briefly, four Swiss male mice (20-25g) were injected i.p. with increasing doses of the 10µg crude venom and 10µg of TL-Leuc (dissolved in 50µL of saline); control animals received 50µL of saline. After 3 h, animals were anesthetized (ketamina® 10% (0,05ml/kg) + xilasina® 2% (0,025ml/kg) and bled by cardiac puncture. Whole blood was placed in glass tubes and kept at 25-30°C until clotting occurred.

Plasma fibrinogen determination

Four Swiss male mice (20-25 g) were injected i.p. with increasing doses of the 10µg crude venom and 10µg of TL-Leuc (dissolved in 50µL of saline); control animals received 50µL of saline. After 3 h, animals were anesthetized (ketamina® 10% (0,05ml/kg) + xilasina® 2% (0,025ml/kg) and bled by cardiac puncture. The plasma fibrinogen concentration was determined by clotting time (in an equipment coagulometric Quick-Tmer- DRAKE Ltda.) after obtained the plasma in the presence of citrate, using the kit commercial Hemostat Fibrinogen produced by Human Gmbh and distributed by Human of Brazil.

Prothrombin time (PT) and Partial thromboplastin time (aPTT)

To determine the parameters of blood clotting, were inoculated 10µg enzyme TL-Leuc intraperitoneally into male Swiss mice of (20-25g; n=3) and the animals used as controls received saline. After 3h the animals were anesthetized (ketamina® 10% (0,05ml/kg) + xilasina® 2% (0,025ml/kg) and bled by cardiac puncture. The collected blood was centrifuged at 360xg for 15 minutes at 4°C and plasma obtained was used for determination of (PT) and (aPTT) by the commercial kit of PT and aPTT company`s BIOS and Diagnostic Industry Ltda. (www.clot.com.br).

Activity on chromogenic substrates

The ability of the different TL-leuc concentrations (0.01µg/µL; 0.015µg/µL; 0.02µg/µL; 0.03µg/µL; 0.04µg/µL; 0.05µg/µL; 0.06µg/µL) to hydrolyze the 150µL chromogenic substrates was performed on the substrates S-2238 (substrate for enzyme thrombin-like) 1 mmol/L; S-2222 (substrate for factor Xa) 0.6 mmol/L; S-2266 (substrate for glandular kalikrein) 0.2 mmol/L; S-2302 (substrate for plasma kalikrein) 0.1 mmol/L; S-2251 (substrate for plasmin) 0.3 mmol/L. The solutions were diluted in Tris-HCl 50mmol/L, pH 7.4 contend 5mmol/L de $CaCl_2$ incubated for 20 minutes at 37 ° C and then we performed reading at 405nm in a microplat reader (BioTeK – Elx50). We used a control solution under the same condictions and the addition of 0.01 of Abs/ 405nm was considered as one unit of activity.

To test the inhibition of enzymatic activity of TL-leuc, the substrate S-2238 (for thrombin-like enzymes) was used.

Revista Biociências - Universidade de Taubaté - v.32 - n.1 - p. 18-36, 2026 - ISSN: 14157411
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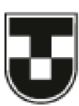

The enzyme was previously incubated for o ne hour with 10µL of EDTA and PMSF 20mmol/L. Activity was performed as previously described.

Hemorrhagic activity

Hemorrhagic activity was determined by the method of NIKAI et al., (1984) with modifications. In these assays, different doses of enzyme or crude venom were injected intradermically into the skin of Swiss male mice (20-25 g). After 3h the animals were anesthetized (ketamina® 10% (0,05ml/kg) + xilasina® 2% (0,025ml/kg) and sacrificed. The skins were removed, and the area of hemorrhage on the underside of the skin was measured.

Myotoxic activity

Groups of three male Swiss mice (20-25g) were administered via intramuscular (i.m.) injection into the right gastrocnemius muscle of the animal. Were injected with 10µg of crude venom of Bothrops leucurus and 50µg of TL-leuc diluted in 50µL de PBS. The control animals received 50µL of PBS. After 3h the animals were anesthetized (ketamina® 10% (0,05ml/kg) + xilasina® 2% (0,025ml/kg) and bled by cardiac puncture. The level of CK was determined by Kit CK-UV kinetic (BIOCLIN).

Histological analysis

Histological alterations induced by TL-Leuc and *B. leucurus* crude venom was analyzed in groups of three male Swiss mice (20-25g). For examination of myonecrosis groups of mice received i.m. injection of 50 µg of TL-Leuc or 10µg of crude venom/50 µL saline. Control animals received i.m. injections of 50 µL saline. Animals were anesthetized (ketamina® 10% (0,05ml/kg) + xilasina® 2% (0,025ml/kg) and sacrificed and a small section of the central region of the gastrocnemius muscle was excised and soahed in fixing solution (10% formaldehyde in PBS, v/v). The material was then dehydrated by increasing concentrations of ethanol and processed for inclusion in paraffin. The resulting blocks were sliced in 2.5 µm thick sections, stained with 0.25% (v/v) hematoxylin-eosin and examined under a light microscope.

Statistical Analysis

The results were presented as means ± standard deviation (S.D.). Statistical significance of results was evaluated using Kruskal-Wallis tests. Values of $p< 0.05$ were considered significant.

RESULTS AND DISCUSSION

Snakebites constitute a public health problem due to its prevalence. According to the Ministry of Health in 2018 in Brazil there were more than 30.000 accidents involving bites by snakes, and of these 88% were caused by snake genus *Bothrops* (Viperidae families) (http://portal.saude.gov.br/portal/saude/).

Accidents with bothropics are characterized by a strong action locally represented by pain,

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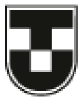

edema, hemorrhage and necrosis, followed by changes in blood clotting (CAMEY et al., 2002; GUTIÉRREZ et al., 2005; GUTIÉRREZ et al., 2007; OLIVEIRA et al., 2009; BALDO et al., 2010). Consequently, *Bothrops* venom is intensely investigated for the presence of molecules with the potential to cause specific physiological alterations in the hemostatic system and blood clotting. Among the various biologically active substances that make up the bothropic venom the metalloproteinases (SVMPs) and serine proteinases (SVSPs) are the proteases that have more interaction with the coagulation cascade and alterations in the hemostatic system. Therefore, these proteases are intensively studied to elucidate their action—a prerequisite for understanding the pathophysiology of envenomations—and ultimately exploiting their capacity for developing new antithrombotic drugs (GUTIÉRREZ et al., 2005; KOH et al., 2006; SANCHEZ et al., 2007; RAFAEL et al., 2008; JIA et al., 2009).

In this work, we purify a new thrombin-like enzyme from snake venom of Bothrops leucurus and seek to show the action of this protease with the blood clotting cascade, as well as investigating its toxicity its systemic action. Initially about 170mg of the crude venom of *B. leucurus* were diluted in 2.0 mL of 50 mM ammonium bicarbonate pH 7.8 centrifuged at 10.000xg for 10 minutes and applied to ion exchange resins CM-Sepharore fast flow. In this first step we obtained fractions called CM1 to CM7 (figure 1A).

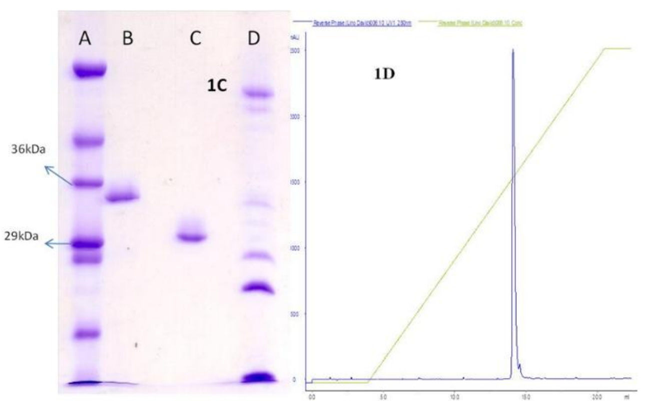

Figure 1: Purification of TL-Leuc: (1A) Chromatography of the crude venom Bothrops leucurus on CM-Sepharore fast flow, eluted with ammonium bicarbonate buffer (AMBIC) 50mM, pH 7.8, setting up a gradient with convex AMBIC 50-500mM from the fraction 50. The fractions were collected in a flow of 2.0 mL/fraction and 20mL/h. (1B) CM6 fraction was rechromatographed in HiPrep Sephacryl S-300, eluted with buffer AMBIC 50mM, pH 7.8. The fractions were coleted in a flow of 2.0mL/fraction and 12mL/h. (1C) SDS-PAGE (12%) with agents denaturants. Lanes: (A) molecular weight markers (with β-mercaptoethanol); (B) 10 μg of TL-Leuc analyzed under reducing conditions to show subunit molecular weight. (C) 10 μg of TL-Leuc analyzed under non-reducing conditions to show the native oligomeric form; (D) 10μg of B. leucurus crude venom. (1D) Analysis of TL-Leuc by RP-HPLC using a C4 column of 2.0x2.5cm (GE Health Care), equilibrated with solvent A (0.1% trifluoroacetic acid and 5% acetonitrile) and eluted with a concentration gradient of solvent B (80% acetonitrile, 0.1% trifluoroacetic acid) from 0 to 100% at a flow rate of

Revista Biociências - Universidade de Taubaté - v.32 - n.1 - p. 18-36, 2026 - ISSN: 14157411
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0,5 mL/min at room temperature.

Figura 1: Purificação de TL-Leuc: (1A) Cromatografia do veneno bruto Bothrops leucurus em fluxo rápido de CM-Sepharore, eluído com tampão de bicarbonato de amônio (AMBIC) 50mM, pH 7,8, estabelecendo um gradiente com AMBIC convexo 50-500mM a partir da fração 50. As frações foram coletadas em um fluxo de 2,0 mL/fração e 20 mL/h. (1B) A fração de CM6 foi recromatografada em HiPrep Sephacryl S-300, eluída com tampão AMBIC 50mM, pH 7,8. As frações foram coletadas em um fluxo de 2,0 mL/fração e 12 mL/h (1C) SDS-PAGE (12%) com agentes desnaturantes. Linhas: (A) marcadores de peso molecular (com β-mercaptoetanol); (B) 10 µg de TL-Leuc analisados em condições redutoras para mostrar o peso molecular da subunidade. (C) 10 µg de TL-Leuc analisado em condições não redutoras para mostrar a forma oligomérica nativa; (D) 10µg de veneno bruto de B. leucurus. (1D) Análise de TL-Leuc por RP-HPLC utilizando uma coluna C4 de 2,0x2,5 cm (GE Health Care), equilibrada com o solvente A (0,1% de ácido trifluoroacético e 5% de acetonitrilo) e eluída com um gradiente de concentração do solvente B (80% de acetonitrilo, 0,1% de ácido trifluoroacético) de 0 a 100% a uma taxa de fluxo de 0,5 mL/min em temperatura ambiente.

CM1 and CM6 fractions showed higher coagulant activity on bovine plasma and bovine fibrinogen. The CM1 fraction showed many protein bands on SDS-PAGE, while the CM6 fraction showed high coagulation activity and few contaminants. After lyophilized, CM6 was rechromatographed on HiPrep Sephacryl S-300 and resolved into two new fractions (figure 1B). The first fraction, named TL-Leuc, was analyzed by reverse-phase HPLC chromatography and showed high purity on SDS-PAGE (figures 1C and 1D). TL-Leuc showed a high in vitro coagulant activity upon bovine plasma and bovine fibrinogen solution (table 1). Serine proteases with thrombin-like activity demonstrate a high plasma clotting capacity through the conversion of fibrinogen to fibrin. However, since they are typically unable to activate Factor XIII to Factor XIIIa, the resulting fibrin clot is unstable (weak).This weak fibrin clot is easily removed by the action of endogenous body itself (CASTRO et al., 2004).

TL-Leuc was analyzed in mass spectrometry (MALDI TOF-TOF), and revealed a molecular mass of 30710.25 Da and when alkylated and reduced, the mass was at 32109.69Da (figure 2). The difference between the masses reveals that this enzyme has 12 cysteine residues. SVSPs present mass of 26-67 kDa, with a variation of the portion of carbohydrates that compose them. These enzymes also generally have 12 cysteines residues (VILCA-QUISPE et al., 2010; SAJEVIC et al., 2011). Still using mass spectrometry, a sample of 300µg TL-Leuc was cleaved by trypsin and the spectrum was analyzed using the software program Matrix Science (Mascot Search Results) and revealed a score signification (Protein scores greater than 73 are significant ($p<0.05$)) showed similarity to Thrombin-like enzyme batroxobin (GI|114837) (figure 3A). For confirmation and more information about the amino acid composition of the enzyme peptides 854 Da (figure 3B), 1416 Da

(figure 3C), 2680 Da (figure 3D), 2366 Da (figure 3E) were subjected to MS/MS fragmentation.

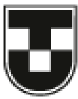
Table 1: Coagulant specific activity and recovery protein of crude venom from *Bothrops leucurus* and fractions: activity on bovine plasma and bovine fibrinogen solution, performed with 10µg of crude venom/CM6 fractions; and 5µg of TL-Leuc.

Tabela 1: Atividade específica do coagulante e proteína de recuperação do veneno bruto de Bothrops leucurus e frações: atividade em plasma bovino e solução de fibrinogênio bovino, realizada com 10 µg de veneno bruto/frações CM6; e 5 µg de TL-Leuc.

	Clotting activity on bovine plasma (seconds)	Standad deviation (σ); n= 5	Clotting activity on fibrinogen (seconds)	Standad deviation (σ); n= 5	Protein mass	Recovery
Crude venom *B. leucurus*	37.5	0.034	33.1s	0.040	170	100
CM6	18.1	0.018	17.6s	0.017	18.9	11.2
TL-leuc	6.2	0.005	6.0s	0.003	2.4	1.41

The peptides obtained by MS \ MS indicate identity or extensive homology with other enzymes in snake venoms. The peptides that showed significant score, were applied in a program BLAST bioinformatics (www.ebi.ac.uk/Tools/blastall/) and showed similarity to many other serine proteases from snake venoms, including: Batroxobin from *Bothrops atrox*, 54% (GI: 114837), *Lachesis stenophrys*, 88% (GI: 123883734), *Bothrops jararaca*, 95% (GI: 13959657), *Bothrops jararacussu*, 81% (GI: 123895619), *Trimeresurus stejnegeni*, 85% (GI: 82239734), *Bothrops asper*, 85% (GI: 123883733), *Trimeresurus flaviviridis*, 91% (GI: 13959616), *Bothrops insularis*, 86% (GI: 82244284), Gyroxin from *Crotalus durissus terrificus*, 91% (GI: 82124461).

The peptide fragments obtained by MALDI-TOF and the Edman degradation method were analyzed in the protein database (https://www.ncbi.nlm.nih.gov/tools/cobalt/cobalt.cgi). The alignment demonstrated in figure 4 revealed that the enzyme is similar to other serine proteases from snake venom. Such as: Thrombin-like enzyme bothrombin from *B. jararaca* (P81661.1); snake venom serine protease HS112 from *B. jararaca* (Q5W960.1); snake venom serine protease BITS01A from *B. insularis* (Q8QG86.1); snake venom serine protease homolog HS120 from *B. jararaca* (Q5W958.1); snake venom serine protease BthaTL



from *B. alternatus* (Q6IWF1.1); Thrombin-like enzyme BjussuSP-1 from *B. jararacuçu* (Q2PQJ3.1).

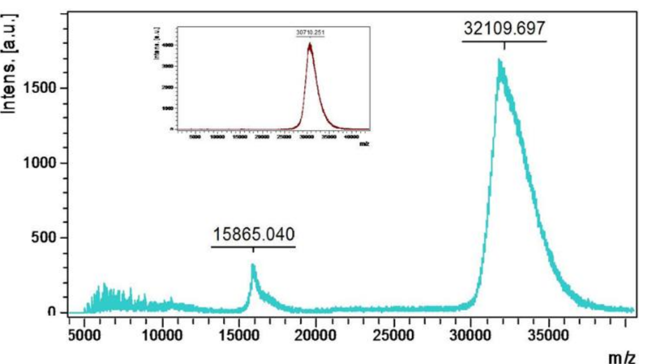
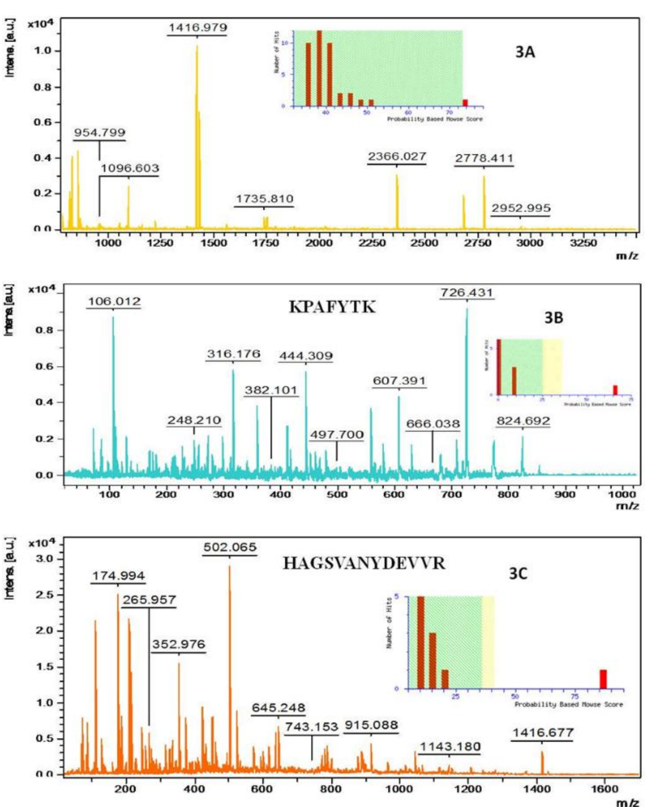
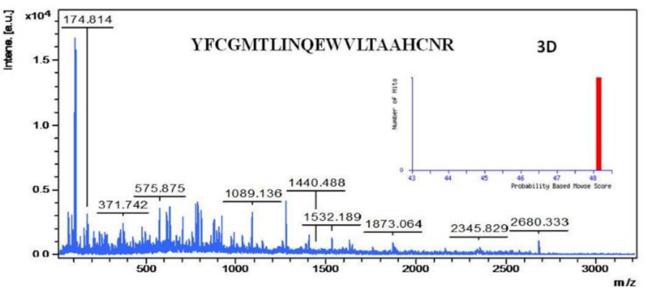


Figure 2: Molecular weight of TL-Leuc determined by matrix-assisted laser desorption ionization-time-of-flight (MALDI- TOF) mass spectrometry (Autoflex II, Bruker Daltonics, Germany).

Figura 2: Peso molecular do TL-Leuc determinado por espectrometria de massa com dessorção laser assistida por matriz - tempo de voo (MALDI- TOF) (Autoflex II, Bruker Daltonics, Alemanha).

The fragments also aligned with the sequence of Leucurobin (MAGALHÃES et al., 2007) using the program EMBOSS-Pairwise Alignment Algorithms (http://www.ebi.ac.uk/Tools/emboss/align/), which shows differences between many amino acids (figure 5). Showing this is a new serine protease from snake venom of *B. leucurus*.





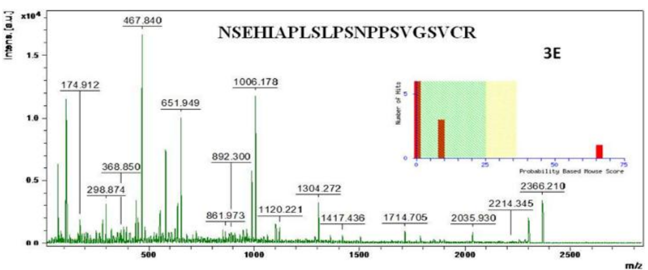


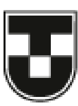
Figure 3: (3A) Spectrum of peptides (PMF: peptide mass fingerprinting) of TL-Leuc after enzyme digestion with trypsin in MALDI TOF/TOF. Revealed a score signification (Protein scores greater than 73 are significant (p<0.05)). (3B) Fragmentation of the

peak 854.47Da in MALDI TOF/TOF (MS/MS fragmentation); (Individual ions scores > 31 indicate identity or extensive homology ($p<0.05$). (3C) Fragmentation of the peak 1416.68Da; Individual ions scores > 41 indicate identity or extensive homology ($p<0.05$); (3D) Fragmentation of the peak 2680.25Da; Individual ions scores > 35 indicate identity or extensive homology ($p<0.05$); (3E) Fragmentation of the peak 2366.18Da; Individual ions scores > 36 indicate identity or extensive homology ($p<0.05$).

Figura 3: (3A) Espectro de peptídeos (PMF: impressão digital em massa de peptídeos) de TL-Leuc após digestão enzimática com tripsina em MALDI TOF/TOF. Revelada uma significância do escore (os escores proteicos maiores que 73 são significativos (p<0,05)). (3B) Fragmentação do pico 854,47Da no MALDI TOF/TOF (fragmentação MS/MS); (Escores de íons individuais > 31 indicam identidade ou homologia extensa (p<0,05). (3C) Fragmentação do pico 1416,68Da; Escores de íons individuais > 41 indicam identidade ou homologia extensa (p<0,05); (3D) Fragmentação do pico 2680,25Da; Escores de íons individuais > 35 indicam identidade ou homologia extensa (p<0,05); (3E) Fragmentação do pico 2366,18Da; Escores de íons individuais > 36 indicam identidade ou homologia extensa (p<0,05).

The TLEs from snake venoms can be classified according to ability to cleave fibrinogen as: The venonbins A (who are acting on fibrinopeptide A); venonbins B (who are acting on the fibrinopeptide B) or venonbins A / B (which cleaves both) (COSTA et al., 2010). TL-Leuc was tested on solution fibrinogen. When 5µg of TL-Leuc were incubated with fibrinogen at different time intervals, found out that only showed the action on the chain Aα of fibrinogen (figure 6A). The same happened when incubated 10µg for a time of two hours chains Bβ and γ not were degraded (figure 6C). Thus we can classify TL-Leuc as an alpha-fibrinogenase. The action on fibrinogen was also evaluated in the presence of protease inhibitors. TL-Leuc was inhibited by Benzamidine and aprotinin, and did not change its action in the presence of EDTA and 1,10-phenanthroline (figure 6D), which showed that this is a serine protease. Still regarding the activity on fibrinogen, TL-Leuc lost its proteolytic capacity when pre-incubated with β-mercaptoethanol, demonstrating the importance of disulfide bonds in the stability of tertiary structure of the enzyme (figure 6D).

Blood clotting is a complex sequence of chemical reactions that result in the formation of a fibrin clot. It is an important part of hemostasis in which the damaged blood vessel wall is covered with a fibrin clot to stop bleeding and help repair damaged tissue. Change in blood clotting time is due to deficiency of coagulation cascade factors. There have been identified several proteins in snake venoms that interfere with coagulation, such as components anticoagulant or procoagulant (MARSH, WILLIAMS, 2005; SANCHEZ, SWENSON, 2007; SAJEVIC et al., 2011).



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Query_122654  1         VIGGDECDINEHPFLAFMYYS-----------------PVFCY-LPWVLTHLGKHAGSVANTDEVVRSHYKGATCP  58
P81661.1      1         VIGGDECDINEHPFLAFMYYSpQYFCGMTLINQEWVLTAAHCD-KTYMRIYLGIHTRSVANDDEVIRYPKEKFICP  75
Q5W960.1      1     [24]VIGGDECDINEHRFLAFLYAG-GYYCGGTLINQEWVLSAAHCD-KRIIRIYLGMHTRSVPNDDEEIRYPKEKFICP  98
Q8QG86.1      1     [24]VVGGDECDINEHPFLAFLYSH-GYFCGLTLINQEWVLTAAHCD-RRFMRIYLGIHARSVANDDEVIRYPKEKFICP  98
Q5W958.1      1     [24]VIGGDECNINEHPFLAFLYTG-WIFCSGTLINKEWVLTVKQCNnRRPMRIYLGMHTRSVPNDDEEIRYPKEMFICP  99
Q6IWF1.1      1         VIGGDECDINEHRFLAFLYPG-RFFCSGTLINQEWVLTVAHCD-TISMRIYLGLHTRSVPNDDEEIRYPMEKFKCP  74
Q2PQJ3.1      1         VLGGDECDINEHPFLAFLYSH-GYFCGLTLINQEWVVTAAHCD-STNFQMQLGVHSKKVLNEDEQTRNPKEKFICP  74

Query_122654  59   NKN--------------------SEHIAPLSLPSNPPSVGSVCRIMGWGAITTSEDTYPDVPHCANINLFNNTVCREAYN  118
P81661.1      76   NKKKNVITDKDIMLIRLNRPVKNSTHIAPISLPSNPPSVGSVCRIMGWGAITTSEDTYPDVPHCANINLFNNTVCREAYN  155
Q5W960.1      99   NKKKNVITHKDIMLIRLNRPVKNSEHIAPLSLPSNPPSVGSVCRIMGWGSITTPDETSPNVPHCANINLFNNTVCREAYN  178
Q8QG86.1      99   NKNMSDEKDKDIMLIRLNRPVKNSTHIAPISLPSNPPSVGSVCRVMGWGSITIPNDTYPDVPHCANINLVNDTVCRGAYK  178
Q5W958.1      100  NKKKN-----DIMLIRLNRPVNNSEHIAPLSLPSNPPSVGSVCRIMGWGTITPSKATYPDVPHCANINLFNYTVCRGAHA  174
Q6IWF1.1      75   NRKRSYIKDKDIMLIRLNRPVNDSPHIAPLSLPSNPPSVGSVCHVMGWGTTSPSKATYPDVPHCANINLVNDTMCHGAYN  154
Q2PQJ3.1      75   NKNMSEVLDKDIMLIKLDKPISNSKHIAPLSLPSNPPSVGSVCRIMGWGSITIPNETYPDVPYCANINLVDYEVCQGAYN  154

Query_122654  119  GLPA----------KTLIDTC-----GPLIC--------------CKEMQKPAFYTKVFDYLPWIQSIIAGLKTATTG-  167
P81661.1      156  GLPA--KTLCAGVLQGGIDTCGGDSGGPLICNGQFQGILSWGSDPCAEPRKPAFYTKVFDYLPWIQSIIAGNKTATCPP  232
Q5W960.1      179  GLPA--KTLCAGVLQGGIDTCGGDSGGPLICNGQFQGILSWGGIPCAQPRKPAFYTKVFDYLPWIQSIIAGNKTATCPP  255
Q8QG86.1      179  RFPAKsRTLCAGVLQGGKDTCVGDSGGPLICNGTFQGIVSWGGKVCARPRKPALYTKVFDYLPWIQSIIAGNKTATCPP  257
Q5W958.1      175  GLPVTsRKLCAGVLEGGIDTCSADSGGPLICNGQLQGIVSWRGGSCAQPHKPGLYTKVFDYLPWIQSIIAGSTTATCPP  253
Q6IWF1.1      155  GLPVTsRKFCAGVLQGGIDTCVGDSGGPLICNGQFQGIVSWGGKVCARLPRPALYTKVFEYLPWIQSIIAGNTTATCPL  233
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Figure 4: Multiple sequence alignments of SVSP. Query 122654 stands for TL-Leuc; (P81661.1) stands for Thrombin-like enzyme bothrombin from *Bothrops jararaca*; (Q5W960.1) stands for Snake venom serine protease HS112 from *B. jararaca*; (Q8QG86.1) stand for Snake venom serine protease BITS01A from *Bothrops insularis*; (Q5W958.1) stand for Snake venom serine protease homolog HS120 from *B. jararaca*; (Q6IWF1.1) stand for Snake venom serine protease BthaTL from *Bothrops alternatus*; (Q2PQJ3.1) stand for Thrombin-like enzyme BjussuSP-1 from *Bothrops jararacuçu*.

Figura 4: Múltiplos alinhamentos sequenciais de SVSP. Query 122654 significa TL-Leuc; (P81661.1) significa a enzima semelhante à trombina botrombina de Bothrops jararaca; (Q5W960.1) significa protease de serina venenosa de cobra HS112 de B. jararaca; (Q8QG86.1) significa protease de serina de veneno de cobra BITS01A de Bothrops insularis; (Q5W958.1) significa Serin venom protease homolog HS120 de B. jararaca; (Q6IWF1.1) significa Snake venom serine protease BthaTL de Bothrops alternatus; (Q2PQJ3.1) significa enzima semelhante à trombina BjussuSP-1 de Bothrops jararacuçu.

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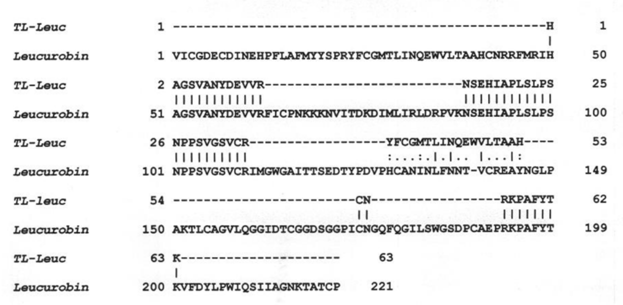


Figure 5: Alignment of the TL-Leuc with with the sequence of Leucurobin (Magalhães et al., 2007) using the program EMBOSS-Pairwise Alignment Algorithms (http://www.ebi.ac.uk/Tools/emboss/align/).

Figura 5: Alinhamento do TL-Leuc com a sequência de Leucurobin (Magalhães et al., 2007) utilizando o programa EMBOSS-Pairwise Alignment Algorithms (http://www.ebi.ac.uk/Tools/emboss/align/).

The administration of $20 \mu \text{g}$ of TL-Leuc resulted in a significant reduction in plasma fibrinogen and rapid defibrinogenation, demonstrating its potency in inducing an incoagulable state in mice (figure 6B). Moreover, this enzyme was able to interfere with the prothrombin time (PT) and partial thromboplastin time (aPTT), suggesting that this enzyme can alters both intrinsic and extrinsic pathways of coagulation cascade. The animals that received doses of 20 µg of TL-Leuc showed a prolongation in prothrombin time (> 120s). While animals that received saline the plasma clots with 22.3 seconds. Changes in the intrinsic system, was also observed using the activated partial thromboplastin time (aPTT), which showed a clotting time of plasma obtained from control animals 9.03seconds of the animals inoculated with 20µg of TL-Leuc > 240s (all experiments performed n = 3). Therefore, this enzyme exhibits thrombin-like activity by directly targeting the blood coagulation cascade, leading to prolonged clotting times and a marked decrease in plasma fibrinogen concentration.

Thrombin-like and fibrinogenolytic enzymes target plasma fibrinogen, leading to its depletion and consequently reducing blood viscosity and coagulation capacity. These results are indications of a possible clinical application of the thrombin-like enzyme TL-Leuc in pathological disorders involving blood clotting (EBLE, 2010; SAJEVIC et al., 2011).

TL-Leuc was tested on various chromogenic substrates showed an activity and the substrates S-2238 (for thrombin-like), S-2266 (for glandular kalikrein) and S-2302 (for plasma kalikrein), demonstrated a thrombin-like and kallikrein-like activities (figure 7A). Once kallikrein cleaves high molecular weight kininogen (HMWK) and releases peptides vasodilators (KOH and KINI, 2012), the action of kallikrein-like of TL-Leuc is a focus of study seeking a clinical application for this enzyme. However, TL-Leuc was incapable to cleave the substrates S-2222 (for factor Xa) and S-2251 (for plasmin). In confirmation this enzyme is devoid of action on fibrin. It was strongly inhibited by PMSF and did not alter its action in the presence of EDTA when assayed on the substrate S-2238 (figure 7B). This inhibition was also showed for other thrombin-

like (VILCA-QUISPE et al., 2010).

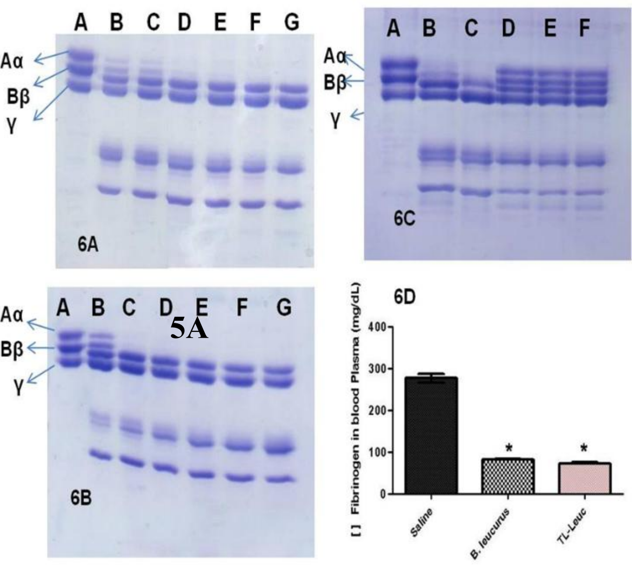


Figure 6: Proteolytic activity of TL-Leuc upon fibrinogen and Quantification of fibrinogen in blood plasma (6A) SDS-PAGE at 12% of bovine fibrinogen degradation. Samples of 50 μL fibrinogen (1,5mg/mL) were incubated with TL-Leuc (5μg) at different intervals of time, Lane A: Fibrinogen control, Lanes B-G: Fibrinogen incubated with TL-Leuc by 5, 10, 15, 30, 60 and 120 min, respectively. (6B) SDS-PAGE to 12% of bovine fibrinogen, Lane A: Fibrin control; B-G: solution of bovine fibrinogen incubated with dose of 1μg, 2μg, 4μg, 6μg, 8μg and 10μg TL-Leuc 2 hours, respectively. (6C) SDS-PAGE to 12% of proteolytic activity upon bovine fibrinogen, Lane A: Fibrin control; B-G: 5μg of TL-Leuc pre-incubated for one hour with protease inhibitors 1,10-phenanthroline, EDTA, β-mercaptoethanol, aprotinin and benzamidine, respectively (all 10mM). (6D) Plasma fibrinogen determination after i.p. injections of crude venom or TL-Leuc (10 and 10μg/50μL Saline) respectively. Control animals received only Saline. The plasmatic fibrinogen concentration was determinate by Hemostat Fibrinogen Commercial kit. Bars represent the mean ±SD (n=3). Statistical significance related to control Saline ($P<0.005$ Test t Student*).

Figura 6: Atividade proteolítica de TL-Leuc sobre o fibrinogênio e quantificação do fibrinogênio no plasma sanguíneo (6A) SDS-PAGE a 12% da degradação do fibrinogênio bovino. Amostras de 50 μL de fibrinogênio (1,5mg/mL) foram incubadas com TL-Leuc (5μg) em diferentes intervalos de tempo, Faixa A: controle de fibrinogênio, Faixas B-G: fibrinogênio incubado com TL-Leuc por 5, 10, 15, 30, 60 e 120 minutos, respectivamente. (6B) SDS-PAGE para 12% de fibrinogênio bovino, Lane A: controle de fibrina; B-G: solução de fibrinogênio bovino incubada com doses de 1μg, 2μg, 4μg, 6μg, 8μg e 10μg de TL-Leuc 2 horas, respectivamente. (6C) SDS-PAGE a 12% da atividade proteolítica sobre fibrinogênio bovino, Lane A: controle de fibrina; B-G: 5μg de TL-Leuc pré-incubado por uma hora com inibidores de protease 1,10-fenantrolina, EDTA, β-mercaptoetanol, aprotinina e benzamidina, respectivamente (todos 10mM). (6D) Determinação de fibrinogênio plasmático após injeções i.p. de veneno bruto ou TL-Leuc (10 e 10μg/50μL Salina), respectivamente. Os animais de controle receberam apenas solução salina. A concentração plasmática de fibrinogênio foi determinada pelo kit comercial Hemostat Fibrinogen. As barras representam a média da DP (n=3). Significância estatística relacionada ao controle de solução salina ($P<0,005$ Teste t student).*

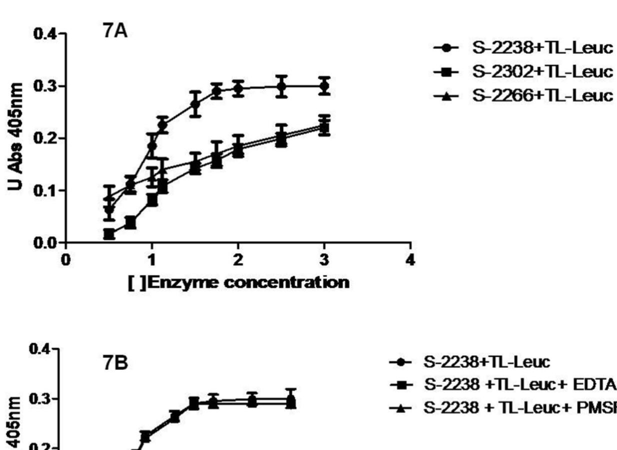


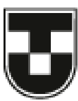
Figure 7: Activity on chromogenic substrates (7A) Different concentration of the TL-Leuc pre incubated with 150µL the solution of each substrate by 20 minutes at 37 ° C. (7B) Activity different concentration of the TL-Leuc (Pre-incubated by 60 minutes with solution 10mM of the EDTA and PMSF) upon substrate S-2238. Performed reading at 405nm in a microplat reader (BioTeK – Elx50) (n=3).

Figura 7: Atividade em substratos cromogênicos (7A) Concentração diferente do TL-Leuc pré-incubado com 150µL da solução de cada substrato por 20 minutos a 37 °C. (7B) Atividade concentração diferente do TL-Leuc (pré-incubado 60 minutos com solução 10mM de EDTA e PMSF) sobre substrato S-2238. Leitura realizada a 405nm em leitor de microplacas (BioTeK - Elx50) (n=3).

The toxins of snake venoms cause severe damage, such as edema, hemorrhage and necrosis. These damages are mainly from the synergistic action of enzymes metalloproteinases, serine proteases and phospholipases A2 (CAMEY et al., 2002; GUTIÉRREZ et al., 2005; GAY et al., 2009; OLIVEIRA et al., 2009). Tl-Leuc was devoid of hemorrhagic action with dose of up to 100µg, when injected intradermally in mice (figure 8). It was also devoid of significant myotoxic activity measured by creatine kinase levels (CK) (data not shown). The damage caused by the crude venom of *B. leucurus* and enzyme TL-Leuc, also was analyzed by histopathology of gastrocnemius muscle of mice (figure 9). The photomicrographs showed that 10µg of crude venom *B. leucurus* provoked local tissue damage evidenced by the degeneration of muscle fibers, proeminente hemorrhagic action and leukocyte infiltration, which demonstrated the existence of many potent toxins in the crude venom. of TL-Leuc apparently induced low myotoxicity evidenced by leukocyte infiltration and necrosis in some localized regions.

The muscle is the main region of the body affected by the bites of snakes and with these results we can propose that TL-leuc not contribute significantly to the toxicity envenonation caused by snake of *B. leucurus*.

In conclusion, TL-Leuc is a new thrombin-like with fibrinogenolytic and kallicrein like activities isolated from snake venom of *B. leucurus*. This enzyme provokes systemic alterations by its action on blood coagulation by degrading fibrinogen. Many reports suggest therapeutic application of toxins isolated from snake venoms (KOH et al., 2006; EBLE, 2010; VILCA-QUISPE et al., 2010; SAJEVIC et al., 2011; KOH et al., 2012).



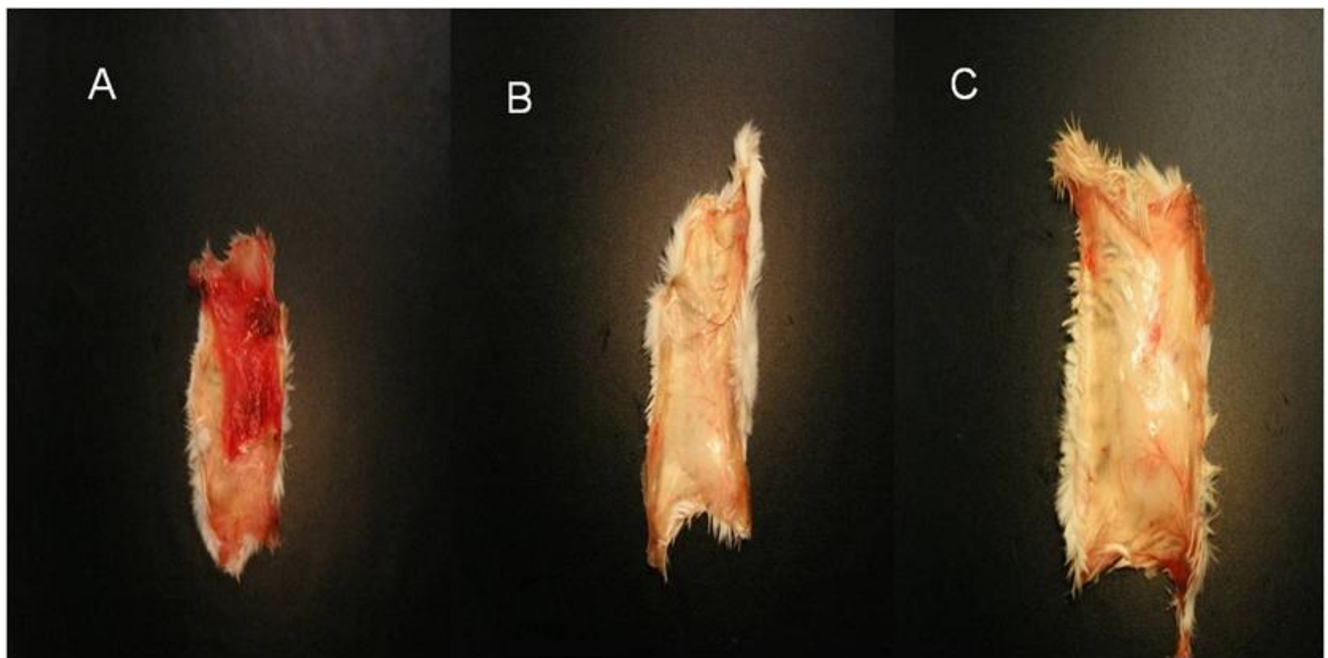


Figure 8: Hemorrhagic activity induced by the crude venom of *Bothrops leucurus* (A) 10µg of crude venom *B. leucurus*; (B and C) 50µg end 100 µg of TL-Leuc, respectively.

Figura 8: Atividade hemorrágica induzida pelo veneno bruto de Bothrops leucurus (A) 10 µg do veneno bruto de B. leucurus; (B e C) 50 µg e 100 µg de TL-Leuc, respectivamente.

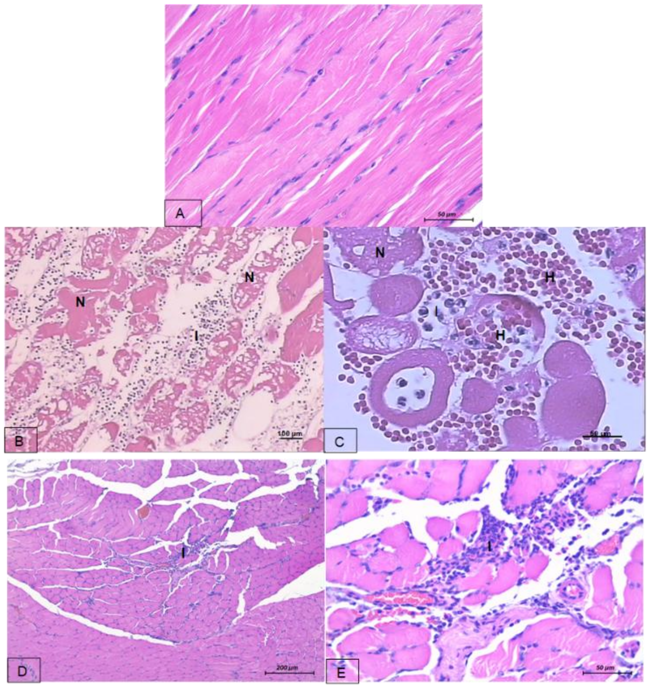
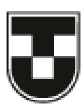


Figure 9: Light micrographs of sections of mouse gastrocnemius muscle 24 h after i.m. injection of 10 µg of crude venom *Bothrops leucurus* and 50 µg of TL-Leuc /50 µL saline, respectively, stained with hematoxylin-eosin. (A) Saline alone: normal integer fibers are seen (B-C) 10µg of crude venon *B. leucurus*: (N) necrosis evidenced by the degeneration of muscle fibers; (I) leukocyte infiltration; (H) Hemorrhage; (D-E) 50µg of TL-Leuc: apparently low evidenced of leukocyte infiltration (I).

Figura 9: Micrografias luminosas de seções do músculo gastrocnemio do rato 24 h após a injeção por IM de 10 µg de veneno bruto Bothrops leucurus e 50 µg de solução salina TL-Leuc/50 µL, respectivamente, coradas com hematoxilina-eosina. (A) Apenas solução salina: fibras inteiras normais são



observadas (B-C) 10 µg de venon bruto de B. leucurus: (N) necrose evidenciada pela degeneração das fibras musculares; (I) infiltração de leucócitos; (H) hemorragia; (D-E) 50 µg de TL-Leuc: aparentemente baixa evidência de infiltração de leucócitos (I).

Thus the preliminary data of the TL-Leuc we led us to propose a possible therapeutic application in disorders related to blood coagulation.

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