Research Article

Biochemistry of the Thrombin-Like TLBpic and Its Purification from *Bothrops pictus* “Jergon de la Costa” (Reptilia: Viperidae)

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The venom of snakes is composed of a heterogeneous mixture of simple and complex substances, with inflammation and hyperalgesia being the first symptom caused by the action of *Bothrops* venom, generating processes such as leukocyte infiltration, hemorrhage, and the intravascular formation of thrombi. Within the simple substances, we have free amino acids, peptides, nucleotides, carbohydrates, lipids, and biogenic amines (organic molecules) as well as cations and anions (inorganic constituents). Of the ions, we can highlight calcium, which is an important cofactor of some proteolytic enzymes as well as phospholipases A₂. And magnesium and zinc are important cofactors of venom metalloproteases. Complex substances are related to proteins and enzymes. Studies related to the total venom of snake present in several organic substances act as pain mediators and are called biogenic amines, such as bradykinin, histamine, 4-hydroxytryptamine, *N*-methyl-5-hydroxytryptamine, *N'*-*N'*-dimethyl-5-hydroxytryptamine, and serotonin. In the present study, a fraction with serinoprotease and coagulant activity has been purified on fibrinogen, called TLBpic, using a cationic ion exchange chromatographic system coupled to an HPLC system. The main characteristic of our protocol is the speed, and the high recovery of the fraction with optimal terms gave result of evidence in the SDS-PAGE gel. The ESI (electrospray ionisation), corresponding to the electrophoresis of proteins in polyacrylamide gels and to their denaturing solubilization in the presence of the SDS ionic detergent, uniting the proteins, breaking hydrophobic interactions, showing a molecular mass of ~30 kDa, demonstrating high molecular homogeneity that exists in this family of proteins, is a soft ionization method, in which the samples were ionized by the addition or removal of a proton, with very little extra energy to cause fragmentation of the produced ions. Samples with molecular masses greater than 1200 Da originate multicharged ions ($M + nH$) $n+$ in the positive ionization mode; this methodology guarantees that the purified material has a high degree of purity.

1. Introduction

Fifty years ago, when the first serpent venom protease was purified, several researchers have been reporting the purification of this kind of enzyme, finding in the biochemistry the relevant information in the understanding of certain metabolic regulations and the specific inhibition of fibrinogen coagulation and the ability to degrade to TLBm [1]. The venom of snakes causes processes such as hemorrhages that

can lead to severe tissue destruction [2]. The hypotension, the incoagulability of the blood, with the consumption of the fibrinogen, and the signal of increase in breathing are simultaneous systemic symptoms. Depending on the amount of poison injected, the kidney may be compromised with irreversible results [3]. The proteases of snake venom act in the conversion of fibrinogen [4], in factors I and X of the blood coagulation cascade, as well as in enzymes that activate the coagulation protein factors, being probably the most

important inducers of damage and symptoms caused by *Bothrops* poison such as thrombin that induces the direct coagulation of fibrinogen and prothrombin activators and factor X of metalloproteinases [5].

Within the total poison are several organic substances called biogenic amines, such as bradykinin, histamine, 4-hydroxytryptamine, *N*-methyl-5-hydroxytryptamine, *N'*-*N'*-dimethyl-5-hydroxytryptamine, and serotonin, which would act as pain mediators caused by the venom of snakes [6]. Snake venom possesses several enzymes [7], and at least 26 types of enzymes have already been described, many of which are hydrolytic in nature. Of this total, 12 are commonly found in varying proportions in the different groups of snakes, and the rest is distributed in a timely manner.

There are complex substances referred to as proteins and enzymes similar to the thrombin of other snakes that show coagulant activities [5]. And simple compounds are referred to as organic molecules and inorganic constituents are referred to as cations and anions, highlighting calcium within the ions, being an important cofactor of some proteolytic enzymes as well as phospholipases A₂ and magnesium and zinc that are also important cofactors of metalloproteinases of poison [8]. Citrate is found in high concentrations in poisons of certain snakes, and experimentally, it was shown that citrate acts as an inhibitor of several enzymes, such as nucleotidases, esterases, proteases, and phospholipases A₂ [9] noting that poisons are not individual compounds but a complex mixture of proteins and their activity varies between each species of snake [10]; in this case, it would act as a cofactor of endogenous neutralization of the snake against the present enzymes of the venom. In the present investigation, a fraction with serinoprotease-coagulant activity has been purified on fibrinogen, a fraction denominated TLBpic, using a cationic ion exchange chromatographic step coupled to an HPLC system. This methodology guarantees that the purified material has a high degree of purity. The main feature of our protocol is the speed and high recovery of the fraction, which can be translated into optimal cost-benefit terms.

2. Materials and Methods

For the purification of *Bothrops pictus* serinoprotease, a protein-pack SP 5 PW (0.78 × 8 cm) column (Waters Inc.) was used. The chromatographic system used was the APPS LC650E (Waters), equipped with a pump for four solvent channels. The column and chromatographic system were previously equilibrated with initial buffer (0.05 M ammonium bicarbonate, pH 7.6). The elution of the samples was performed using a linear gradient of ammonium bicarbonate concentration (1.0 M, pH 7.9). The chromatographic run was monitored at 280 nm, and the fractions were collected in a Foxy 200 automatic collector. The samples obtained were grouped and then lyophilized and stored at -20°C. Polyacrylamide gel electrophoresis was performed according to the methodology described by Laemmli [11]. The polyacrylamide plates were made discontinuously, showing a gel of concentration of 5% and a running gel of 12.5%. The plates were prepared using a stock solution of

acrylamide (30% T, 0.8% C). The concentration gel was made using a 0.5 M Tris-HCl buffer of pH = 6.8 and running gel using a 1 M Tris-HCl buffer of pH = 8.8. Both gels were added enough SDS at 20%, to reach a final concentration of 0.5% (v/v). The SDS-PAGE was carried out in a double system of miniplates SE 250 Mighty Small II (Hoeffer Scientific Instruments). Samples and molecular mass markers were dissolved in the sample buffer (0.075 M Tris-HCl, pH = 6.8, 10% glycerol, 4% SDS, 0.001% bromophenol). The electrophoretic run was performed at 30 mA. The gels were colored with 0.05% Coomassie Blue solution at 37°C, and the excess dye was removed with 7% acetic acid. For the analysis of the molecular homogeneity degree of the samples, PAGE-SDS Tricine gel electrophoresis was also used, according to the method described by Schagger and Von Jagow [12] through a discontinuous system in a 10% run gel. The synthetic substrate BAPNA was used to measure the amidase activity of proteolytic enzymes such as trypsin, chymotrypsin, factor Xa, human plasma kallikrein, thrombin, and human plasmin. The amount of 20 µl of the sample was placed in an incubation medium containing 1000 µl of substrate solution previously dissolved in dimethyl sulfoxide (Fisher Scientific Company USA) as stock solution, to be used in the proportion of 1/10 (10 µl) in the 10 mM Tris-HCl buffer, 10 mM·CaCl₂, 100 mM·NaCl, pH = 7.8, in addition to containing 250 µL more buffer, for a final volume of 1850 µL. After 30 minutes, the reaction was blocked with 30% acetic acid (500 µL). The absorbance changes were read at 405 nm.

2.1. Automatic Amino Acid Analysis. The amino acid analysis was performed in an automatic amino acid analyzer Pico-Tag (Waters System), and the phenylthiocarbamyl-amino acid product was identified through the HPLC equipment, product derived from the derivatization with phenylisothiocyanate of the amino acids obtained by acid hydrolysis with reference to the activity of substrates hydrolyzed by poisons to proteinase activity the influence of metal ions [13]. This form of chromophores can be detected in concentrations of 1 pmol according to the methodology described [14].

2.2. Solubilization and Acid Hydrolysis. 300 µg of the sample were dissolved in 50 µl of buffer B (66% acetonitrile + 0.025% TFA). It was centrifuged at 200 G for 15 minutes, and the supernatant was divided into two aliquots of 25 µl each and subsequently dried with the help of the work station (dryer-lyophilizer coupled with a barometer) up to 65 millitorr. Subsequently, for the acid hydrolysis, the samples were placed (in duplicate) in 25 µl each, in a reaction bottle in their own tubes; then, 100 µl of a 6N-HCl solution in addition to 1 mg/ml phenol was placed in the bottom of the reaction bottle to prevent the formation of chlorotyrosine. A vacuum of approximately 1-2 torr was then made until the start of HCl bubbling. Created the vacuum was allowed to enter nitrogen (SS ultra pure) for 5 seconds. This stage was repeated three times. Next, the lid of the reaction bottle was removed, and the temperature was raised to 105°C for hydrolysis for 24 hours.

After this period, the reaction tube was placed in vacuum up to 65 millitorr to dry the hydrolyzed sample.

2.3. Derivatization. To the sample was added 20 μl of a mixture of methanol, water, and triethylamine in the ratio of 2 : 2 : 1 (v/v). Each tube was agitated and centrifuged at 250 g for 15 minutes and placed in vacuum up to 65 millitorr for its evaporation. This procedure removed the salts and solvents adsorbed by the amino acids. A fresh derivatization solution was prepared with methanol, triethylamine, water, and phenylisothiocyanate (PITC) in the ratio of 7 : 1 : 1 : 1 (v/v), and 20 μl was added to each reaction tube; these were left at room temperature environment for 30 minutes. After derivatization, the sample was dried under vacuum until 50 millitorr to complete the removal of the entire PITC. The sample was dissolved in 50 μl of a 0.4 mM sodium phosphate buffer solution containing 5% acetonitrile. The amino acid-PITC analysis was performed on HPLC using a μ -Bondapak C-18 reverse-phase column with a linear gradient of 0 to 100% and 66% acetonitrile for 21 minutes. The identification of each amino acid was made in relation to a run pattern of amino acids-PITC [15]. For the quantification of cysteine and methionine, the samples were previously oxidized with performic acid. The hydrolysis and derivatization of the oxidized samples of these amino acids followed the described methodology.

2.4. Ion Exchange HPLC Analysis Method. For the purification of *Bothrops pictus* serinoprotease, a protein-pack SP 5PW (0.78 $\times$ 8 cm) column (Waters Inc.) was used. The chromatographic system used was the APPS LC650E (Waters), equipped with a pump for four solvent channels. The column and chromatographic system were previously equilibrated with initial buffer (0.05 M ammonium bicarbonate, pH 7.6). The elution of the samples was carried out using a linear gradient of concentration of ammonium bicarbonate (1.0 M, pH 7.9). The chromatographic run was monitored at 280 nm, and the fractions were collected in a Foxy 200 automatic collector. The samples obtained were grouped and then lyophilized and stored at -20°C .

2.5. Electrophoresis on SDS-PAGE. Polyacrylamide gel electrophoresis was performed according to the methodology described [16]. The polyacrylamide plates were made discontinuously showing a gel of 5% concentration and a running gel of 12.5%. The plates were prepared using a stock solution of acrylamide (30% T, 0.8% C). The concentration gel was made using a 0.5 M Tris-HCl buffer of pH = 6.8 and running gel using a 1 M Tris-HCl buffer of pH = 8.8. Both gels were added enough SDS at 20%, to reach a final concentration of 0.5% (v/v). The SDS-PAGE was carried out in a double system of miniplates SE 250 Mighty Small II (Hoeffer Scientific Instruments). Samples and molecular mass markers were dissolved in the sample buffer (0.075 M Tris-HCl, pH = 6.8, 10% glycerol, 4% SDS, 0.001% bromophenol). The electrophoretic run was performed at 30 mA. The gels were colored with 0.05% Coomassie Blue solution at 37°C , and the excess dye was removed with 7% acetic acid. For the

analysis of the molecular homogeneity degree of the samples, PAGE-SDS Tricine gel electrophoresis was also used, according to the method described by [17], through a discontinuous system in a 10% run gel.

2.6. Determination of Proteolytic Activity. The synthetic substrate BApNA was used to measure the amidase activity of proteolytic enzymes such as trypsin, chymotrypsin, factor Xa, human plasma kallikrein, thrombin, and human plasmin. 20 μl of the sample was placed in an incubation medium containing 1000 μl of substrate solution previously dissolved in dimethyl sulfoxide (Fisher Scientific Company, USA) as a stock solution, to be used in the ratio of 1/10 (10 μl) in 10 mM Tris-HCl buffer, 10 mM $\cdot\text{CaCl}_2$, and 100 mM $\cdot\text{NaCl}$, pH = 7.8, in addition to containing 250 $\text{m}\mu\text{l}$ more of buffer, for a final volume of 1850 μl . After 30 minutes, the reaction was blocked with 30% acetic acid (500 μl). The absorbance changes were read at 405 nm.

2.7. Determination of Fibrinogenolytic Activity. It was determined by the method described in [18] and the genetic regulation of fibrinogen synthesis and its assembly of clot properties and its variability [19], adapted to our experimental condition. Bovine fibrinogen (fraction I from bovine plasma) was used with 40–60% sodium citrate from Armor Pharmaceutical Company (USA). The concentration of the fibrinogen solution used was 2 mg/ml. The purified TLBpic fibrinogenolytic fraction was diluted in 0.1 M Tris-HCl buffer (pH 7.4, 20 μg). The experimental run time to achieve the identification of fibrin formation was up to 10 minutes at 37°C . Subsequently, the temperature decreased, and the samples were applied to a 12% SDS-PAGE electrophoresis gel together with a standard solution of bovine fibrinogen, in order to show the type of chain that was able to hydrolyze. According to the results, it was shown that it is an alpha-type thrombin-like. The fibrinogenolytic activity (coagulant) was determined by measuring the coagulation time at the first fibrin network formation signal after the addition of 20 μl of sample on the fibrinogen substrate previously incubated with 10 mM $\cdot\text{CaCl}_2$ at 37°C for 10 minutes (m/v, depending on coagulable proteins) in 0.1 M Tris-HCl buffer, pH 7.4. The time of the linear relationship between the intervals of the coagulation times and the respective protein concentrations in the fibrin network formation reaction was after 2 minutes. The number of coagulation units is defined as the amount of enzyme that coagulates 1.0 ml. of a standard solution of as fibrinogen in 15 seconds at 37°C .

2.8. Determination of the Molecular Mass of the Thrombin-Like by Mass Spectrometry Electrospray (ESI). Referring to ionization sources by mass spectrometry, identification by relevant electron ionization was established [19]. An aliquot of the sample (4.5 μl) was injected into a C18 reverse-phase UPLC column (nanoAcquity UPLC, Waters) coupled to a nanoelectrospray tandem mass spectrometry system on a Q-ToF Ultima API mass spectrometer (MicroMass/Waters) at a flow of 600 nl/min. The gradient was 0–50% acetonitrile

in 0.1% formic acid for 45 minutes. The instrument was operated in the continuous MS mode, and the data acquisition was of 27000–31000 m/z , at a speed of 1 second with a delay of 0.1 second. The spectra were accumulated in approximately 200 scans, and the multiple loaded data produced by the mass spectrometer on the m/z scale were converted to the mass scale (molecular mass) using the Maximum Entropy software, provided with the MassLynx 4.1 package. The parameters of processing were a strip of mass of exit 400–1800 Da with a resolution of 0.1 Da/channel, and the proportion of minimum intensity between successive peaks is 20% (left and right). The deconvolved spectrum was then smoothed (2×3 channels, Savitzky–Golay smooth), and mass values were obtained using 80% peak and minimum peak length as the average height of four channels.

2.9. Determination of the N-Terminal Sequence. To determine the amino acid sequence of the amino terminal portion of the sample obtained in the RP-HPLC, 40 nmol of the reduced sample was applied with 1 M dithiothreitol (DTT) in an automatic sequencer model 477 of the Applied Biosystems (California, USES).

2.9.1. Edman's Automatic Degradation. This method was used for the determination of the N-terminal portion of the sample. The automatic sequencer uses the degradation technique [21], to remove and identify amino acids from the N-terminal portion of a polypeptide. After the activation of a filter composed of paper and fiberglass, the protein was covalently bound to this support and then placed in the reaction chamber. At the end of each degradative cycle, the N-terminal amino acid is removed from the side chain in the form of a more stable derivative, such as phenylthiohydantoin of the corresponding amino acid (PTH). The PTH-amino acid is transferred to a high efficiency liquid chromatography system, where the identification is made by comparison with the chromatography of a PTH-amino acid standard. Reagents and buffer were transferred to the reaction chamber and converted by automatic control through a microprocessor, allowing automatic sequences with a high sensitivity between 10 and 500 picomoles of protein or peptides. The reagents used were as follows: R1: 5% phenylisothiocyanate (PITC) in *n*-heptane; R2: 12.5% trimethylamine (TMA) in water; R3: trifluoroacetic acid (TFA), with 0.002% dithiothreitol (DTT); R4: 25% TFA in water with 0.01% DTT; R5: acetonitrile, with 0.001% DTT; S1: *n*-heptane; S2: ethylacetate; S3: 1-chlorobutane; and S4: 20% acetonitrile in water.

3. Results and Discussion

3.1. Chromatographic Fractionation of *Bothrops pictus* Total Venom by Column Ion Exchange. As a first chromatographic step for the purification of thrombin-like fraction from the total venom of *Bothrops pictus* (coastal pallet). From total snake venom, 100 mg was dissolved in 25 ml of a buffer solution of 0.2 M ammonium bicarbonate buffer pH 8.0, and

FIGURE 1: Ion exchange (cationic) chromatography.

an SP 5PW ion exchange column (1.8×100 cm) was inserted. The various fractions that elute were subjected to a test to determine enzymatic activity using synthetic and chromogenic substrate $N\alpha$ -p-tosyl-L-arginine methyl ester (TAME), with the enzymatic activity detected in peak 12, which was named as TLBpic, as can be seen in Figure 1.

An SP 5 PW (Waters) column coupled to an HPLC system of *Bothrops pictus* total venom is observed. The run was performed with bicarbonate ammonium buffer (0.1 M and pH 8.0), at a constant flow of 0.3 ml/min.

3.2. Electrophoresis in SDS-Page. To determine the molecular mass of the enzyme, polyacrylamide gel electrophoresis was performed at a concentration of 12.5% in the presence of SDS (SDS-PAGE). Figure 2 shows the results of the polyacrylamide gel electrophoresis of the TLBpic fraction from *Bothrops pictus* venom, where the said fraction has a molecular mass of ~ 30 kDa, which demonstrates the high molecular homogeneity that exists in this family of proteins.

Electrophoresis is observed in 12.5% polyacrylamide gel (m/v) MM (molecular mass markers: rabbit muscle phosphorylase B (97 kDa), bovine albumin (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (30 kDa), soybean trypsin inhibitor (20 kDa), and bovine milk lactalbumin (14 kDa) and TLBpic fraction with thrombin-like activity from the venom of *Bothrops pictus*, colored with Coomassie blue.

3.3. Fibrinogenolytic Activity of the TLBpic Fraction. The TLBpic fraction exhibited a fibrinogenolytic activity against bovine fibrinogen, revealing that it is an alpha-type thrombin-like, being able to degrade the fibrinopeptide alpha of the fibrinogen molecule, evidenced in Figure 3.

Related to the TLBpic fraction using bovine fibrinogen, the concentration of fibrinogen solution used was 2 mg/ml (0.2%). The purified TLBpic fibrinogenolytic fraction was diluted in 0.1 M Tris-HCl buffer, pH 7.4 (20 μ g). The maximum waiting time for the formation of the fibrin network was 2 minutes at 37°C. A SDS-PAGE gel (12%) was used for 20 μ g of sample.

3.4. Measurement of Proteolytic Activity. The proteolytic activity was determined according to the methodology described in the previous chapter. The amidase hydrolysis was measured on the synthetic substrate DL-BAPNA ($N\alpha$ -

FIGURE 2: Electrophoresis analysis.

FIGURE 3: Fibrinogenolytic activity.

FIGURE 4: Determination of proteolytic activity.

benzoyl-DL-arginine p-nitroanilide) (Sigma Chemical Company USA), 54 mM for the dosing of the fractions (20 μ g), in Tris-HCl buffer, 10 mM, 10 mM-CaCl₂, and 100 mM-NaCl, pH = 7.8. The absorbance changes were read at 405 nm. It was observed that the TLBpic fraction from ion exchange

TABLE 1: Amino acid composition of the TLBpic fraction.

Amino acids	Molar reason (<i>n</i> moles residue/mol of protein)	Molecular weight calculated
Asp	33	3798.6
Glu	19	2453.5
Ser	23	2003.1
Gly	30	1712.1
His	10	1371.6
Arg	9	1405.9
Thr	14	1555.7
Ala	24	1777.3
Pro	23	2234.0
Tyr	11	1255.7
Val	14	2284.1
Met	3	393.6
Cys	12	1237.7
Ile	18	1857.1
Leu	15	1697.6
Phe	9	1324.7
Lys	12	1538.3
Total	270	29834.3

(cationic) chromatography was the one that exhibited a significant proteolytic activity, as shown in Figure 4.

54 mM of chromogenic substrate DL-BAPNA (N α -benzoyl-DL-arginine p-nitroanilide) was used per 20 μ g of sample. The absorbance changes were read at 405 nm.

3.5. Amino Acid Composition of the TLBpic Fraction. The serinoprotease TLBpic was subjected to acid hydrolysis (6N-HCl) and derivatization to obtain the global amino acid composition, as shown in Table 1. The overall composition analysis showed a high concentration of amino acids such as Asp and Glu, a high concentration of hydrophobic amino acids such as Pro, Val, and Tyr, and a low content of basic amino acids like His and Arg. The residue that corresponds to Trp due to the method (Pico-Tag) was not determined.

3.6. Determination of the Intact Mass of the Thrombin-Like TLBpic by Electrospray Mass Spectrometry (ESI). The intact mass of the thrombin-like protein purified from *Bothrops pictus* venom was determined by mass spectrometry electrospray (ESI) (Figure 5(a)).

The results show that the molecular mass of the protein is 29854.78 Da, confirming the degree of molecular homogeneity of thrombin.

3.7. N-Terminal Sequence of the Thrombin-Like TLBpic and Sequential Homology. The N-terminal sequence of the thrombin-like protein TLBpic from the venom of *Bothrops pictus* was VIGGDECNINEHRFLVFMYYSP. Thrombin-like sequential homology analysis of TLBpic was performed through the home page <http://www.pubmed.com/switprot/bblast>. Figure 6 shows the N-terminal sequence of the serinoprotease with fibrinogenolytic activity TLBpic, compared with a data bank of the proteins with already determined sequences. The TLBpic fraction showed a high homology in relation to other serinoproteases (~90%).

FIGURE 5: Mass spectrum intact. Differences of the intact mass spectrum analysis in deconvolved conditions (a) and in the spectrum showing the multicarga (b) ions of the thrombin-like protein TLBpic, which are observed in this spectrometry methodology, through electrospray mass (ESI).

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TLPic  VIGGDECNINEHRFLVFMYYSP...
114837  .....D....P..A.....
14285806.....D....P..A.....
461512  .....ALV.....
13959660.....AL.T.....
13959630.....AL.N.....

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FIGURE 6: Analysis of sequential homology. Sequential homology for the thrombin-like protein TLBpic from *Bothrops pictus* venom is shown (114837 (batroxobin, Itoh et al. [22]); 14285806 (bothrombin, Nishida et al. [23]); 461512 (Ancrod, Kisiel et al. [24]); 13959660 (pallabin, Fan et al. [25]); 13959630 (calobin, Hahn et al. [9])).

4. Discussion

The hypotension, the incoagulability of the blood with the consumption of fibrinogen, and the signal of increase of the breathing are simultaneous systemic symptoms. Snake venom proteases act on the conversion of fibrinogen [4]. These are probably the most important inducers caused by *Bothrops* poison. These proteases have fibrinogenolytic activity, acting in blood coagulation, fibrinolysis, platelet aggregation, and in the processes of thrombosis and hemostasis [26]. Commonly, the purification of these serinoproteases with thrombin-like activity has been carried out using various conventional chromatographic processes, such as molecular exclusion or ion exchange with a time demand [27]. Studies on thrombin-like activity in *Bothrops atrox*, which is eluted in volumes after the main protein fraction of Brazilian origin, have been reported in [28]. According to studies, amino acid composition is determined by partially calculating nonactive chromatographic analysis and resulting in free thromboplastin activity, differing its different conditions to the activity of purity; there are no studies of purification and characterization of thrombin-like fractions from the total venom of *Bothrops pictus*, which is

observed to purify a serinoprotease with fibrinogenolytic activity called thrombin-like TLBpic from total poison of *Bothrops pictus*, so the study places the bases in the identification of a fraction with thrombin-like activity, obtained in a combination of conventional liquid chromatography and high efficiency, resulting in the purification with high molecular homogeneity, as evidenced in the SDS-PAGE gel. ESI (electrospray ionisation) is a soft ionization method, in which the samples are ionized by the addition or removal of a proton, with very little extra energy to cause fragmentation of the produced ions. Samples with molecular masses greater than 1200 Da originate multicharged ions $(M + nH)^{n+}$ in the positive ionization mode. Figure 5(a) shows the spectrum of the deconvolved protein obtained with the help of the Maximum Entropy software, thus confirming the accuracy of the thrombin-like mass of TLBpic. Figure 5(b) shows the molecular mass spectrum of the ionized form of the thrombin-like TLBpic, by mass spectrometry ESI (positive ionization). Each peak represents the protein molecule carrying a different number of charges (protons) [29]. Some of the studies carried out have been referred to a fraction called Atroxin, which is a proteolytic enzyme that acts on fibrinogen but is not inhibited by PMSF (phenyl methyl sulphonyl fluoride) or iodoacetate, as are serinoproteases with thrombin activity, like being able to hydrolyze the alpha chain of the fibrinogen molecule, thus being a metalloproteinase would be involved in the reduction of fibrinogen levels and then exert an anticoagulant function [30]. 15 fractions were obtained, respectively (Figure 1). Fraction 12 recorded proteolytic activity against BApNA and fibrinogenolytic activity (coagulant) and was termed TLBpic. Serinoproteases have a relative molecular mass of 25 to 35 kDa [31], as we can verify for flavoxobin (23.5 kDa) from *Trimeresurus flavoviridis* [32] and Calobin (34 kDa) of *Gloydus ussuriensis* [33]. The increase in the molecular mass of the serine protease thrombin-like snake venom is due to the fact of forming aggregates

because of its glycoprotein nature [34]. In the biochemical characterization of the proteolytic activity of the thrombin-like fraction, TLBpic BApNA was used as a substrate, which has been characterized as a serinoprotease I of the family of proteases. In this way, it shows similarity in the proteolytic behavior with the two thrombin-like TLE1 and TLE2 of *Bothrops atrox* from Brazil purified by Pirkle [4], as well as the thrombin-like BaIII-4 from *Bothrops atrox* of Peruvian origin [35]. The fibrinogenolytic (coagulant) activity of snake venom has been evaluated using bovine fibrinogen [36–39]. There are some groups of snake venoms that have factors with the ability to coagulate fibrinogen depending on the type of fibrinopeptide released from enzymatic catalysis [40]. Thus, one group releases fibrinopeptides A, another group releases both fibrinopeptides A and B, and a third group releases fibrinopeptides B, most of which are those that release fibrinopeptide [4, 39, 41]. These enzymes are serinoproteases that, unlike thrombin, only release a single type of fibrinopeptide and all are single-chain glycoproteins with an approximate molecular weight of 30 to 35 kDa [2]. These enzymes have an esterase activity with small basic substrates of esters such as benzoyl-L-arginine ethyl ester. The coagulant and esterase activity of these enzymes are inhibited simultaneously by serinoprotease inhibitors such as diisopropyl fluorophosphate (DIPF). The thrombin-like TLBpic was able to act on the fibrinogen with the corresponding clot formation, and the type of fibrinopeptide released was determined according to the electrophoresis in SDS-PAGE (Figure 3). Among the serinoproteases with purified thrombin-like activity are many that have a pI (isoelectric point) acid between 3 and 4 [27, 31]; due to this, the purification and characterization of acid serinoproteases is currently intense. It is not uncommon to find serinoproteases from different sources that have a different pI and similar biological activity. However, the assumption that serinoprotease activity depends on the isoelectric point is inappropriate. According to the determination in the composition of amino acids according to Table 1, there was a high number of acidic amino acids such as Asp and Glu, as well as a high content of hydrophobic amino acids such as Pro, Val, and Tyr, which was evidenced in the elution of reverse-phase HPLC chromatography, when eluted in 60% buffer B. The hydrophobic interactions are responsible for the molecular arrangement of the protein, once they expose the hydrophilic (polar) amino acids and conserve inside the hydrophobic (apolar) amino acid. The crotalase isolated from *Crotalus adamanteus* [4] records 12 cysteines probably involved in the formation of six disulfide bridges. The primary structures of all these thrombin-like enzymes have been determined [4, 22, 42] based on homology with mammalian serinoproteases. However, the biological activity, such as the knowledge of its conformational structure in the thrombin-like TLBpic, needs to be better elucidated by means of other biological models, such as platelet aggregation and hemolysis, as well as physical-chemical studies such as mass spectrometry, MALDI-tof, nuclear magnetic resonance (NMR), and circular dichroism (DC), among others, and additionally, crystallographic studies to determine its tertiary structure.

5. Conclusions

Through an ion exchange chromatographic step coupled to an HPLC system on an SP 5 PW (high performance liquid chromatography) column, it has been possible to purify a serinoprotease with fibrinogenolytic activity called thrombin-like TLBpic from *Bothrops pictus* total venom. The thrombin-like serinoprotease TLBpic showed a high degree of molecular homogeneity in 12.5% SDS-PAGE gel, with a relative molecular mass of ~30 kDa. The thrombin-like TLBpic exhibited a proteolytic activity against BApNA (N α -benzoyl-DL-arginine p-nitroanilide) and fibrinogenolytic against bovine fibrinogen, revealing that it is a thrombin-like alpha-type because it is able to degrade the alpha fibrinopeptide of the fibrinogen molecule, evidenced in the electrophoresis gel. The TLBpic fraction showed a high number of acidic amino acids such as Asp and Glu, as well as a high concentration of hydrophobic amino acids such as Pro, Val, and Tyr, revealing that it is an acidic protein, suggesting that its behavior in solution is highly stable due to its high degree of compaction. The TLBpic fraction presented in its N-terminal region the consensus sequence of the invariable residues ... VIGGDECNINEHRFLAFMYISP ... similar to that of the serinoproteases compared according to the databases. Serinoproteases with thrombin-like activity show a high sequence homology in the N-terminal region. The purified TLBpic of *Bothrops pictus* reveals a great homology (~90%) with other serinoproteases: batroxobin, bothrombin, Ancrod, and others.

Data Availability

The data used to support the findings of this study are available from the corresponding author upon request.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

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