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Protein Expression and Purification 30 (2003) 32–42

Protein
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Kallikrein-like proteinase from bushmaster snake venom

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Received 11 November 2002, and in revised form 28 January 2003

Abstract

A kallikrein-like proteinase of *Lachesis muta muta* (bushmaster) venom, designated LV-Ka, was purified by gel filtration and anion exchange chromatographies. Physicochemical studies indicated that the purified enzyme is a 33 kDa monomeric glycoprotein, the Mr of which fell to 28 kDa after deglycosylation with PNGase F. Approximately 77% of the protein sequence was determined by sequencing the various fragments derived from digestions with endoproteases. The partial sequence obtained suggests that LV-Ka is of a similar size to other serine proteinases (i.e., approximately 234 amino acid residues). Sequence studies on the NH₂-terminal region of the protein indicate that LV-Ka shares a high degree of sequence homology with the kallikrein-like enzymes EI and EII from *Crotalus atrox*, with crotalase from *Crotalus adamanteus* and significant homology with other serine proteinases from snake venoms and vertebrate serum enzymes. LV-Ka showed kallikrein-like activity, releasing bradikinin from kininogen as evidenced by guinea pig bioassay. In addition, intravenous injection of the proteinase (0.8 µg/g) was shown to lower blood pressure in experimental rats. In vitro, the isolated proteinase was shown to have neither fibrin(ogeno)lytic activity nor coagulant effect. LV-Ka was active upon the kallikrein substrates S-2266 and S-2302 (specific activity = 13.0 and 31.5 U/mg, respectively; crude venom = 0.25 and 6.0 U/mg) but had no proteolytic effect on dimethylcasein and insulin B chain. Its enzymatic activity was inhibited by NPGB and PMSF, indicating that the enzyme is a serine proteinase. Interestingly, one of the other reactions catalyzed by plasma kallikrein, the activation of plasminogen was one of the activities exhibited by LV-Ka.

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Keywords: Kallikrein-like protease; Snake venoms; Bushmaster; Kinin

Viper snake venoms contain a great variety of different proteins that are toxic. These components mediate their toxicity by either stimulating or inhibiting the hemostatic system of human victims or experimental animals, resulting in common clinical complications of blood clotting or uncontrolled hemorrhage [1,2]. This apparent paradox in that the venom acts in vivo as an anticoagulant whereas in vitro it coagulates blood has been attributed to the presence of fibrinogenolytic enzymes in snake venoms [2,3]. Administration of Viperidae venoms in experimental animals usually produces a precipitous fall in systemic blood pressure, terminating in cardiac arrest. Therefore, circulatory shock with internal hem-

orrhage is the frequent cause of death in the case of true viper (Viperinae) or pit viper (Crotalinae) bites [4,5]. Current interest is directed towards some fibrinolytic proteinases including metalloproteases as well as the clotting enzymes and the RGD²-containing peptides be-

² Abbreviations used: S-2266, H-D-Val-Leu-Arg-pNA; S-2302, H-D-Pro-Phe-Arg-pNA; S-2238, H-D-Phe-Pip-Arg-pNA; PMSF, phenylmethanesulfonyl fluoride; TLCK, N- α -p-tosyl-L-lysine chromomethyl ketone; NPGB, p-nitrophenyl-p-guanidinobenzoate; SBTI, soybean trypsin inhibitor; EDTA, ethylenediaminetetraacetic acid; HMW kininogen, high molecular weight kininogen; Bk, bradikinin; RGD, Arg-Gly-Asp; PRP, platelet-rich plasma; TFA, trifluoroacetic acid; LV-PA, plasminogen activator enzyme from *Lachesis muta muta*; LV-Ka, kallikrein enzyme from *L. m. muta*; DL-BAPNA, N- α -benzoyl-L-arginine-p-nitroanilide; pNA, p-nitroanilide; RCM, S-reduced and carboxymethylated; ADP, adenosine diphosphate; PBS, phosphate buffered saline.

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¹ In memoriam.

cause of their potential clinical application in the treatment of vascular thrombotic diseases [4,6,7]. The proteinases so far described are generally classified as serine proteases and metalloproteinases. There is only weak or indirect evidence for the presence of thiol and aspartic proteases in snake venoms. A number of them seem to degrade mammalian tissue proteins at the site of bites in a nonspecific manner to immobilize the victims. Several of these proteinases, however, cleave some of the plasma proteins of the victims in a relatively specific manner to give potent effects, as either activators or inhibitors of their hemostatic and coagulation systems [1–3,8,9]. Some of these enzymes hydrolyze fibrinogen specifically and release fibrinopeptides A or B or both [2], and other enzymes act upon other substrates, e.g., kininogen [10], plasminogen [11–13], and protein C [14]. Based on the sequence comparisons of 40 serine proteinases isolated from snake venoms, a phylogram was constructed in which three clusters of serine proteases were grouped together and were designated as coagulating enzymes, kininogenases, and plasminogen activators [15].

Studies on the venom of the large pit viper *Lachesis muta muta* indicate that it contains a variety of pharmacologically active peptides and proteins with diverse biological activities. These compounds can produce activating or inactivating effects on several of the various interactions of the hemostatic system. They include the metalloproteinases, designated mutalysins I and II, which exhibit direct fibrin(ogeno)lytic activities that are known to inhibit blood coagulation [16,17]. A fibrinogen-clotting enzyme/gyroxin analogue which releases fibrinopeptide A rapidly from human fibrinogen and minor amounts of fibrinopeptide B but was devoid of kallikrein-like activity and did not activate factor XIII was also reported ([18,19], Magalhaes et al., submitted). Recently, another serine proteinase with plasminogen activating activity called LV-PA was isolated and some of its biochemical and pharmacological properties have been investigated [13]. A 27.9-kDa serine proteinase capable of releasing bradikinin from low molecular weight kininogen was also reported from the same *L. muta* venom [20]. However, the mode of action of this proteinase is unclear and details of its structure are as yet unavailable. It has been proposed that the venom kallikreins along with other hypotensive components in the venom participate in the production of the venom shock [5,21]. Therefore, it is deemed important to isolate the active component(s) from bushmaster venom with kinin-releasing (kallikrein-like) activity. In this report, we describe the purification procedure and several of the chemical and enzymatic properties of a kallikrein-like enzyme from *L. m. muta* venom named hereby LV-Ka. Furthermore, the purpose of this report was to detail our studies on the remarkable similarities we have identified between kinin-release and the activation of plasminogen.

Materials and methods

Venom of *L. m. muta* was obtained from the serpentarium of the Ezequiel Dias Foundation, Belo Horizonte, Brazil. Chromogenic substrates S-2266, S-2302, and S-2238 were from KabiVitrum, Stockholm, Sweden. PNGase F was from New England BioLabs, Beverly, MA, USA. Human fibrinogen essentially plasminogen free, plasmin, thrombin, and the peptide *N*-*p*-Tos-Gly-Pro-Lys-pNA were from Sigma Chemical (St Louis, MO, USA). α 2-Macroglobulin of human origin was from Calbiochem (San Diego, CA, USA). All other chemicals were of analytical reagent grade.

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE)

SDS–PAGE without reduction or after reduction with 4% β -mercaptoethanol was performed by the method of Laemmli [22], using 12% gels and stained with Coomassie brilliant blue R-250. Apparent molecular masses were determined from reduced gels, by comparison with a protein calibration mixture (Pharmacia) containing bovine serum albumin (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (30 kDa), and soybean trypsin inhibitor (20.1 kDa).

Deglycosylation of the proteinase

LV-Ka (100 μ g) was dissolved in 90 μ l denaturing buffer (0.5% SDS, 1% (v/v) β -mercaptoethanol). The sample solution was denatured by boiling for 5 min. After addition of 10 μ l reaction buffer (50 mM phosphate, pH 7.2), 10 μ l of 10% NP-40, and 100 U recombinant PNGase F, the sample was incubated at 37 °C for 20 h. The reaction was terminated by boiling for 5 min and PAGE loading buffer was added to the reaction mixture. The molecular mass and the purity of the venom proteinase with or without deglycosylation were analyzed by SDS–PAGE (12% gel).

Purification of native LV-Ka

LV-Ka was purified from *L. m. muta* snake venom by a combination of gel filtration on Sephacryl S-200 and ion-exchange chromatography on DEAE–Sephacryl CL-6B at pH 8.0 and 7.3, respectively. For the first step of purification, 1.6 g crude venom was dissolved in 10 ml of 50 mM ammonium acetate buffer (pH 7.4) containing 0.3 M NaCl and centrifuged at 6000g to remove the insoluble material. The solution (1200 mg protein) was applied to two 2.5 \times 100 cm columns in series packed with Sephacryl S-200 equilibrated and eluted with the same buffer. The flow rate was 8.0 ml/h and 7.0 ml fractions were collected at 4 °C. For all chromatography procedures, protein concentration was monitored by

measuring the absorbance at 280 nm. Amidase activity on the substrate S-2302 was also determined and SDS-PAGE was performed on selected fractions. For the second step, active fractions from the gel filtration were pooled, dialyzed against distilled water and lyophilized. This material (125.3 mg) was applied to a DEAE-Sephacrose CL-6B column (1.0 × 19 cm) equilibrated with 20 mM Hepes buffer (pH 8.0). The column was developed with a linear gradient of NaCl (0–0.3 M). The flow rate was 14 ml/h. For the third step of purification, rechromatography on the DEAE column at pH 7.3 was utilized to remove minor contaminants. Active fractions from the previous step (39.0 mg) were dissolved in 2.0 ml of 20 mM Hepes buffer, pH 7.3, and applied to the same DEAE column, equilibrated with the same buffer. The column was developed with a linear gradient from 0 to 0.3 M NaCl at a flow rate of 14 ml/h. A symmetrical peak, associated with amidolytic activity, was obtained.

Chromogenic assays

The amidolytic activity was measured with a Shimadzu spectrophotometer using 1-cm path cuvettes. Assays were performed in 50 mM Tris-HCl, pH 7.6, containing 0.15 M NaCl and 80 µl peptide *p*-nitroanilides: Val-Leu-Arg-pNA (2 mM), Pro-Phe-Arg-pNA (2 mM), Phe-Pip-Arg-pNA (2 mM), and Tos-Gly-Pro-Lys-pNA (0.2 mM) in a total volume of 1.0 ml. The reactions were initiated by the addition of the enzyme to a final concentration of 20 to 34 nM and incubated at 37 °C. The initial rate of *p*-nitroanilide release was determined and the enzyme activity was expressed as Δ4405 nm/min/mg protein. The increase in absorbance at 405 nm is proportional to the enzymatic activity.

Plasminogen activation

Plasminogen activation was assayed, as described previously [13]: human plasminogen was incubated at a final concentration of 2 µM in 50 mM Hepes buffer, pH 7.6, containing 0.1 M NaCl with the sample tested (1.0 µg). Aliquots were taken at various time intervals and then assayed for plasmin activity formed on Tos-Gly-Pro-Lys-pNA (0.2 mM).

Effects of pH and temperature on the purified enzyme

The stability of the enzyme at various pH values was examined by incubating it (1.5 µg LV-Ka) in various buffers (50 mM Hepes, pH 7.0 and 8.0; 50 mM Tris-HCl, pH 9.0, and 6.0 mM borate, pH 10.0 and 11.0) for 2 h at room temperature. After this time, the enzyme was mixed with the synthetic substrate S-2302 (2 mM) and amidolytic activity was then determined in a spectrophotometer by measuring the absorbances at 405 nm. The thermal stability of the proteinase was examined by

heating it (1.5 µg) in 50 mM Hepes buffer, pH 8.0, at various temperatures (30, 40, 50, 60, 70, and 80 °C) for 15 min. After this time, the samples were cooled in an ice bath and the remaining amidase activity on the synthetic substrate S-2302 (2 mM) was determined.

Inhibition studies

The effects of various protease inhibitors specific for different classes of proteinases were examined. Stock solutions of inhibitors were freshly prepared as follows: NPGb, PMSF, and D-Val-Phe-Lys-Chromomethyl ketone were dissolved in dimethyl sulfoxide, while benzamidine, *p*-aminobenzamidine, SBTI, aprotinin, and TLCK were dissolved in 20 mM Hepes buffer, pH 7.5, containing 5 mM NaCl. LV-Ka (45 nM) was incubated for 30 min at 37 °C in 20 mM Hepes buffer, pH 7.5, containing 5 mM NaCl in the presence or absence of the inhibitors at the indicated concentrations. The initial rate of amidolysis was measured after the addition of 60-µl (2 mM) S-2302. The tubes were incubated for 10 min at 37 °C and the reaction was stopped by the addition of 100 µl of 60% acetic acid. The activity was expressed as percentage of control (LV-Ka alone with no inhibitors).

Determination of partial amino acid sequence of LV-Ka

The purified proteinase (1.5 mg) was reduced and S-carboxymethylated (RCM), as described in [27]. The modified protein was desalted by HPLC on a µ-Bondapak C₁₈ column eluted with a linear gradient of 0–100% acetonitrile in 0.1% TFA at a flow rate of 1 ml/min over a period of 50 min. A sample of the RCM enzyme was digested with the Glu-specific endoproteinase from *Staphylococcus aureus* V₈ (2% w/w enzyme:substrate in 0.1 M ammonium bicarbonate, pH 8.0, for 24 h at 37 °C) and trypsin (2% w/w enzyme:substrate in 0.1 M ammonium bicarbonate buffer, pH 8.0, for 3 h at 37 °C). The peptides produced were separated by HPLC on a µ-Bondapak C₁₈ column using a linear gradient of 0–70% acetonitrile in 0.1% TFA, over 160 min at a flow rate of 1 ml/min. The sequences of the intact native proteinase, RCM-enzyme, and peptides from the digestion with endoprotease were determined by automatic sequencing in an Applied Biosystems Model 477A pulsed liquid-phase sequencer/Model 120A PTH HPLC analyzer system using a standard Edman degradation program.

Fibrin(ogeno)lytic activity studies

The fibrinogenolytic activity was measured by incubating 500 µl fibrinogen (5 mg/ml, essentially plasminogen free) in 25 mM Tris-HCl buffer, pH 7.6, containing 75 mM NaCl with 4.5 µg LV-Ka at 37 °C. At different time intervals (15, 30, 60, and 120 min), 50-µl aliquots of the incubation mixture were withdrawn and mixed with

an equal volume of denaturing solution (8 M urea, 4% β -mercaptoethanol, and 4% SDS). After reduction and denaturation overnight at room temperature, the samples were submitted to SDS-PAGE (10%). The fibrinolytic activity was conducted in the presence or absence of plasminogen. To measure the ability of LV-Ka to dissolve fibrin clot, a fibrin plate lysis assay was conducted in a 24-well plate (Costar, Cambridge, MA), as described previously [13]. Plasmin (Sigma) was used as control.

Fibrinogen clotting assay

Clotting activity was measured by mixing appropriate concentration (20 μ l) of sample tested with 200 μ l human fibrinogen (3 mg/ml) in 0.154 M NaCl at 37 °C. The clotting activity was expressed in NIH thrombin-equivalent units/mg protein [23].

Hypotensive effect

Blood pressure was assayed on male Wistar rats (260–280 g) anesthetized with urethane (140 mg/100 g, intraperitoneally). The femoral vein and femoral artery were isolated and cannulated with polyethylene catheters (PE 50 intramedic tubing, 0.58 inner diameter). For measurement of arterial blood pressure, the femoral artery catheter was attached to a pressure transducer (Codas, Dataq Instruments, USA). The femoral vein catheter was used for infusion of LV-Ka or saline. Once the arterial blood pressure was stabilized, baseline pressure (systolic, diastolic, and mean) measurements were obtained. The sample (220 μ g LV-Ka in saline or saline alone, 100 μ l) was then administered intravenously.

Kinin-releasing activity

Kinin-releasing activity was performed on isolated ileum of male guinea pigs weighing 180–200 g. A strip of ileum approximately 2.5 cm was removed and mounted longitudinally in an organ bath containing Tyrode's solution (10 ml), a mixture of 95% O₂ plus 5% CO₂ was bubbled into the bath. Resting tension was adjusted to a load of 0.3–0.5 g and ileum was allowed to equilibrate for at least 60 min. Isometric contractions were measured with a force-displacement transducer (Grass TF 0.3) on a computer through a data acquisition system (Codas, Dataq Instruments, USA). Bovine kininogen (10 mg/ml) was mixed with LV-Ka (2.4 and 3.8 μ g) in a final volume of 530 μ l of 20 mM Hepes buffer, pH 8.0, and incubated at 37 °C for 10 and 20 min. After that, 200- μ l aliquots were removed and added into the bath to measure the kinin formed. Under these conditions, the amount of kinin released was proportional to the incubation time. The heights of the recorded contractions were compared with those produced by increasing amounts of bradikinin within the linear portion of the

dose–response curve. The ileum was washed and allowed to rest for 10 min between each test dose.

Other biological assays

Hemorrhagic activity assay was evaluated by the method of Kondo et al. [24] and [25] in adult guinea pigs instead of rabbits by the determination of the minimum hemorrhagic dose (MHD).

Platelet aggregation assay. The proteinase LV-Ka was tested for platelet aggregation inhibitory activity using fresh human platelet rich plasma (PRP) according to [13]. A PACKS-4 platelet aggregation chromogenic kinetic system (Helena Laboratories, Beaumont, TX) was used to monitor platelet aggregation. Inhibition of ADP-, epinephrine-, or collagen-induced platelet aggregation was conducted at 37 °C by adding LV-Ka (50–2000 nM final concentration) 3 min, prior to the addition of the agonist (final concentrations: ADP, 20 μ M; epinephrine, 200 μ M; and collagen, 10 μ g/ml).

Proteolytic activity was also determined using dimethylcasein as substrate according to [26].

Enzyme-linked immunoabsorbent assay (ELISA)

We have used the ELISA test with the intention of investigating the cross-reactivity of rabbit serum anti-LV-PA (*Lachesis* venom plasminogen activator) with a related proteinase LV-Ka and with snake venoms of *L. m. muta*, *Bothrops leucurus*, *Bothrops neuwiedi*, and *Bothrops jararacussu*. Microtiter plates (Hemobag Produtos Cirúrgicos, Ribeirão Preto, São Paulo) were coated overnight at 5 °C with 0.5 μ g/ml enzyme or venom samples in 50 mM sodium bicarbonate buffer, pH 9.6 (100 μ l, standard volume). After washing with 0.05% Tween/saline, the wells were blocked with 2% casein in PBS for 1 h at room temperature. Rabbit serum against a serine proteinase LV-PA diluted (1:400 to 1:12800) was added and incubated for 1 h at 37 °C. The plates were then washed and the binding was visualized by incubation with peroxidase-coupled antirabbit IgG (diluted 1:4000) for 1 h at 37 °C and further addition of *o*-phenylene diamine (0.2 mg/ml in citrate buffer, pH 5.2 in the presence of 0.04% hydrogen peroxide). Absorbance was read at 492 nm. The absorbance of preimmune serum (control) was subtracted. All measurements were made in triplicate.

Results

Purification of Lachesis venom kallikrein (LV-Ka)

The progress of purification was followed by determining the following for each fraction: amidolytic activities on DL-BAPNA, S-2302, S-2266, S-2238, and

Tos-Gly-Pro-Lys-pNA, coagulant activity on human fibrinogen, proteolytic activity on dimethylcasein, fibrin (ogeno)lytic activity, hemorrhagic activity, phospholipaseA₂ activity, and effect on the platelet function. S-2302 was used as the substrate to follow the purification of LV-Ka.

Nine peaks (P1, P2, P3, P4, P5, P6, P7, P8, and P9) were obtained when the crude venom (1.6 g) was applied to Sephacryl S-200 column (data not shown, first step). The venom enzymes that released pNA were predominantly associated with peak P5 (tubes 109–124), showing 3.6% of the amidolytic activity, and were separated from coagulant, hemorrhagic, and dimethylcasein hydrolyzing activities. Phospholipase A₂ activity was also predominantly localized in peak P4, and hemorrhagic and proteolytic activities were concentrated in peaks P1 and P6. Inhibition of platelet aggregation induced by ADP was found between P6 and P7 (not shown). SDS-PAGE revealed that the pooled fraction (peak 5) contained the major band at ~33 kDa, plus several contaminants. The pooled fractions were dialyzed against distilled water and

lyophilized. For the second step of purification, a DEAE column at pH 8.0 was employed. Chromatography of peak 5 (125.3 mg) on the DEAE column resulted in four main protein fractions (D, E, F, and G, not shown). Amidolytic activity on S-2302 was predominantly concentrated in peak F. The fractions comprising peak F, which eluted at a NaCl concentration of 0.16 M (tubes 127–136) and contained 4.2% amidolytic activity of the crude venom, were pooled, dialyzed, and lyophilized. PAGE carried out in native conditions revealed, however, the presence of other small contaminants. Therefore, this material (peak F, 39.0 mg) was again applied to a DEAE column at pH 7.3 (third step). A symmetrical protein peak was obtained, which had constant specific amidolytic activity across the peak (not shown). This peak gave a single band on SDS-PAGE under reducing or nonreducing conditions (Fig. 1A, lanes 1 and 2, and Fig. 1B, lane 1) and was called LV-Ka. The purification scheme of LV-Ka is summarized in Table 1. The final step resulted in an overall yield of 11% and the purification factor was 5.3 with respect to amidolytic activity

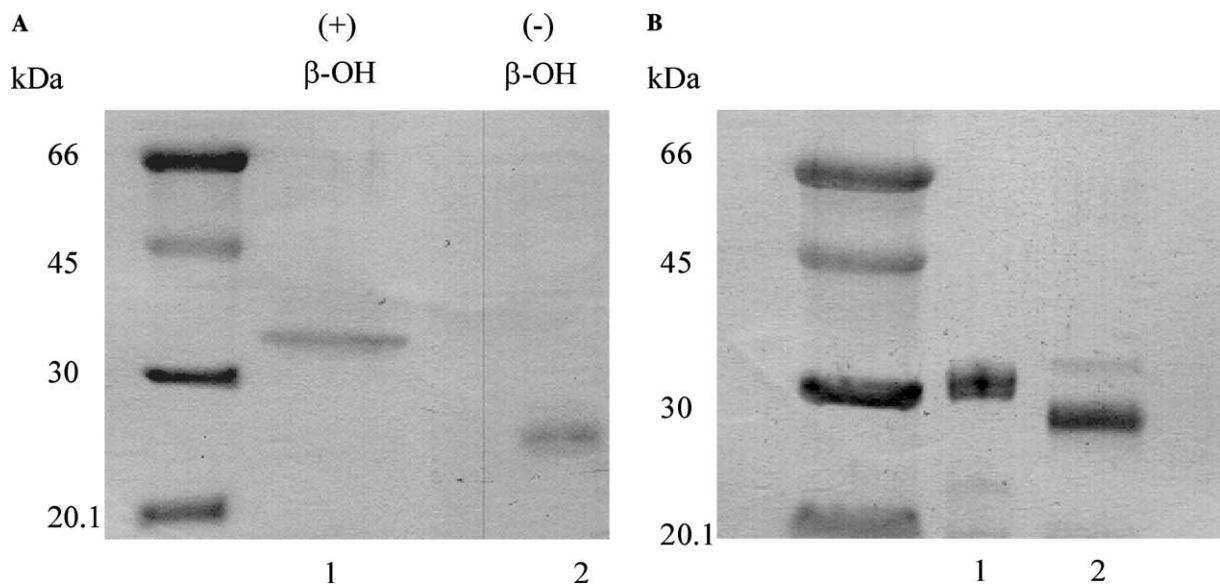


Fig. 1. SDS-PAGE electrophoresis (12% gels) of LV-Ka. (A) Lanes 1 and 2, purified LV-Ka (5 µg) under reducing (β-mercaptoethanol, 4% v/v) and nonreducing conditions, respectively. (B) Lanes 1 and 2, native LV-Ka (5 µg) and after treatment with PNGase F (reducing conditions). The positions of molecular mass markers are indicated at the left.

Table 1
Purification of kallikrein-like enzyme (LV-Ka) from *L. m. muta* venom

Step	Protein		Enzymatic activity		Yield (%)	P.F. ^b
	mg	%	Specific (U/mg) ^a	Total (U)		
Crude venom	1200.0	100	6.0	7200	100	1.0
Sephacryl S-200	125.3	10.4	21.5	2694	37.4	3.6
DEAE-Sepharose CL 6B (pH 8.0)	39.0	3.3	25.0	975	13.5	4.2
DEAE-Sepharose CL 6B (pH 7.3)	25.0	2.1	31.5	788	11.0	5.3

^a One unit of activity was defined as $\Delta 4405$ nm/min. The enzyme activity was expressed as units/mg of protein. The progress of the purification was followed by determining the amidolytic activity on S-2302.

^b P.F., purification factor.

on S-2302. The proteinase represents about 2.1% of total venom proteins.

Physicochemical properties

The molecular masses of the purified LV-Ka were estimated by SDS-PAGE (12%) as 33,000 and 28,000 Da under reducing and nonreducing conditions, respectively (Fig. 1A, lanes 1 and 2). The enzyme migrated as a single band under both conditions, indicating that it is a monomer consisting of a single polypeptide chain. However, following incubation with *N*-Glycosidase F, the proteinase was observed to migrate on SDS-PAGE (under reducing conditions) with an apparent molecular mass of 28,000 (Fig. 1B, lane 2). In addition, the protein band on SDS-PAGE was stained with Schiff's reagent after periodate oxidation (data not shown). These observations indicated that LV-Ka is a glycoprotein, and based on the SDS-PAGE difference the enzyme contains approximately 15% carbohydrate. Furthermore, immunoelectrophoresis of the purified LV-Ka against horse antivenom to *L. m. muta* venom resulted in a single precipitin arc that migrated slightly toward the anode at pH 8.6, suggesting that LV-Ka is an acidic protein (not shown).

pH and heat stability of LV-Ka

As shown in Fig. 2A, LV-Ka exhibited its maximum activity at the temperature range from 30 to 40 °C. Also, the optimal pH for the venom enzyme amidase activity was between 7 and 9 (Fig. 2B).

Effect of protease inhibitors on LV-Ka

Table 2 summarizes the effects of several inhibitors on the S-2302 hydrolyzing activity of LV-Ka. Incubation of

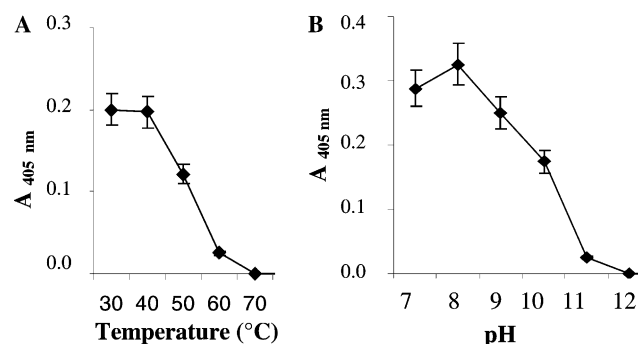


Fig. 2. Effect of temperature and pH on the activity of the purified LV-Ka. (A) The purified enzyme (1.5 μ g) in 50 mM Hepes buffer (pH 8.0) was maintained for 15 min at various temperatures and then quickly cooled in an ice bath. The enzyme activity on S-2302 was then determined: \blacklozenge , LV-Ka. (B) The LV-Ka (1.5 μ g) at various pH values was incubated at room temperature for 2 h. The enzyme activity on S-2302 was then measured: \blacklozenge , LV-Ka. In both cases, the points represent means \pm SE ($n = 4$).

Table 2
Effect of several inhibitors on the amidolytic activity of LV-Ka

Inhibitor	Concentration (mM)	Residual activity (%)
None	—	100 \pm 4.0
NPGB	1	2.0 \pm 0.6
EDTA	5	100 \pm 3.0
PMSF	1	22.0 \pm 1.2
<i>p</i> -Aminobenzamidine	10	8.0 \pm 1.0
Benzamidine	10	52.0 \pm 2.5
SBTI	200 μ g/ml	80.0 \pm 4.0
Aprotinin	11 TIU/ml	46.0 \pm 3.0
D-Val-Phe-Lys-CMK	1	4.0 \pm 0.6
TLCK	1	54.2 \pm 4.0

Note. The purified enzyme (1.5 μ g) was incubated with each compound at the indicated concentrations in 0.940 ml of 20 mM Hepes buffer, pH 7.5, containing 5 mM NaCl at room temperature for 30 min, after which 60- μ l of 2 mM *H*-D-Pro-Phe-Arg-pNA was added and incubated for 10 min at 37 °C. Control was performed for each compound (no LV-Ka). These values are expressed as percentages relative to control and are given as means \pm SD, $n = 4$.

the purified enzyme for 30 min at room temperature with NPGB, a specific reagent for active-site serine residues of serine proteinases, completely abolished its amidolytic activity, indicating that LV-Ka is a serine-type proteinase. The enzyme was also inhibited by PMSF, demonstrating the involvement of a serine residue at the active site for its catalytic effect. The amidolytic activity of LV-Ka was inhibited to some degree by benzamidines; however, SBTI and EDTA (5 mM) had little or no effect.

Enzymatic activity of LV-Ka on protein and peptide substrates

The purified kallikrein-like enzyme possesses amidolytic activity toward several synthetic peptide substrates. Table 3 shows the substrate specificity of the enzyme and it is obvious that S-2302 (substrate for plasma kallikrein) and S-2266 (substrate for glandular kallik-

Table 3
Substrate specificity of LV-Ka, plasmin, and trypsin

Enzyme	Specific activity (U ^a /mg)			
	Substrate			
	S-2302	S-2266	Tos-Gly-Pro-Lys-pNA	S-2238
C.V. ^b	6.0	0.25	0.019	0.625
LV-Ka	31.5	13.0	3.0 (5.2) ^c	3.0
Plasmin	5.25	1.75	23.4	1.625
Trypsin	222.75	47.0	197.75	80.75

^a One unit was defined as ΔA_{405} nm/min. Specific activity was expressed in U/mg.

^b Crude venom. Results are representative of experiments run in triplicate.

^c 5.2 U/mg was obtained after preincubation of LV-Ka with plasminogen (2 μ M) for 30 min before testing the amidase activity.

rein) are the optimal substrates for this enzyme. The specific activities with these substrates were 31.5 and 13.0 U/mg, (5.25- and 52-fold higher than the crude venom), respectively, whereas the hydrolysis with Tos-Gly-Pro-Lys-pNA (substrate for plasmin) and with S-2238 (substrate for thrombin) was relatively low (specific

activity = 3.0 U/mg, respectively, with each substrate). Furthermore, the specific activity with Tos-Gly-Pro-Lys-pNA was increased from 3.0 to 5.2 U/mg after preincubation of LV-Ka (30 nM) with plasminogen (2 μM) for 30 min at 37 °C. In the first 10 min, a 1.73-fold increase of enzyme activity was observed over the

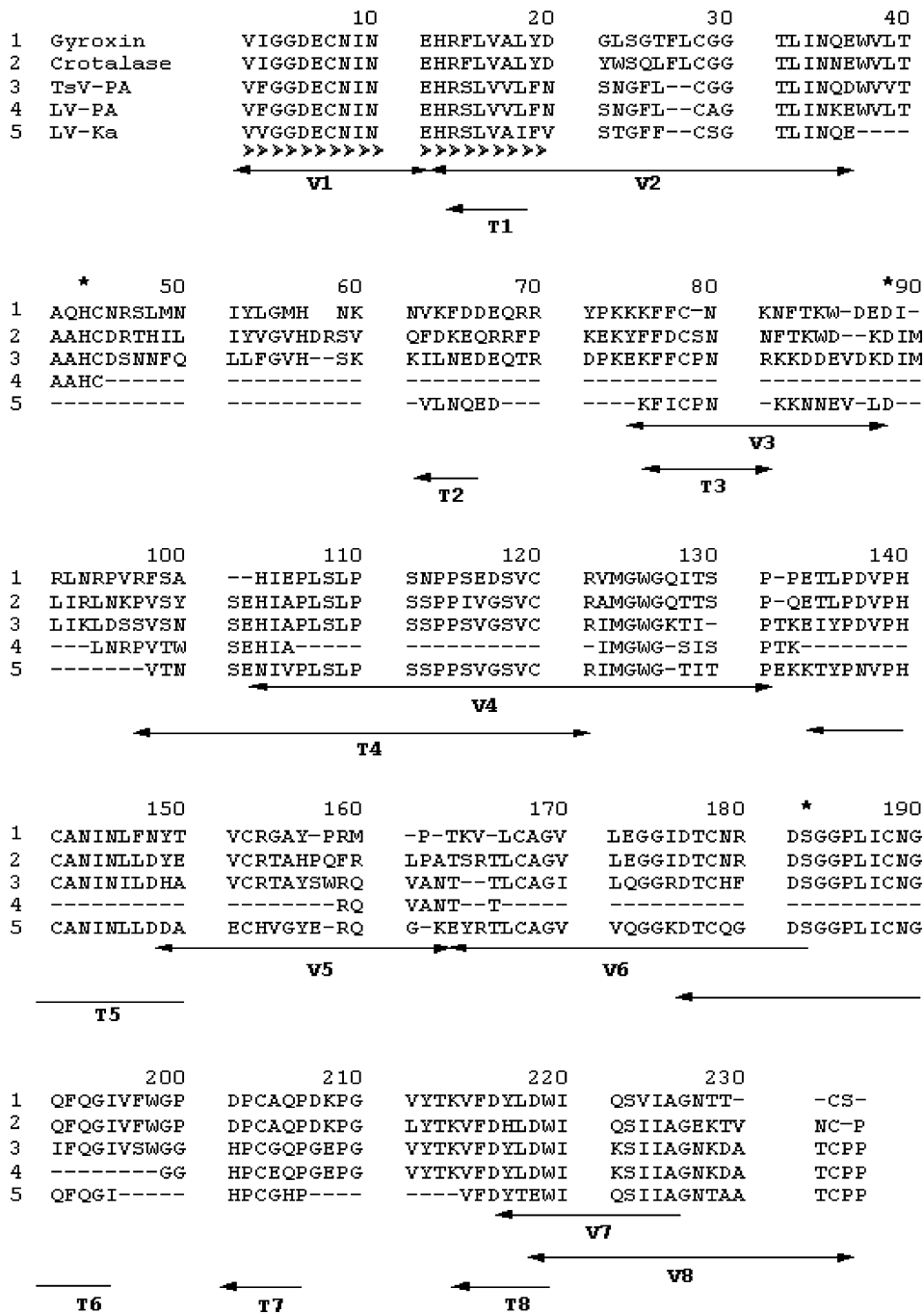


Fig. 3. The amino acid sequence of the kallikrein-like enzyme from the venom of bushmaster snake (*L. m. muta*). Arrows (>) indicate residues determined by automated degradation of the intact S-reduced and carboxymethylated protein. Solid lines indicate fragments derived from enzymatic digestions with trypsin (T) and with Glu-specific endopeptidase from *S. aureus* V₈ (V). Asterisks (*) show catalytic triad residues histidine 43, aspartic acid 88, and serine 182 (anacrod numbering [36]). Alignments of sequences of the clotting enzyme/gyroxin analogue from *L. m. muta* [35], crotalase, defibrinogenating enzyme from *C. adamanteus* [9], TsVPA, plasminogen activator from *T. stejnegeri* venom [11], LV-PA, plasminogen activator from *L. m. muta* venom [13].

zero time control (not shown). For comparative purposes, the amidolytic activity of plasmin (1.0 μg) was also included (Table 3). These results suggest that LV-Ka has a preference for Arg in the P1 position. In addition, proteolytic activities for dimethylcasein and oxidized insulin B-chain were not detected.

Sequence analysis

The N-terminal sequence of the native protein was determined up to the 34th residue. Fragments obtained from the endopeptidase treatments of reduced and carboxymethylated LV-Ka were purified by reverse-phase HPLC. The amino acid sequences of the overlapping fragments were determined; up to 181 amino acid residues, or 77% of LV-Ka, were sequenced (Fig. 3).

Enzyme assays related to blood coagulation factors

LV-Ka did not produce a fibrin clot when incubated with human fibrinogen (essentially plasminogen free, Sigma) at an enzyme/substrate weight ratio of 1:50. Furthermore, SDS-PAGE analysis revealed that incubation of the purified enzyme (1.2 μM) with fibrinogen (2.5 mg/ml, 15.6 μM) at different times (0–120 min) at 37 °C exhibited no fibrinogenolytic effect. The A α -, B β -, and γ -chains of fibrinogen remained intact, even at the end of 120 min (data not shown). In vitro, incubation of LV-Ka (4 μg corresponding to 0.6 μM) with fibrin clot also showed that the enzyme neither dissolved a fibrin clot nor digested any of the chains of fibrin α -, β -, γ - γ chains, and α -polymer when observed on SDS-PAGE (data not shown). The lack of activity toward both native and model substrates indicated that LV-Ka lacks plasmin-like activity. However, when experiments (fibrin plate assay) were conducted in the presence of plasminogen (2 μM) and SBTI in order to prevent the proteolytic effect of the plasmin formed, since the inhibitor

blocks plasmin but not LV-Ka activity (Table 2), traces of fibrinolytic effect were observed at a concentration of 2 μg of LV-Ka compared with streptokinase used as a positive control (not shown).

The effect of the kallikrein-like enzyme on platelet aggregation was also investigated. LV-Ka at a concentration of up to 2 μM did not cause aggregation when incubated with platelet rich plasma. In addition, after exposure to 2 μM LV-Ka, $\sim 90\%$ aggregation was still induced by ADP, collagen, and epinephrine, suggesting that the enzyme did not inhibit platelet aggregation (not shown).

The hypotensive action of LV-Ka was investigated in cannulated rats. The enzyme induced an abrupt fall in blood pressure when it was injected into the vein of anesthetized rats (0.8 $\mu\text{g/g}$) (Fig. 4A). The hypotensive effect exhibited by LV-Ka is likely to be due to its inherent kallikrein-like activity and this decrease remained for more than 30 min. On the other hand, kinin-releasing activity was tested using the purified enzyme. The reaction mixture, containing 0.24–0.38 μg LV-Ka and partially purified kininogen from bovine plasma, induced the release of kinin of the isolated guinea pig ileum in a concentration-dependent manner (Fig. 4B). The positive control (bradikinin, Bk) showed a typical release curve, as seen in Fig. 4B. By comparison with the dose–response curve obtained with Bk, the kinin formed by the reaction mixture of LV-Ka and plasma was found to be equivalent to 0.23 μg bradikinin. As a negative control, bovine kininogen incubated without LV-Ka produced no kinin release. In addition, no biological effect was observed when only the enzyme (no plasma) was administered (data not shown).

Immunological cross-reaction

It was felt important to examine the immunological cross-reactivity of available polyclonal antibodies raised

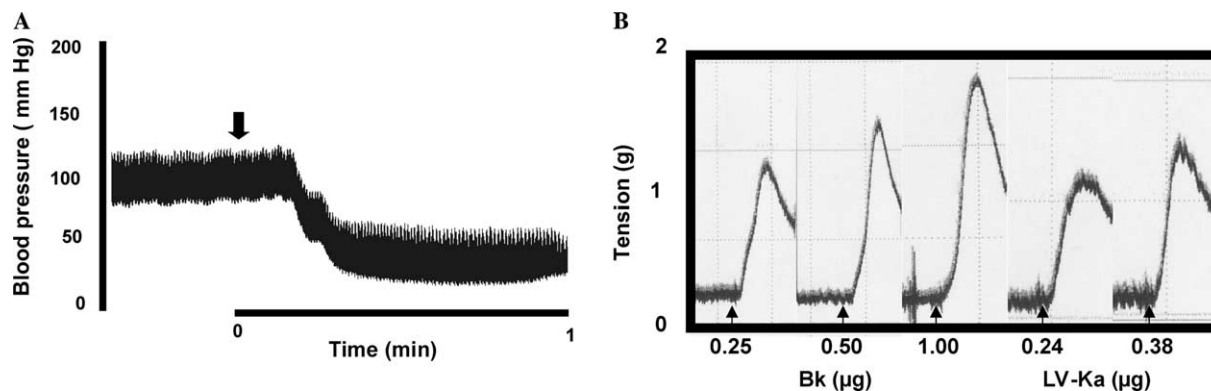


Fig. 4. (A) The effect of LV-Ka on rat blood pressure. Blood pressure tracing showing a typical effect of the venom enzyme (0.8 $\mu\text{g/g}$); arrow indicates the point of sample injection. (B) Kinin released from partially purified kininogen by the kallikrein-like enzyme LV-Ka on isolated ileum guinea pig. Arrows indicate the points of samples injection (Bk, 0.25, 0.5, and 1.0 μg) and incubation mixture containing LV-Ka (0.24 and 0.38 μg). Experimental conditions are described under Materials and methods.

against a related serine protease from the same bushmaster venom, the plasminogen activator (LV-PA) with LV-Ka, as well as with other serine proteinases and snake venoms. Therefore, immunological cross-reaction was investigated by ELISA and immunodiffusion. The anti-LV-PA antibodies cross-reacted with LV-PA, LV-Ka, and with the crude venom of *L. m. muta* (not shown). However, the antibodies showed little or no immunological cross-reaction with venoms of *Bothrops* species. When both proteases, LV-Ka and LV-PA, were confronted with horse antivenom to *L. m. muta* venom and with rabbit serum against LV-PA by immunodiffusion, a continuous precipitin line was observed (data not shown). A continuous precipitin arc was also observed between rabbit serum against LV-PA with LV-Ka and LV-PA (not shown), indicating that both serine proteases do share some common epitopes. By contrast, no reaction was observed with: trypsin, human plasmin, clotting enzyme from *L. m. muta*, venom and with bovine thrombin.

Discussion

The venom of the large new world pit viper *L. m. muta* has been documented as a complex mixture of proteinases exhibiting proteolytic, fibrinogen-clotting, hemorrhagic, and neurotoxic effects [28]. Furthermore, envenomings by bushmasters were characterized by severe local and systemic effects with complete defibrinogenation without thrombocytopenia and hypotension [29,30]. The present paper details the purification scheme and the biochemical characterization of the kallikrein-like enzyme from *L. m. muta* venom, named LV-Ka. The enzyme has been purified to a high degree of homogeneity, as indicated by electrophoresis in acrylamide and SDS acrylamide gels, immunodiffusion, and immunoelectrophoresis using horse antivenom to *L. m. muta* and rabbit serum against LV-PA. Finally, sequence analysis shows a single amino-terminal residue for LV-Ka, ruling out the possibility of a contaminant unless it has a blocked amino-terminal residue. The native enzyme is an acidic glycoprotein. Its Mr was determined by SDS acrylamide gel electrophoresis, and values of 33,000 and 28,000, respectively, were obtained for a single polypeptide chain for the native and deglycosylated LV-Ka. The role of carbohydrates in kallikrein-like enzymes has not been sufficiently clarified, but in some cases the glycans seem to be needed for protein stabilization rather than for catalytic function of the venom enzymes [32]. Thus, in the neuraminidase treatment of crotalase, the clotting enzyme with kallikrein-like effect of *Crotalus adamanteus* venom does not alter the amidolytic and clotting activity [32], suggesting that sialic acid does not play a functional role in the enzyme reaction under the in vitro assay. On the other hand,

LV-Ka lost approximately 90% amidase activity after treatment with PNGase F to remove all carbohydrate (data not shown). The enzyme possesses relatively specific and strong activity on the model kallikrein substrates S-2302 and S-2266, but it had no proteolytic activity towards dimethylcasein or oxidized insulin B chain. However, Tos-Gly-Pro-Lys-pNA, which is a specific substrate for plasmin, and S-2238, a substrate for thrombin, were demonstrated to be poor substrates for LV-Ka, attesting to some distinct features of this enzyme as compared with conventional serine proteases involved in the process of blood coagulation and fibrinolysis. Moreover, the present study reveals that the activation of plasminogen is another possible biological function of this protease, as evidenced by the increased amidolytic activity on Tos-Gly-Pro-Lys-pNA by LV-Ka preincubated with plasminogen at an enzyme/substrate weight ratio of 1:66. Other related proteinases such as tissue kallikrein and factor Xa may also participate in the extrinsic pathway of human fibrinolysis [33]. Unlike the kallikrein-like enzymes from snake venoms, gila-toxin, a kallikrein-like protease from the Mexican beaded lizard (*Heloderma h. horridum*) did not hydrolyze the substrate S-2302 [34]. The differences in specificity of kallikrein-like enzymes for synthetic substrates and inhibitors are presumed to be dependent on the structure of the substrate-binding site and/or active center [10].

The venom enzyme was identified as a serine proteinase by its rapid inhibition by PMSF and NPGB. Interestingly, we found that D-Val-Phe-Lys-CMK, a selective inhibitor of plasmin, is also an effective inhibitor of the enzyme. Moreover, LV-Ka does not clot fibrinogen, nor does it show fibrin(ogeno)lytic activity in vitro. In comparison with the *Lachesis* enzyme, the majority of kallikrein-like enzymes hydrolyze fibrinogen which could result or not in fibrin clots [31–34].

After LV-Ka had been purified, 77% of its amino acid sequence was solved by automated protein sequencing (Fig. 3). The proteinase showed extensive sequence similarity with the clotting enzymes from *L. m. muta* (60.8%) [35], 60.4% sequence identity with crotalase from *C. adamanteus* [9], and with other venom serine proteinases such as the TsVPA from *Trimeresurus stejnegeri* (74.4% homology) [11], and 72.4% sequence homology with LV-PA from the same bushmaster venom [13]. There is also some homology evident with other serine proteinases from snake venoms and mammalian kallikrein [10,31]. This homology facilitates the identification of the Asp⁸⁸ and Ser¹⁸³ as two of the catalytic-triad residues common to serine proteinases. As observed the sequences of flanking residues are highly conserved, suggesting that these residues are necessary for the spatial constraint of the active site residues. As it might be expected the first residue of the catalytic-triad would be His⁴³. Like crotalase and TsVPA, LV-Ka exhibits a conserved C-terminal extension (Fig. 3), which is highly

conserved among snake venom serine proteinases as well as in other members of the trypsin-like proteinases.

In conclusion, the kallikrein-like enzyme (LV-Ka) shares some structural homology and enzymatic properties with other members of the trypsin/kallikrein family of serine proteases. The bradikinin-releasing and hypotensive effect of LV-Ka may result from the structural similarity to kallikrein. The comparison of structural data and activity with other enzymes which interfere with the hemostatic system indicates that LV-Ka may have great potential as an antihypertensive agent.

Acknowledgments

This work was supported by the Brazilian agencies Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) and Fundação de Amparo a Pesquisa do Estado de Minas Gerais (FAPEMIG), Processo EDT-24.000/01.

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