Cytotoxicity and inhibition of platelet aggregation caused by an l-amino acid oxidase from *Bothrops leucurus* venom

Gustavo B. Naumann [a], Liliana F. Silva [a], Luciana Silva [a], Gilson Faria [a], Michael Richardson [a], Karla Evangelista [a], Markus Kohlhoff [a], Celia M.F. Gontijo [b], Alexei Navdaev [c], Flavia F. de Rezende [c], Johannes A. Eble [c], Eladio F. Sanchez [a],*

[a] *Research and Development Center, Ezequiel Dias Foundation, 30510-0103, Belo Horizonte, Brazil*
[b] *Research Center Rene Rachou, Belo Horizonte, Dept. Vascular Matrix Biology Excellence Cluster CardioPulmonary System, Frankfurt University Hospital, Frankfurt am Main, Germany*
[c] *Center for Molecular Medicine, Dept. Vascular Matrix Biology Excellence Cluster CardioPulmonary System, Frankfurt University Hospital, Frankfurt am Main, Germany*

ARTICLE INFO

ABSTRACT

Background: Multifunctional l-amino acid oxidases (LAAOs) occur widely in snake venoms.
Methods: The l-AAO from *Bothrops leucurus* (*Bl*-LAAO) venom was purified using a combination of molecular exclusion and ion-exchange chromatographies. We report some biochemical features of *Bl*-LAAO associated with its effect on platelet function and its cytotoxicity.
Results: *Bl*-LAAO is a 60 kDa monomeric glycoprotein. Its N-terminal sequence shows high homology to other members of the snake-venom LAAO family. *Bl*-LAAO catalyzes oxidative deamination of l-amino acids with the generation of H_2O_2. The best substrates were: l-Met, l-Norleu, l-Leu, l-Phe and l-Trp. The effects of snake venom LAAOs in hemostasis, especially their action on platelet function remain largely unknown. *Bl*-LAAO dose-dependently inhibited platelet aggregation of both human PRP and washed platelets. Moreover, the purified enzyme exhibited a killing effect *in vitro* against *Leishmania* sp., promastigotes, with a very low EC_{50} of 0.07 μM. Furthermore, the cytotoxicity of *Bl*-LAAO was observed in the stomach cancer MKN-45, adeno carcinoma HUTU, colorectal RKO and human fibroblast LL-24 cell lines. The enzyme released enough H_2O_2 in culture medium to induce apoptosis in cells in a dose- and time-dependent manner. The biological effects were inhibited by catalase.
Conclusion: *Bl*-LAAO, a major component of *B. leucurus* venom, is a cytotoxin acting primarily via the generation of high amounts of H_2O_2 which kill the cells.
General significance: These results allow us to consider the use of LAAOs as anticancer agents, as tools in biochemical studies to investigate cellular processes, and to obtain a better understanding of the envenomation mechanism.

1. Introduction

l-amino acid oxidases (LAAOs) are flavoproteins which are able to catalyze the oxidative deamination of l-amino acids to produce the corresponding α-keto acids along with the concomitant release of hydrogen peroxide (H_2O_2) and ammonia. Although they occur in many different organisms from invertebrates to vertebrates, their functions in vivo are uncertain. LAAO (EC 1.4.3.2) is widely distributed in venomous snakes including the viperids and elapids and is thought to contribute to their toxicity, possibly through H_2O_2 formed as a result of reoxidation of the transiently reduced FAD cofactor by molecular oxygen [1]. The enzyme is the major component of snake venoms, and in some venom species this enzyme alone constitutes approximately 30% of the total protein content [2,3]. It has been reported to exert a multiplicity of effects on biological organisms including the induction or inhibition of platelet aggregation [4–6]. These enzymes also exhibit antibacterial and antiviral properties, abolishing the growth of both Gram-positive and -negative pro-karyotes as well as HIV [7–9]. Furthermore, venom LAAO has been shown to induce cell death in several mammalian cell lines [10,11]. The effect was attributed to the formation of localized high concentrations of H_2O_2, a known reactive oxygen specie (ROS). It is interesting to note that the LAAO-induced apoptosis has been reported to be different from that caused by exogenous H_2O_2, suggesting that the mode of delivery of H_2O_2 is an important factor [12]. In addition, snake venom LAAOs appear to be cytotoxic against

promastigotes of *Leishmania* sp. [13]. Recent studies indicate that the individual LAAOs differ in their structure, substrate specificity as well as in their biological properties. Thus, ophidian LAAO has become an attractive target for research in molecular biochemistry, physiology and medicine due to its multiple effects on several cells.

The pit viper *Bothrops leucurus* (White-tailed-jararaca) is a poisonous snake habitating areas in the northeast of Brazil. In those regions *B. leucurus* is the leading cause of human accidents and constitutes a relevant public health issue. The venom exerts diversified local and hematological effects and the bites are followed by hemostatic failure. The LD_{50} by ventricular, intraperitoneal and intravenous routes were 5.7, 85.6 and 18.3 µg, respectively, per 20 g mouse [14]. The venom contains hemorrhagic and anticoagulant activities associated with active toxins such as P-I and P-III metalloproteinases [15–17] and procoagulant activity related to thrombin-like enzyme [18]. Phospholipases A_2 reported as K49 and D49 [19] have been isolated from this venom and are under investigation. In addition, the venom has effects on a wide variety of physiological and pathological processes, including inflammation, edema, hemolysis and cancer metastasis. However, compared to other Brazilian *Bothrops* snakes, the toxinology of *B. leucurus* venom is still poorly studied, and so far, no component from this venom with LAAO activity has been reported. In view of the lack of information, we have purified an LAAO from *B. leucurus* venom named *Bl*-LAAO and have studied some of its biochemical and pharmacological properties. Our results have important implications for understanding the envenomation by *B. leucurus* as well as the mechanisms of cell death triggered by this group of enzymes and provide a foundation for further investigations.

2. Materials and methods

2.1. Materials

Venom of *B. leucurus* was from the Ezequiel Dias Foundation Serpentarium, Belo Horizonte, Brazil. Horseradish peroxidase, catalase, o-phenylenediamine, ʟ-leucine and hydrogen peroxide were from Sigma (St. Louis, USA). Collagen and ADP were from Helena Laboratories, USA. N-glycosidase F (PNGase F) was from New England Biolabs, USA and o-glycosidase from Boehringer Mannheim, Germany. Live/Dead viability/cytotoxicity kit for mammalian cells (Molecular Probes, Inc. USA); Other chemicals were of analytical grade. The experiments reported here were conducted according to the guidelines established by the Brazilian College for Animal Experimentation and approved by the local Ethics Committee.

2.2. Purification of Bl-LAAO

Lyophilized *B. leucurus* venom (2 g) was dissolved in 12 ml of 50 mM ammonium acetate buffer (pH 7.4) containing 0.3 M NaCl and centrifuged at $6000 \times g$ (10 min at 4 °C) to remove the insoluble material. In the first step, the solution (1850 mg protein in 12 ml) was applied to two 2.5×100 cm columns in series packed with Sephacryl S-200, equilibrated and eluted with the same buffer. The flow rate was 7 ml/h and 7 ml fractions were collected at 4 °C. The fractions were monitored by spectrophotometry at 280 nm and tested for LAAO activity. For the second step, fractions with LAAO activity were concentrated in a Microcon concentrator (Millipore) to 4 ml (275 mg) and applied to a Sephacryl S-300 (1.5×100 cm) column equilibrated and eluted with 25 mM Tris–HCl buffer (pH 8.0) containing 10 mM NaCl. Active fractions were pooled, dialyzed against distilled water and lyophilized. This material (83 mg) containing protein impurities was applied to a DEAE Sepharose CL 6B (1.0×19 cm) column, equilibrated with 25 mM Hepes buffer, pH 7.5. The bound proteins were eluted with a 0–0.3 M NaCl gradient in the same buffer at a flow rate of 14 ml/h.

2.3. Protein characterization

For purity analysis, 200 µg of the *BL*-LAAO obtained from the DEAE Sepharose column was applied onto a RP HPLC C_4 (4.6×250 mm) column, equilibrated with 0.1% aqueous TFA, which was eluted with a gradient of ACN (0–60%) in 0.1% TFA. The molecular mass (Mr) of purified *BL*-LAAO was determined by gel filtration using a Sephacryl S-200 (1.0×37 cm) column eluted with 20 mM Tris–HCl buffer (pH 7.5) containing 0.15 M NaCl at a flow rate of 6 ml/h. The Mr markers were BSA, (66 kDa), ovalbumin (45 kDa), trypsinogen (25 kDa) and α-lactalbumin (14.4 kDa). The Mr was also determined by polyacrylamide gel electrophoresis (12%) according to [20] and by MALDI TOF mass spectrometry. The N-terminal amino acid sequence was determined by automated Edman degradation using a Shimadzu PPSQ-21 protein sequencer according to the manufacturer's instructions.

2.4. MALDI-TOF mass spectrometry

Protein masses were determined by Matrix assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry. Spectra were recorded and analyzed using a Bruker Autoflex III Smartbeam instrument in the linear positive mode controlled by the proprietary COMPASS™ 1.2 software package. The Nd-YAG-laser power (355 nm) was manually adjusted for optimal signal appearance. A freeze-dried salt- and detergent-free sample was dissolved in few microliters of 30% ACN in 0.1% TFA. 0.5 µl was spotted on a ground steel target plate, mixed with 0.5 µl matrix solution (10 mg/ml sinapinic acid in 50% ACN, 0.1% TFA) and left to dry at room temperature. Commercially available standard protein mixtures were spotted on the same target for calibration purposes prior to sample analyses.

2.5. 2D-electrophoresis (2-DE) and image analysis

The proteins (60 µg) of *B. leucurus* venom were separated by 2-DE. Prior to running the first dimension, the IPG strips were placed in the rehydration tray and the proteins were dissolved in the De Streak Rehydrate solution (GE Health Care, Sweden), 0.5% IPG buffer pH 3–10 (GE Health Care). First dimension IEF was carried out in an Ettan IPGphor 3 (GE Health Care) as described by the manufacturer. Immobiline strips 7 cm, pH 3–10 linear (GE Health Care) were employed for the first dimension separation at 20 °C using a four-phase electrophoresis program: 300 V to reach 200 Vh; 1000 V to reach 300 Vh; 5000 V to reach 4000 Vh; and 5000 V to reach 1250 Vh (total accumulated 5800 Vh with 50 µA/strip). Prior to running the second dimension the proteins in the strip were reduced and alkylated by sequential incubation in the following solutions: 75 mM Tris HCl pH 8.8, 6 M urea, 3% glycerol, 2% SDS, 0.002% bromophenol blue (equilibration buffer-EB), 10 mg/ml DTT for 20 min; and then a solution of 25 mg/ml iodoacetamide in EB for 20 min. Also, SDS-PAGE was done in a mini gel 7.5 cm 12.5% polyacrylamide gel. Coomasie blue was employed for protein staining. Direct scanning and image analysis was performed using an Image Master 2D Platinum 7 (GE Health Care).

2.6. Glycosylation studies

The estimation of the carbohydrate content in *Bl*-LAAO was performed by using a glycoprotein carbohydrate estimation kit (Pierce) according to the manufacturer's instructions. For the determination of N-linked sugars, the enzyme was submitted to treatment with PNGase F. 200 µg of native *Bl*-LAAO was dissolved in 90 µl of denaturing buffer (0.5% SDS, 1% β-mercaptoethanol). The sample was denatured by boiling for 10 min. After addition of 10 µl of reaction buffer (0.05 M phosphate, pH 7.5), 10 µl of 10% NP-40 and 5 units of recombinant PNGase F, the sample was incubated at 37 °C for 15 h. Deglycosylation was subsequently detected by size reduction in

SDS-PAGE. Similarly, the enzyme was treated with o-glycosidase. Furthermore, protein-linked glycosylation was measured using the DIG Glycan Differentiation Kit (Roche Diagnostics, Mannhein, Germany) according to the manufacturer's instructions.

2.7. Enzymatic assays

To measure LAAO activity, a reaction mixture (1 ml) containing 10 μl horseradish peroxidase (1 mg/ml), 10 μM OPD, and 100 μM L-leucine in 0.1 M Tris–HCl buffer, pH 8.5 was incubated at 37 °C. The reaction was started by adding crude venom or purified Bl-LAAO (1–2 μg) and monitored at 436 nm over 20 min. Reactions were stopped by the addition of 100 μl of 60% acetic acid. One unit of the substrate activity was defined as the oxidation of 1 μM of L-leucine per min. To test the enzymatic specificity of BL-LAAO, L-leucine was replaced with other L-amino acids under identical assay conditions. The amount of purified Bl-LAAO in the reaction mixture was 0.66 μM. Protein concentration was determined using a BCA protein assay kit (Pierce) with bovine serum albumin as standard.

2.8. Stability assays

To determine the optimum pH of Bl-LAAO, the purified enzyme was incubated at 25 °C for 30 min under different pH conditions (sodium acetate buffer pH 4.5–5.5; Hepes buffer pH 6.5–7.5; Tris–HCl buffer pH 8.5; borate buffer pH 9.5–10.5) and enzyme activity was determined using L-Leu as substrate. To investigate the effect of lyophilization and freezing, aliquots (1 mg) of the native Bl-LAAO in 25 mM Hepes buffer pH 7.5 were stored for one month at 4 °C and −20 °C. Separate aliquots (3 ml) were dialyzed overnight against 25 mM Hepes buffer, pH 7.5, containing 50 mM KCl. After dialysis, the sample was placed in 50% glycerol and stored at 4 °C. Since the enzyme is susceptible to inactivation/reactivation, in each assay the enzyme (frozen-, maintained at 4 °C or lyophilized) was pre-incubated in acetate buffer, pH 5.0 for 30 min at 37 °C.

2.9. Generation of antibodies specific for Bl-LAAO

Purified Bl-LAAO (250 μg/ml in phosphate buffered saline, pH 7.4) was emulsified with 1 ml of Freunds complete adjuvant for the first immunization and with Freunds incomplete adjuvant for the booster immunizations. Aliquots of 0.5 ml of this emulsion were injected (subcutaneously) into four sites in the back of one rabbit (New Zealand 2.5 kg). Injections were repeated biweekly and serum was obtained after the third injection. The IgG fraction was purified by affinity chromatography on protein A-Sepharose.

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

For analysis of the immunological reactivity of several different pit viper venoms against the anti-Bl-LAAO IgG, western immunoblotting and ELISA were used. The enzyme Bl-LAAO (4 μg) was subjected to SDS-PAGE (12% gel) under reducing conditions, then eletrophoretically transferred onto a nitrocellulose membrane according to the instruction manual (Bio Rad Laboratories). One gel of 2-DE containing separated proteins from B. leucurus venom was also used for immunoblot assay under similar experimental conditions. ELISA plate was coated with 100 μl of 0.5 μg/well of each antigen (Bl-L-AAO or crude venoms) in 0.05 M carbonate buffer, pH 9.6. After washing with 0.05% Tween-saline, a blocking solution (2% casein in phosphate buffered saline-PBS) was added (1 h at room temperature). After two washes with the same solution, anti-Bl-L-AAO antibody previously diluted in PBS containing 0.25% casein and 0.05% Tween 20 (0.015 to 2 μg/well) was added and incubated for 1 h at 37 °C. After six washes, peroxidase-coupled anti-rabbit IgG (Sigma, diluted 1:12000) was added and incubated for 1 h at room temperature. The wells were washed and 100 μl of an OPD solution (0.33 mg/ml in citrate buffer, pH 5.0, in the presence of 0.012% H_2O_2) was added (15 min at room temperature). The reaction was stopped by adding 20 μl of a 1:20 dilution of sulfuric acid. Absorbance values were measured at 492 nm.

2.11. Platelet aggregation studies

Venous blood was collected with informed consent from healthy volunteers who denied taking any medication in the previous two weeks. Aggregation in citrated platelet-rich plasma (PRP) was measured in a PACKS-4 platelet aggregation system (Helena Laboratories, USA). PRP was prepared by centrifugation of citrated blood at 1000 rpm for 10 min. After removal of PRP, the remaining blood was centrifuged at $3000 \times g$ for 10 min and platelet poor plasma (PPP) was collected. The platelets were left for approximately 40 min at room temperature to recover their sensitivity to aggregating agents. Inhibition of ADP- or collagen-induced platelet aggregation was monitored by adding Bl-LAAO (0.1–2.0 μM) for 3 min prior to the addition of agonist (5–10 μM ADP or 2.5–10 μg/ml collagen). Human washed platelets were prepared as described [5]. Washed platelets were suspended in Tyrode's solution and adjusted to about 3×10^8 platelets per ml. The inhibition of platelet aggregation was normalized to the maximum aggregation in the absence of enzyme.

2.12. Cell lines and cell culture

Human gastric carcinoma cell line MKN-45 was obtained from the Institute of Pathology, University of Porto, (Portugal), cultured and maintained in RPMI 1640 medium (Sigma). The human cell lines HUTU (adeno carcinoma), RKO (colon carcinoma) and fibroblast LL-24 were from American Type Culture Collection (ATCC, USA) maintained in EMEM (Sigma) and HAMF12K (Sigma) medium respectively, supplemented with 10% (v/v) fetal bovine serum, 4% glutamine, 50 units/ml penicillin, 50 μg/ml streptomycin, and kept in controlled atmosphere (5% CO_2 incubator at 37 °C).

2.13. Flow cytometry

To investigate whether Bl-LAAO binds to cells, 5×10^{-5} of HUVEC (Human Umbilical Vein Endothelial Cells: PromoCell, Heidelberg, Germany) and the endothelial cell line Eahy926 [21] were incubated with or without 10 μM/ml of Bl-LAAO for 30 min at 4 °C after the blocking step using 1% horse serum in PBS. Rhodocetin, an antagonist of α2β1 integrin [22] isolated from the venom of the Malayan pit viper (Calloselasma rhodostoma) was used as a positive control in a concentration of 5 μg/ml under the same conditions. After this procedure, cells were washed twice with PBS and primary antibodies against rhodocetin (1:1000) and Bl-LAAO (2 μg/ml), both raised in rabbit, were added. IgG from rabbit serum (1:1000) (Sigma, Germany) was used as an isotype control. After 30 min of incubation at 4 °C, cells were washed again and stained with the secondary Alexa Fluor 568-conjugated antibody against rabbit (Molecular Probes, USA) was incubated using also the same conditions. Cells were analyzed using a CyFlow® device (Partec, Germany) and acquired data were plotted as histograms using the WinMDI2.9 program.

2.14. Measurement of H_2O_2 production

EMEM cell culture medium (100 μl/sample) was incubated with Bl-LAAO at various concentrations (1, 10, 20, 50, 100 μg), 50 μg/ml peroxidase and 0.1 mg/ml OPD at 37 °C for several intervals. Then, the quantity of the generated H_2O_2 was determined by a peroxidase-coupled assay [12].

2.15. Measurement of cytotoxicity

Cytotoxicity of native Bl-LAAO and B. leucurus crude venom was tested against LL-24, MKN-45, RKO and HUTU cell lines by MTT assay and the LIVE/DEAD viability/cytotoxicity Kit for mammalian cells (Molecular Probes, USA) assay according to the manufacturer's instruction. The test provides a two-color fluorescence cell viability assay that is based on the simultaneous determination of live and dead cells with two probes that measure recognized parameters of cell viability-intracellular esterase activity and plasma membrane integrity. In brief, 10^5 cells/well were plated in a 96-well microtiter plate. Then cells were exposed to the test samples (Bl-LAAO or crude venom: 1, 2, 4, 10 and 20 μg/ml) for 12 or 24 h at 37 °C. Furthermore, MKN-45 and RKO (10^5 cells) in RPMI 1640 medium (150 μl) were added to 96-well microtiter plates for 12 h before 50 μl of various concentrations (MKN 45 = 1.25, 2.5, 5,10 and 20 μg/ml; RKO = 0.1, 0.25, 0.5, 1, 2, and 4 μg/ml) of Bl-LAAO or crude venom were added. After 24 h of incubation, 50 μl of 50 mg/ml MTT in PBS was added to each well for 4 h. After removing the medium, 100 μl of DMSO was added to each well for 10 min at room temperature to solubilize the MTT-formazan product. After 30 min at room temperature the plate was read with a microplate reader at 503 nm. All the measurements were performed in triplicate. The EC_{50} value was calculated and compared with the control cell culture.

2.16. Characterization of Bl-LAAO action on promastigotes

Promastigotes of Leishmania chagasi (MHOM/BR/74/PP75) and L. braziliensis (MHOM/BR/75/M2903), 4×10^5/well were incubated with crude venom or Bl-LAAO (0.5, 1, 2, 4, and 8 μg/ml) in absence or presence of catalase (100 μg/ml) for 18 h at 25 °C in a microplate assay. Control groups without Bl-LAAO, with or without catalase, and with or without H_2O_2 (5 mM) were also tested. Parasites were incubated in Schneider Insect Medium' (Sigma) supplemented with 10% fetal bovine serum (Invitrogen), and penicillin 100 units/ml, streptomycin 50 μg/ml. The viability was determined by staining with Trypan Blue (1:1) and counting viable cells using a Neubauer counter camera. Values of EC_{50} were determined from a dose–response curve.

2.17. Other assays

Proteolytic activity was measured with dimethylcasein (DMC) as substrate, and clotting activity on human fibrinogen was tested as reported previously [14]. Hemorrhagic activity was assessed by injecting (subcutaneosly) doses of 10, 20 and 50 μg/mouse (18–22 g) as described [23]. To evaluate if the native enzyme is a lethal component different concentrations of Bl-LAAO (10, 30, 50 and 100 μg), were injected (i.p.) into mice (male CF strain, groups of 5 animals at each dose).

3. Results

3.1. Isolation of B. leucurus venom LAAO

LAAO was purified from 2 g (1850 mg protein) of dry venom according to the procedure described under Materials and methods. First, the venom was separated in ten fractions (1 to 10) by gel filtration on Sephacryl S-200 column [17] of which L-amino acid oxidase activity eluted in fraction 2 (Fig. 1, 1st step). For the second step of purification, the proteins of fraction 2 with Mr of approximately 60 kDa were concentrated with a Microcon centrifugal filter unit (Millipore Corp.), the active material (275 mg) was applied to a Sephacryl S-300 column (Fig. 1, 2nd step). The major fraction 2-II (tubes 22–24) which possessed oxidase activity was collected, desalted and lyophilized. Analysis of fraction 2-II by SDS-PAGE (12% gel) showed minor contaminants. Therefore, this material (83 mg)
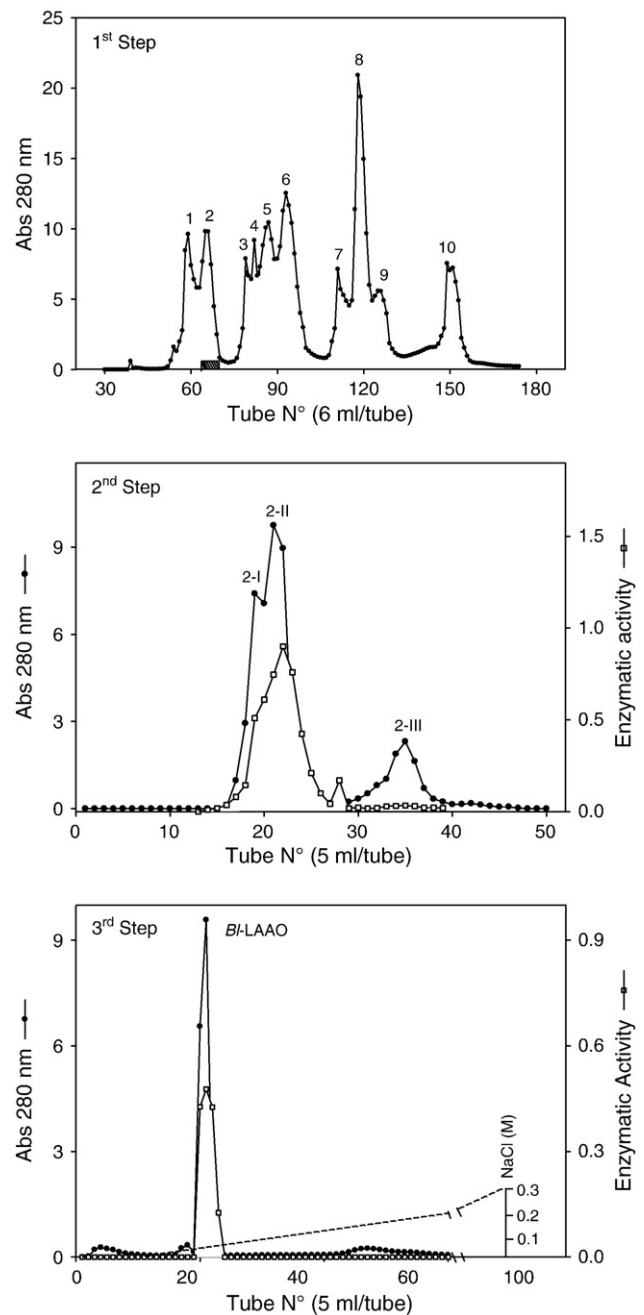

Fig. 1. Purification of an L-amino acid oxidase from the venom of Bothrops leucurus. First step: Lyophilized B. leucurus venom (2 g, 1850 mg protein) was loaded onto two (100×2.5 cm each) columns in series of Sephacryl S-200. The columns were equilibrated and eluted with 50 mM ammonium acetate buffer (pH 7.4) containing 0.3 M NaCl, at a flow rate of 7 ml/h. Active fraction 2 indicated by a bar was concentrated for the next step. Second step: Fraction 2 was subsequently applied to a Sephacryl S-300 (100×1.5 cm) column, which was equilibrated and eluted with 25 mM Tris–HCl buffer (pH 8.0) containing 10 mM NaCl. The active fraction 2-II was dialyzed against distilled water and lyophilized. Third step: 83 mg protein from Sephacryl S-300 gel filtration column was applied to a DEAE ion-exchange column (1.6×20 cm), equilibrated with 50 mM Tris–HCl buffer, pH 8.5 and eluted with 0–0.3 M NaCl linear gradient in the same buffer at a flow rate of 12 ml/h.

was further purified by anion-exchange chromatography on a column of DEAE Sepharose CL 6B, at pH 8.5, using a gradient of NaCl as eluent. Chromatography of fraction 2-II on DEAE column resulted in one peak with high LAAO activity (Fig. 1, 3rd step). An aliquot of this fraction gave one peak in RP-HPLC on a C4 column (not shown), and a unique sequence of amino acids at the N-terminal (Fig. 2a) a single peak in
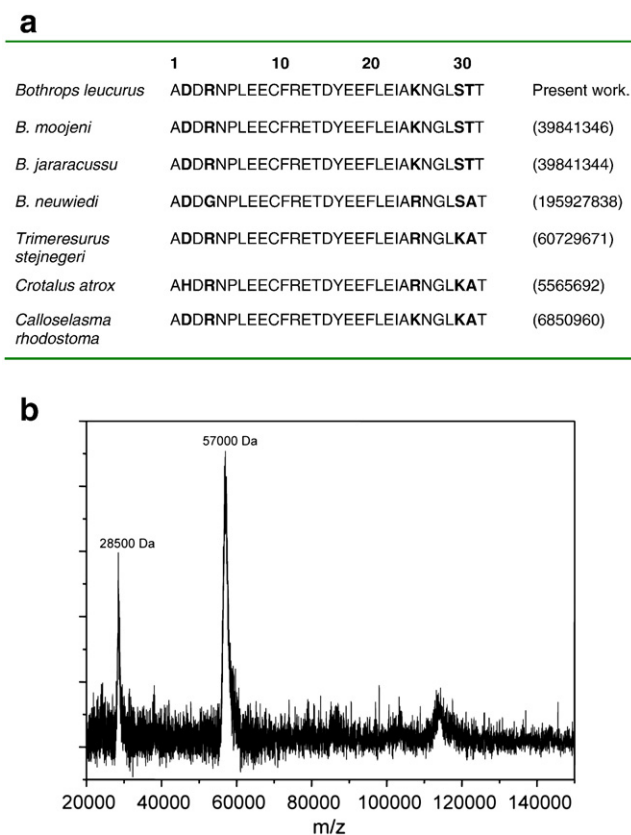
a

	1	10	20	30	
Bothrops leucurus	ADDRNPLEECFRETDYEEFLEIAKNGLSTT				Present work.
B. moojeni	ADDRNPLEECFRETDYEEFLEIAKNGLSTT				(39841346)
B. jararacussu	ADDRNPLEECFRETDYEEFLEIAKNGLSTT				(39841344)
B. neuwiedi	ADDGNPLEECFRETDYEEFLEIARNGLSAT				(195927838)
Trimeresurus stejnegeri	ADDRNPLEECFRETDYEEFLEIARNGLKAT				(60729671)
Crotalus atrox	AHDRNPLEECFRETDYEEFLEIARNGLKAT				(5565692)
Calloselasma rhodostoma	ADDRNPLEECFRETDYEEFLEIAKNGLKAT				(6850960)

b

Fig. 2. (a) Alignment of N-terminal sequence of *Bl*-LAAO with those of other venom LAAOs. Different or similar residues are in bold. The GenBank accession numbers of the N-terminal sequences of LAAOs used in the alignment analysis are presented in parenthesis. (b) MALDI-TOF mass spectrum of native *Bl*-LAAO from *B. leucurus* venom.

MALDI Tof MS (Fig. 2b), and only one band in SDS-PAGE under reducing and non-reducing conditions (Fig. 3b). We named the purified protein *Bl*-LAAO. The final step resulted in an overall yield of 21.4% and *Bl*-LAAO represented 3.7% (w/w) of the total venom proteins. Table 1 summarizes the purification procedure of *Bl*-LAAO.

3.2. Physico chemical and immunological properties

The apparent Mr of native (non-denatured) *Bl*-LAAO was approximately 56 kDa determined by size-exclusion chromatography (Fig. 3a), which suggest that *Bl*-LAAO is a monomer protein. Furthermore, the mass of 57,000 Da was detected by MALDI TOF mass spectroscopy (Fig. 2b), and the purified proteinase appeared as an estimated Mr of 60 kDa in SDS-PAGE under reducing conditions (Fig. 3b). *Bl*-LAAO is a glycoprotein as analyzed by SDS-PAGE in which the enzyme showed an approximately 4 kDa lower value Mr after treatment with PNGase F (Fig. 3c, lane 2). However, incubation with o-glycosidase did not affect the mobility of the protein in SDS-PAGE (Fig. 3c, lane 3) indicating the absence of high mannose structure and little or no o-glycosylation. Furthermore, after blotting onto a nitrocellulose membrane we tested the *Bl*-LAAO for its glycoconjugates using a panel of lectins with different capacities to recognize sugar residues typical for N- and o-glycoconjugates (Fig. 4). In comparison with the respective positive controls (fetuin, carboxypeptidase Y, transferrin, asialofetuin), the purified enzyme (lane E) gave a positive reaction only with the lectin DSA (Fig. 4, lane E, upper panel), which recognizes the sugar motif galactose-β (1–4)-N-acetylglucosamine. The disaccharide moiety is found in complex and hybrid N-glycan structures. Interestingly, no sialic acids or other
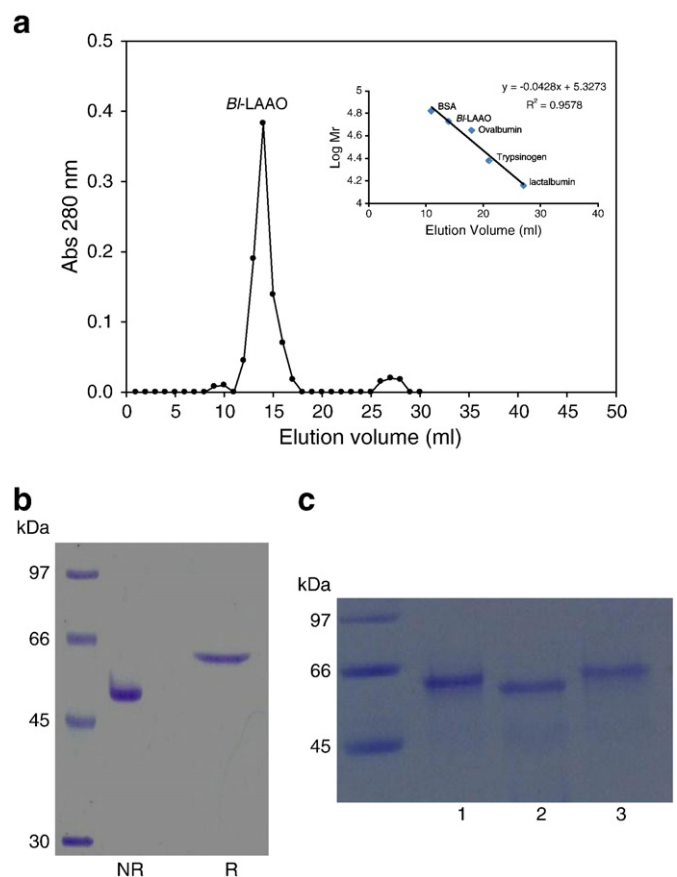
Fig. 3. Molecular mass determination of *Bl*-LAAO. (a) Size-exclusion gel filtration chromatography of *Bl*-LAAO on Sephacryl S-200 shows that *Bl*-LAAO elutes as a single peak. An equation ($y = -0.0428x + 5.3273$) was deduced according to values of log Mr and elution volume of protein standards. The inset shows elution volumes of protein standards: BSA, 66 kDa; ovalbumin, 45 kDa; trypsinogen, 25 kDa, and α-lactalbumin, and 14.4 kDa, demonstrating that native (non-denatured) *Bl*-LAAO has Mr of ~56 kDa. (b) SDS-PAGE (12% gel) of *Bl*-LAAO under non reducing (NR) and reduced (R) conditions. (c) SDS-PAGE (12% gel) of undigested (1), and of *Bl*-LAAO after treatment with PNGase F (2) or with o-glycosidase (3). Molecular mass markers are shown on the lane of left.

common sugar motif typical of o-linked or complex type N-linked glycoconjugates could be detected within *Bl*-LAAO indicating the absence of common o-linked sugar residues within *Bl*-LAAO.

Although the enzyme appears pure by some physical criteria, it showed some heterogeneity in mass spectroscopy (not shown) and in 2-DE, the latter one of which separated it into three major components with pI of 5.8 to 6.1 (Fig. 5a). All three dots were immunologically recognized by the antiserum against *Bl*-LAAO (Fig. 5b). The same Mr-band (at approximately 60 kDa) was also immunologically detected in the crude venom (Fig. 5c), ruling out a

Table 1
Purification of an *Bl*-LAAO from *Bothrops leucurus* venom.

Step	Protein		Enzymatic activity			
	(mg)	(%)	Sp. activity (U/mg)[1]	Total (U)	Yield (%)	PF[2]
Crude venom	1850	100	5.75	10,637	100	1
Sephacryl S-200	275	14.9	7.9	2172	20.4	1.4
Sephacryl S-300	83	4.5	32	2656	25	5.6
DEAE Sepharose CL-6B	69	3.7	33	2277	21.4	5.7

[1] One unit of enzymatic activity was defined as the oxidation of 1 μM L-leucine per minute. Specific activity was expressed as U/mg.
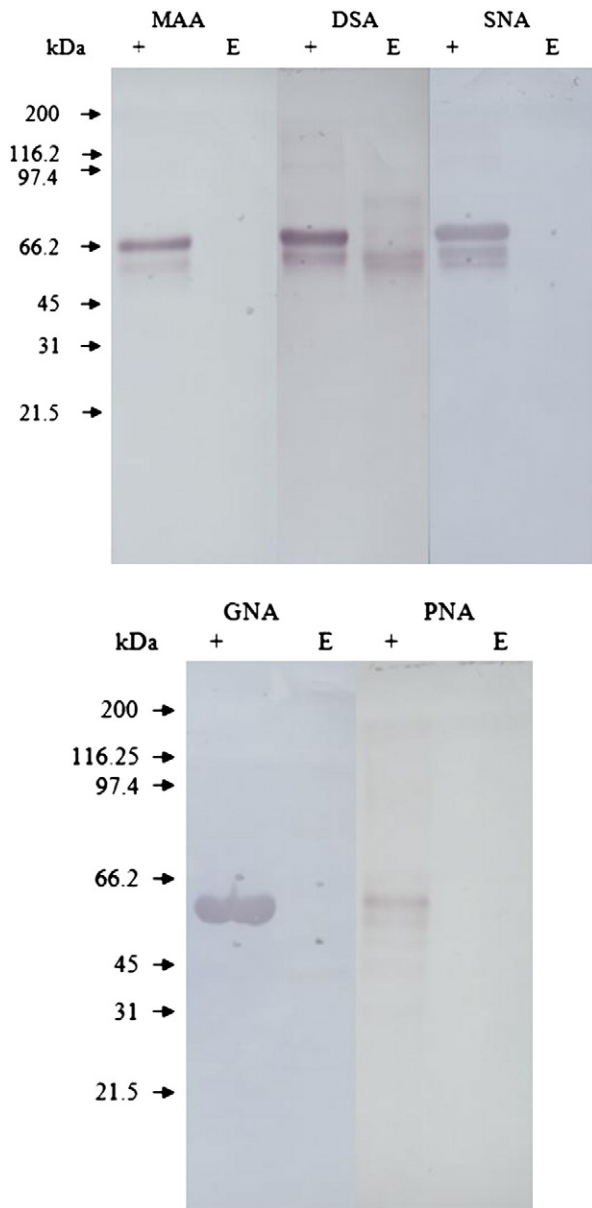
[2] PF, purification factor.

Fig. 4. Glycoprotein detection on the *Bl*-LAAO. The purified enzyme (denoted E), with N- and o-glycosylated control proteins (denoted +) were transferred to a nitrocellulose membrane after SDS-PAGE and stained with digoxigenin-labeled lectins and enzyme-conjugated anti-digoxigenin-antibodies. The following lectins were used (target structures given in parenthesis): *Galanthus vivalis* agglutinin (GNA) (mannose in high mannose- type N-glycoconjugates), *Sambuca nigra* agglutinin (SNA) ((2–6)-linked sialic acid in complex type N-glycoconjugates), *Datura stramonium* agglutinin (DSA) (galactose-β(1–4)-N-acetylglucosamine as core unit of N-glycoconjugates), *Maackia amurensis* agglutinin (MAA) (α(2–6)-linked sialic acid in complex type N-glycoconjugates and o-glycans), peanut agglutinin (PNA) (galactose-β(1–3)-N-acetylgalactosamine of o-Glycans. *Bl*-LAAO is recognized by DSA confirming the presence of N-glycosylation.

proteolytic maturation of a *Bl*-LAAO precursor in the protease-rich venom. When the anti- *Bl*-LAAO IgG was tested against the purified enzyme and with other pit viper venoms by ELISA, the antibody reacted with similar affinities with *B. leucurus, B. atrox,* and *B. barnetti*. Low reactivity was observed with *L.m.muta* (bushmaster) but there was no reaction with *Crotalus durissus terrificus* (white venom, as negative control) (Fig. 5d), which is characterized by its neurotoxic activity.

Bl-LAAO converts an appropriate substrate in the presence of oxygen to the corresponding α-keto-carbonic acid releasing H_2O_2 and NH_3. H_2O_2 can be detected by horseradish peroxidase-catalyzed

conversion of OPD. The photometric OPD signal is linear in a range of up to 100 μM (data not shown). Therefore, the OPD signal can be directly recalculated to H_2O_2 release as a measure for *Bl*-LAAO activity (Fig. 6a). The substrate specificity of the enzymatic activity of *Bl*-LAAO was characterized by using various amino acid substrates (Fig. 6b). The enzyme showed high catalytic activity against the hydrophobic residues L-Met, L-Leu, L-Norleu, L-Trp, L-Phe and was moderately active against L-Tyr. The other amino acids were not affected by the enzyme (Fig. 6b). The optimal pH for enzymatic activity using L-Leu as substrate was found to be between 7.5 and 8.5 (not shown). *Bl*-LAAO in the presence of L-Leu produced H_2O_2 in a dose- and time dependent manner (Fig. 6c), and with 50 μg enzyme approximately 60 μM H_2O_2 was generated in 4 h.

3.3. Cytotoxicity and apoptosis-inducing activity of Bl-LAAO

A number of LAAOs from snake venoms show cytotoxic and antipathogenic activities, and the H_2O_2 produced through the enzymatic reaction appears to be involved in these reactions [9,10,24]. The cytotoxic effect of *Bl*-LAAO on MKN-45 and RKO cell lines was examined after 24 h incubation in a dose-dependent manner, we were able to determine the EC_{50} values using the MTT method. As shown in Fig. 7a and b, the EC_{50} of 0.41 and 0.04 μM, were determined for MKN-45 and RKO cells, respectively. Under similar experimental conditions EC_{50} values of 0.38 and 0.15 μM, were obtained on human fibroblasts LL-24 cells (control) and on HUTU tumor cells (data not shown). Catalase (100 μg), an active scavenger of H_2O_2, inhibited approximately 25% of the cytotoxicity of *Bl*-LAAO on MKN-45 cells (Fig. 7c, lane 4). Indeed, H_2O_2 (5 mM) exhibits a cytotoxic effect higher than that of *Bl*-LAAO and when H_2O_2 was added simultaneously with *Bl*-LAAO to the cells, the death rate was approximately 95% (Fig. 7c, lane 6). To test whether H_2O_2, one of the products of the enzyme reaction, is the main mediator of *Bl*-LAAO-inducing cytotoxicity and apoptosis, we quantified *Bl*-LAAO-generated H_2O_2 in HAM F12 medium. When the medium was incubated with *Bl*-LAAO at 50 μg, approximately 60 μM H_2O_2 was produced within 4 h (Fig. 6c), which is enough to induce apoptosis in MKN-45 cells (not shown). The toxicity of *Bl*-LAAO and its reaction products were also tested toxicity in the Live/Dead viability/cytotoxicity assay in which the polyanionic dye calcein with intense green fluorescence (ex/em ~495/~515 nm) is retained within live cells and another dye, ethidium homodimer (EthD-I) enters dead cells through damaged membranes and binds to nucleic acids with red fluorescence (ex/em ~495/~635 nm). Thus, without *Bl*-LAAO the cells (LL-24, MKN-45 and HUTU) appear intensely green fluorescent (alive) (Fig. 8, −*Bl*-LAAO), while in presence of 10 μg/ml enzyme lead to a bright red fluorescence of dead cells (Fig. 8, +*Bl*-LAAO). These nuclear morphological changes are typical of apoptosis indicating that HUTU cells appears to be more sensitive to effect of *Bl*-LAAO than MKN-45 and LL-24, respectively. These results suggest that *Bl*-LAAO by producing H_2O_2 induced apoptosis and caused cell death.

3.4. Bl-LAAO does not interact with endothelial cells, but affects platelet function

After injection, snake venom components are quickly disseminated through the blood stream. To test whether *Bl*-LAAO is sequestered by the endothelial lining of blood vessels, we tested its interaction with both HUVECs (Fig. 9a) and the endothelial cell line EAhy926 (Fig. 9b). Whereas rhodocetin, an α2β1 integrin specific inhibitor of Malayan Pit viper venom, bound to endothelial cells in flow cytometry (Fig. 9a and b, blue line), *Bl*-LAAO did not interact with these cells (Fig. 9a and b, red line), ruling out a direct effect of *Bl*-LAAO with endothelial cells.

Platelets are another potential and immediate target of blood-borne snake venom components. However, the reported effects of snake venom LAAOs on platelet function are still controversial. For
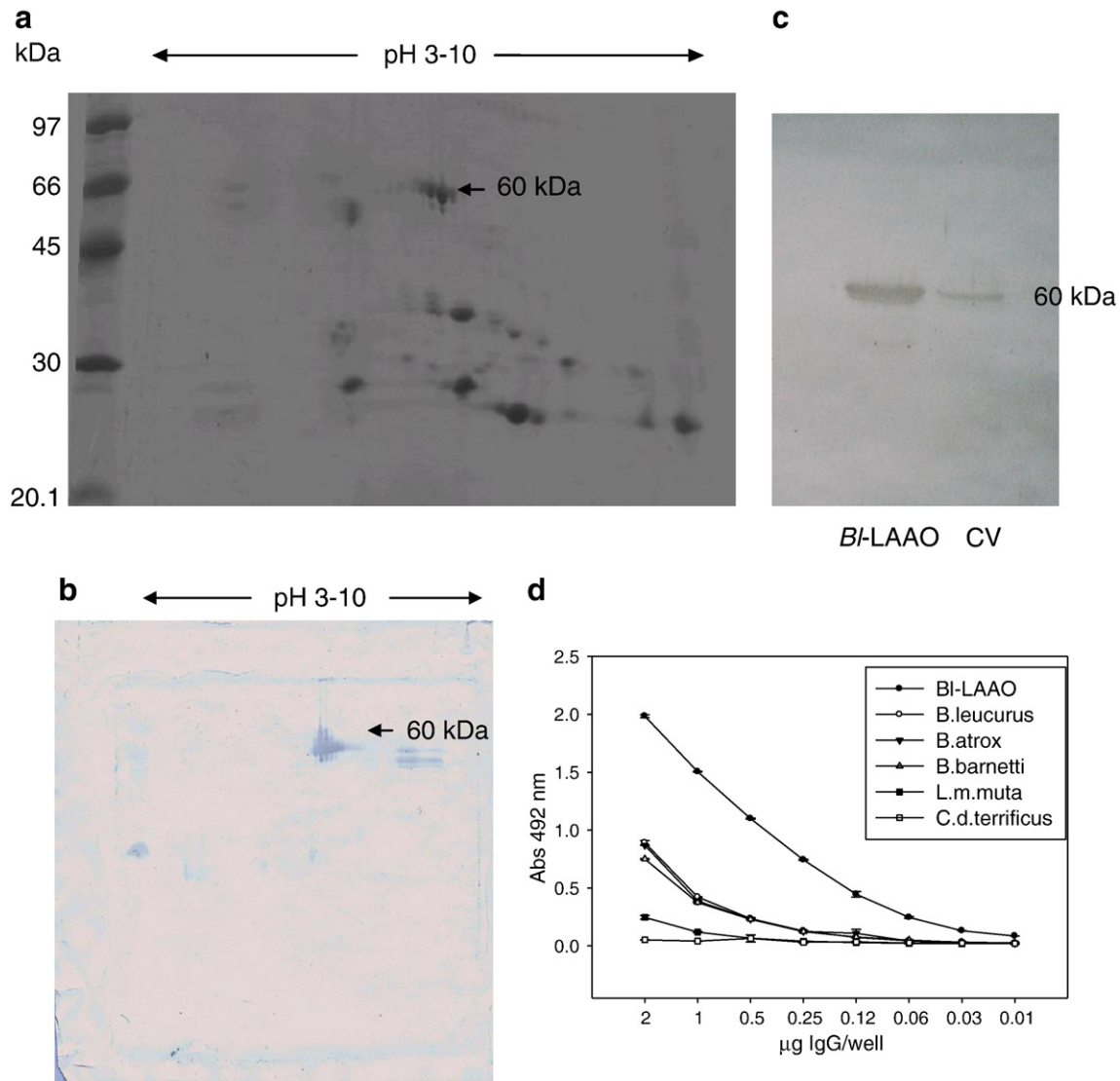
Fig. 5. 2-DE SDS-PAGE pattern of venom proteins from *B. leucurus*. (a) 60 µg of total proteins were isoeletrically focused (pI range 3–10) followed by separation by SDS-PAGE (12.5% gel) and Coomassie blue staining. Protein spot of ~60 kDa is indicated by arrow. Molecular mass markers are shown at the left of the gel. (b) Immunoblotting of anti-*Bl*-LAAO IgG against venom proteins separated by 2-D SDS-PAGE. Arrows indicates the reactivity of the antibody with the 60 kDa *Bl*-LAAO. (c) Immunoblotting of anti-*Bl*-LAAO IgG against purified *Bl*-LAAO and crude venom (CV). (d) Reactivity of anti-*Bl*-LAAO IgG against several pit viper venoms analyzed by ELISA. 96-well microtitration plates were precoated with 0.5 µg/ml of *Bl*-LAAO, *Bothrops species*, *L.m.muta* and *C.d. terrificus* snake venoms. Anti-*Bl*-LAAO IgG was added at different dilutions. Binding was visualized by incubation with peroxidase-coupled anti-rabbit IgG (diluted 1:12000) and subsequent peroxidase-catalyzed conversion of OPD. The absorbance of pre-immune serum (control) was substracted. These values are the average of two experiments run in triplicate.

instance, LAAOs from *Bothrops pirajai* [24] and from *Eristocophis macmahoni* [25] induce platelet aggregation. By contrast LAAOs from *Vipera berus berus* [26] and from *Naja naja oxiana* [27] as well as other venom enzymes inhibit platelet aggregation triggered by several agonists dose-dependently in the range of 0.1–2.5 µM. *Bl*-LAAO did not induce platelet aggregation by itself at a final concentration of 2 µM. But the enzyme dose dependently inhibited the platelet aggregation of human PRP (Fig. 9c) and washed platelets (Fig. 9d) induced by collagen and ADP with an IC$_{50}$ value of 0.21 µM (Fig. 9e).

3.5. Characterization of Bl-LAAO action on promastigotes

Reactive oxygen species among them H$_2$O$_2$ are product of *Bl*-LAAO catalysis and serve in the defense of invaded pathogens. Incubation of promastigotes of *Leishmania braziliensis* and *L. chagasi* for 24 h with the purified *Bl*-LAAO resulted in dose-dependent mortality of parasite numbers with the minimal dose that induces 50% decrease of parasite numbers estimated at 0.07 and 0.08 µM against *L. chagasi* and

L. braziliensis, respectively (Fig. 10a). This effect was suppressed by adding catalase (Fig. 10b, lane 2). In addition, promastigotes of *L. chagasi* showed H$_2$O$_2$ susceptibility, as observed when the H$_2$O$_2$ was added to the incubation medium (Fig. 10b, lane 3). Under the same experimental conditions, similar results were obtained against *L. braziliensis* (not shown).

In vitro, *Bl*-LAAO at 0.4 µM and above significantly delayed the progress of the coagulation of fibrinogen by thrombin. However, the effects of *Bl*-LAAO on human blood coagulation remain largely unknown and is a topic of future investigation. Unlike other snake-venom LAAOs, *Bl*-LAAO did not produce local hemorrhage in mice at 50 µg/mouse and was not lethal in mice with doses up to 100 µg/mouse (i.p.).

4. Discussion

This study reports the isolation and characterization of *Bl*-LAAO, an LAAO from the venom of *B. leucurus*. *Bl*-LAAO is a monomeric and glycosylated flavoprotein similar to other reported ophidian LAAOs
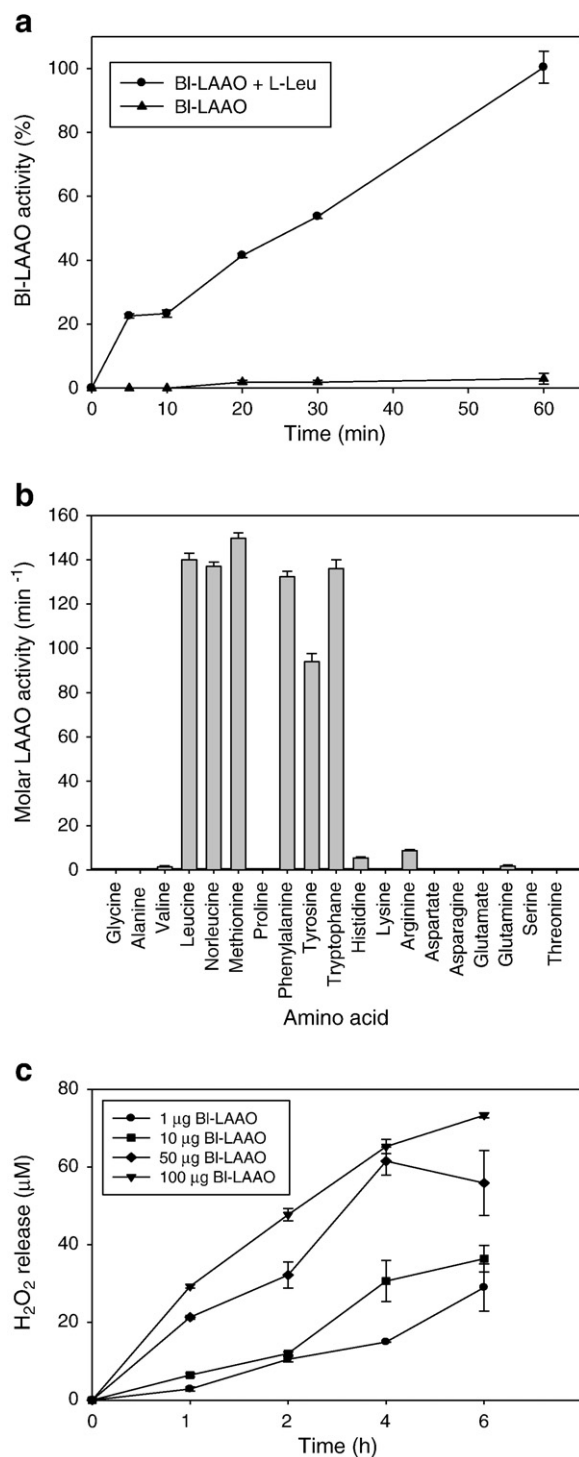
Fig. 6. LAAO activity of *Bl*-LAAO. (a) Time dependent oxidase activity of *Bl*-LAAO. The oxidase activity was measured in the presence (●) and absence (▲) of L-Leu as substrate. Data represent mean ± S.D. (n = 4). (b) L-amino acid species-specific LAAO activity of *Bl*-LAAO. (c) Time and concentration-dependent production of H_2O_2 by *Bl*-LAAO. Generation of H_2O_2 by incubation of *Bl*-LAAO in HAM F12 medium. Native enzyme (1–100 μg/ml) was incubated with HAM F12 medium containing peroxidase and OPD for the intervals indicated as described in Materials and methods. Data shown represent the average of two independent experiments, with error bars indicating the maximum and minimum deviation from the average.

Fig. 7. *Bl*-LAAO induced MKN-45 and RKO carcinoma cells death in a concentration-dependent manner. (a) MKN-45 cells were incubated with the indicated concentrations of *Bl*-LAAO or crude venom for 24 h, (b) RKO cells were treated with different concentrations of *Bl*-LAAO under the same conditions. Cell viability is expressed as the percent of ratio of A503 nm of oxidized formazan MTT of *Bl*-LAAO-treated cells with the one of control cells. Values are given as means ± S.D. of three independent experiments. (c) Characterization of *Bl*-LAAO cytotoxic activity on MKN-45 tumor cells. Cells were incubated with *Bl*-LAAO (10 μg/ml) with or without catalase (100 μg) and H_2O_2 (5 mM). 1, control cells; 2, cells + *Bl*-LAAO; 3, cells + catalase; 4, cells + *Bl*-LAAO + catalase; 5, cells + H_2O_2; 6, cells + *Bl*-LAAO + H_2O_2. The cells were cultured for 24 h. Bars represent mean ± S.D. of three independent experiments.

[1–3,25]. Its Mr of 60 kDa determined by reduced SDS-PAGE is consistent with the data obtained by mass spectrometry, 2-DE and gel filtration chromatography in which the enzyme had a Mr of 57, 60 and 56 kDa, res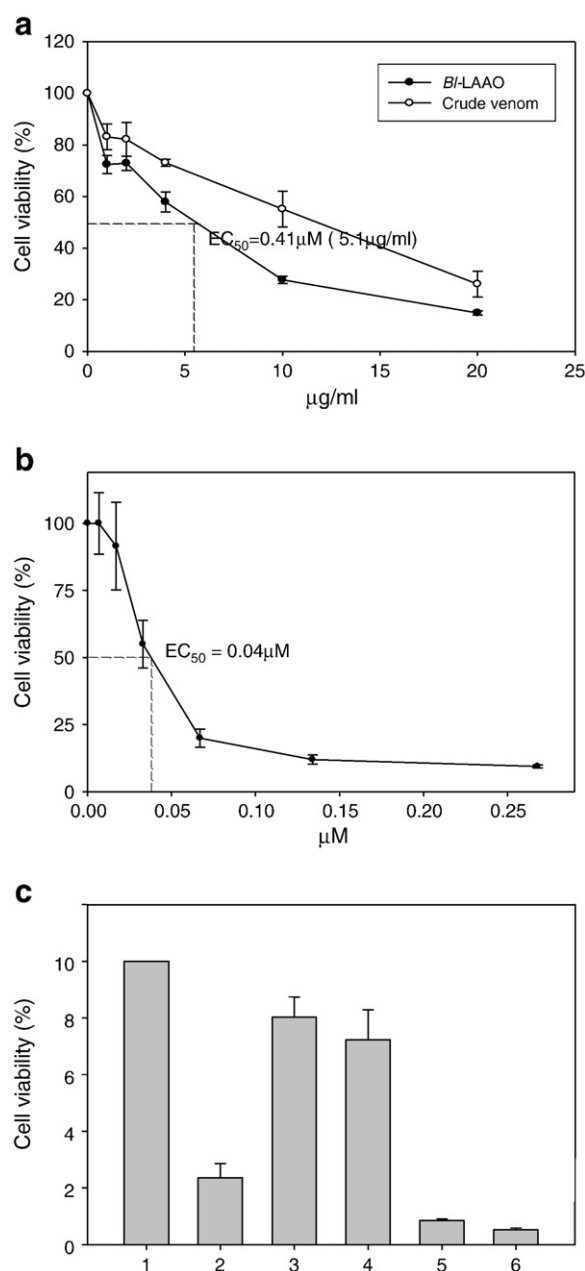pectively. The N-terminal sequence of amino acids shows a high homology to other members of this family of toxins, including the presence of a highly conserved Glu rich motif, which may play a role in substrate binding. In agreement with the structure of LAAO from *C. rhodostoma* [1], this N-terminal region (5–25 residues) constitutes one part of the substrate-binding domain. Our finding of 2-DE that the enzyme has a range of isoelectric points (pI) from about 5.8 to 6.1, reflects the presence of isoforms with distinct pIs similarly to other snake venom LAAOs [4,25,28,31]. However, the existence and the nature of the possible isoforms have to be further investigated.
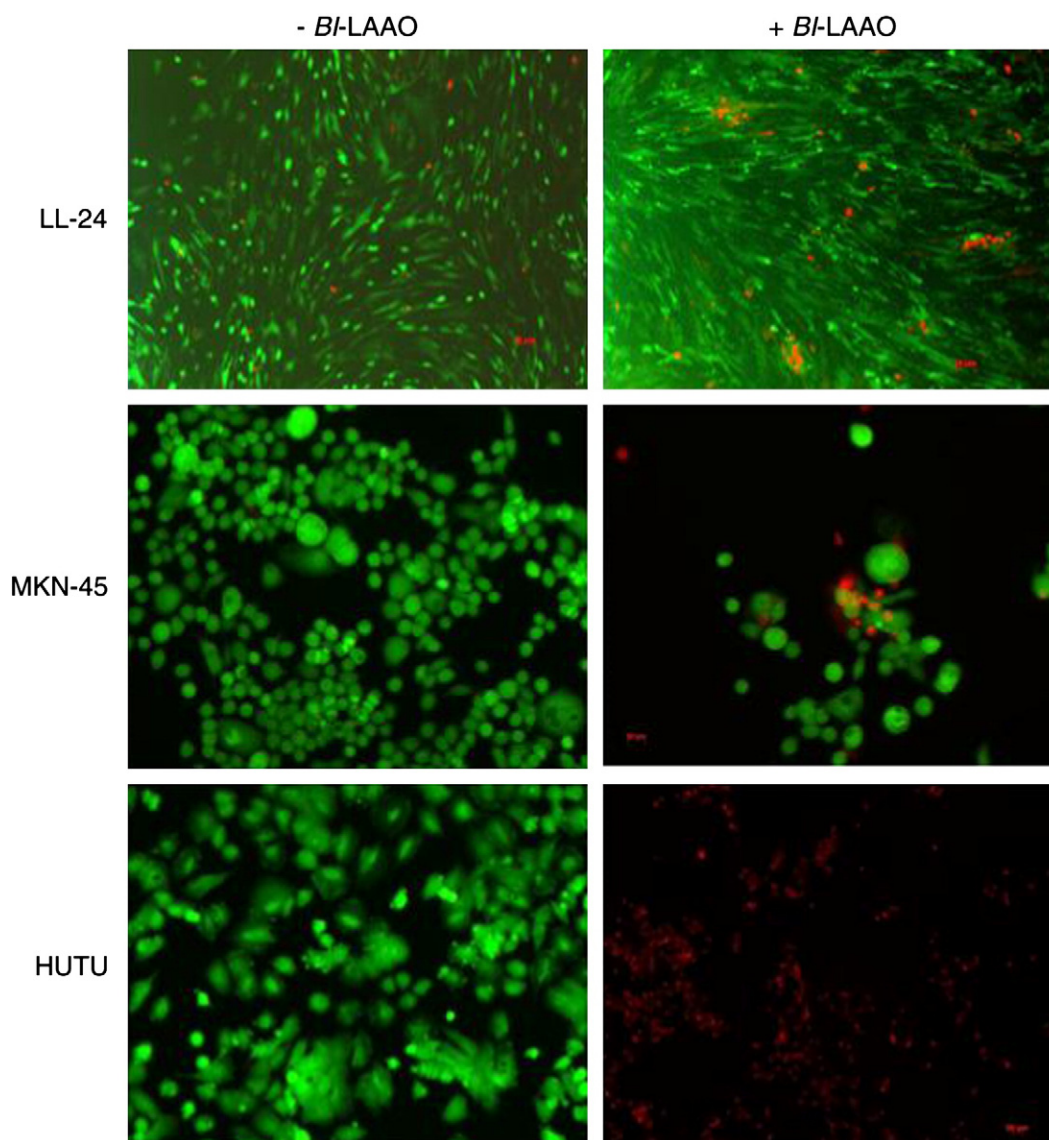
Fig. 8. Analysis of *Bl*-LAAO-induced apoptosis. Human fibroblast LL-24, gastric carcinoma MKN-45 and duodenal carcinoma HUTU cell lines were treated with *Bl*-LAAO (10 μg/ml) for 24 h, and cell cytotoxicity was examined by the LIVE/DEAD Viability/Cytotoxicity assay as described in Materials and methods. As shown, the polyanionic dye calcein is retained within live cells as intense uniform green fluorescence (controls, panels on the left). EthD-I enters cells through damaged membranes thereby highlighting apoptotic cells by red fluorescence (panels on right).

The presence of isoforms may be due to different composition or different glycosylation as revealed for other homologous flavoenzymes [4,6,28], or they may have been synthesized from different genes as was suggested for two distinct LAAOs from *Pseudechis australis* [30]. Moreover, heterogeneous glycosylation of *Bl*-LAAO may also cause this divergence and would not be surprising as the venom used for purification of *Bl*-LAAO was collected from only two snake individuals. Lectins which recognize common structural motifs of high mannose-type and o-glycoconjugates or sialic acid residues failed to bind to *Bl*-LAAO. Only the DSA-lectin with a specificity of recognizing the N-glycoconjugate core structure bound *Bl*-LAAO. In line with this, PNGase F cleaved off a 3.7 kDa large N-glycoconjugate, which however lacks any commonly linked sialic acid residues. In comparison, LAAO from *C. rhodostoma* is a dimeric flavoprotein of 55 kDa per protomer and contains up to 3.7 kDa of glycosylation per protomer [1,31]. Yet, the crystallized structure of LAAO (*C. rhodostoma*) exhibits two N-glycosylation sites at Asn172 and Asn361, both of which seem very similar in the composition and branching of the sugar chains [31].

An examination of the cross-reactivity between pit viper venoms from three different genera showed that antibodies against *Bl*-LAAO cross-reacted with of the venoms of *B. leucurus*, *B. atrox*, *B, barnetti,* and *L.m.muta* (bushmaster) to varying degrees. No cross-reactivity was observed with the white venom from *C.d. terrificus* (South American rattlesnake). Characterization of the proteomes of these snake venoms has identified LAAOs of 55 kDa (*B. atrox*), 60 kDa (*B. barnetti*) and 60 kDa (*L.m.muta*, per monomer) (manuscript in preparation). All of them are yellow venoms and since the purified homogeneous *Bl*-LAAO was bright yellow, its flavin (mainly FAD) cofactor is likely to cause the venom color [32]. The immunoblotting of electrophoretically separated proteins of *B. leucurus* with purified IgG anti-*Bl*-LAAO showed that only one protein species of the crude venom is recognized. Hence, *Bl*-LAAO is not degraded in the venom despite the abundance of distinct proteinases.

Usually the oxidizing activity of snake venom LAAOs is determined with L-Leu as substrate. In the case of pit viper and viper venoms hydrophobic amino acids are the best substrates [26,33]. *Bl*-LAAO oxidizes a variety of hydrophobic amino acids having the highest specificity for Met and Leu when each amino acid is used as substrate. Few exceptions have been reported for these enzymes, e.g. the enzyme from *Bungarus fasciatus* [29] is very active against L-Tyr,
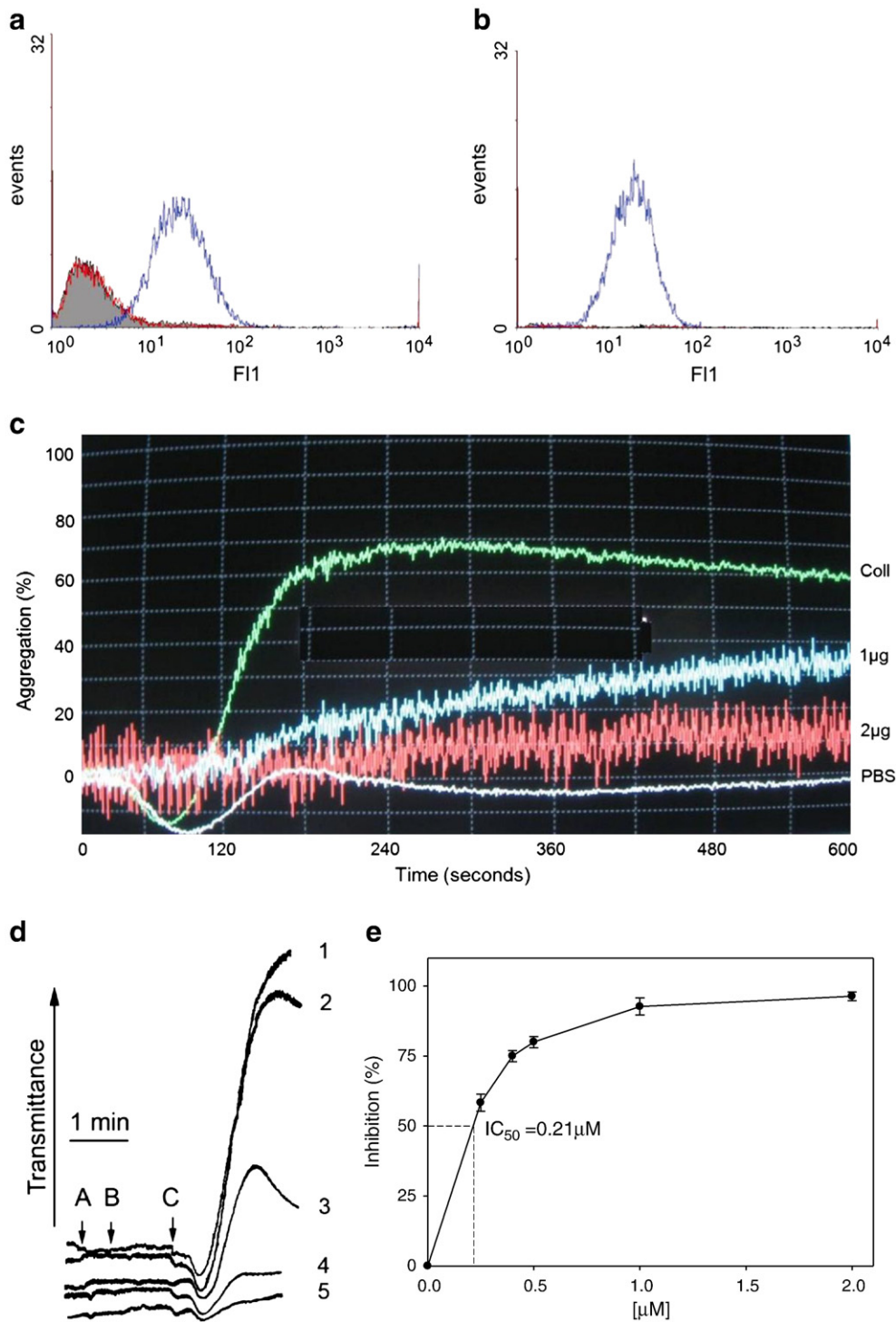
Fig. 9. *Bl*-LAAO does not directly interact with endothelial cells, but affects human platelet aggregation in PRP and washed platelets. (a) and (b) Flow cytometric histograms of HUVEC (a) and EAhy926 cells (b) after treatment with rhodocetin (blue line) and subsequent staining with the respective antibodies, both raised in rabbit. Histogram for the isotype control rabbit antibodies is shown in grey. (c) Aggregometer tracings of collagen-induced PRP aggregation inhibited by *Bl*-LAAO (1 and 2 μM) in comparison with the control group (PRP + collagen 10 μg/ml). PBS was also used as a negative control. (d) Washed platelets suspension were pre-incubated with the indicated concentrations of *Bl*-LAAO (1; collagen control, 2.5 μg/ml; *Bl*-LAAO: 2, 0.25 μg/ml; 3, 1 μg/ml; 4, 4 μg/ml; 5, 16 μg/ml). The time points marked with A, B and C show when L-Leu (2 mM), *Bl*-LAAO and collagen were added to platelet suspension. (e) Concentration dependence of *Bl*-LAAO inhibition of ADP-induced platelet aggregation. The data represent the means ± S.D. (n = 5).

L-Asp, L-Phe, L-Glu, L-Trp, and L-His. In the case of LAAO from the venom of the elapid king cobra *Ophiophagaus hannah,* L-Lys is the best substrate [34]. However, this residue is not oxidized by *Bl*-LAAO or is oxidized very slowly or not at all by the majority of venom LAAOs. It has been reported that LAAOs from different snakes differ in their substrate binding sites [35]. The difference in specificity of the king cobra enzyme has been attributed to differences in its primary structure, since the enzyme has less than 50% identity with the primary sequences of other homologous enzymes [34]. Furthermore, the crystallized structure of *C. rhodostoma* LAAO with L-Phe as
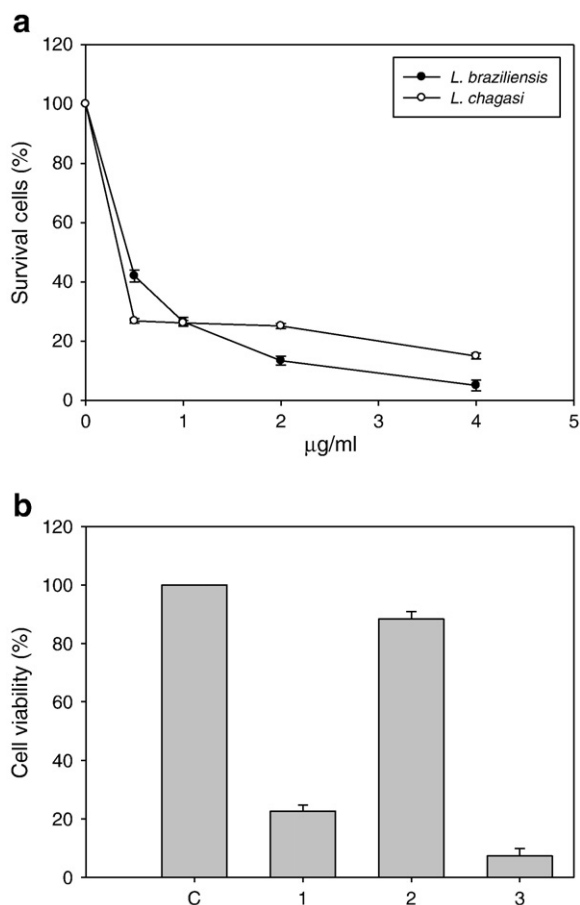
Fig. 10. Characterization of parasiticide effect of *Bl*-LAAO against promastigotes of *Leishmania* sp. (a) Leishmanicide dose-dependent activity induced by *Bl*-LAAO against *L. braziliensis* and *L. chagasi*. (b) C (control), promastigotes 4×10^5 cells of *L. chagasi* in Schneider Insect medium; 1, promastigotes + 10 μg of *Bl*-LAAO; 2, promastigotes + 10 μg *Bl*-LAAO + catalase (100 μg); and 3, promastigotes + H_2O_2 (5 mM). Bars represent S.D. (n = 4).

substrate provides a deeper understanding of the catalytic mechanism, leading to the conclusion that hydrophobic residues fit perfectly into the hydrophobic pocket in the catalytic cavity [36].

It is clear that venom LAAOs affect the function of platelets (activation or inhibition) through the formation of H_2O_2 because catalase, an H_2O_2 scavenger, inhibits these effects. However, any effect of ROS on platelets should be observed under conditions of potential physiological relevance [4]. We have conducted our studies using either the PRP system or washed platelets. In this work we showed that *Bl*-LAAO inhibits collagen-induced platelet aggregation of both human PRP and washed platelets, which may imply that different LAAOs affect platelets differently by a mechanism other than via H_2O_2 production. It would be reasonable to assume that *Bl*-LAAO has some influence on the hemostatic system since the venom of *B. leucurus* causes diverse hematological effects, among them a drastic hemostatic failure. Similar to *Bl*-LAAO, the enzymes from *N. naja kaoutia* [4], *V. berus berus* [26], *V. lebetina* [33], *N. naja oxiana* [27], and *Agkistrodon halys blomhoffii* [38] inhibited ADP- and collagen platelet aggregation in dose-dependent fashion (inhibitory concentrations in the range of 0.1–2.5 μM). Other enzymes such as LAAO from *N. atra* [5], *B. alternatus* [6], and *E. macmahoni* [25] induced aggregation in the same concentration range. The potential reasons for these differences have been discussed by some investigators [4,5,26]. The inhibitory potency may be connected to the interference of H_2O_2 in the interaction between the activated platelet integrin αIIb/β3 and fibrinogen in the PRP system [3] as well as by the reduced binding

for ADP in platelets exposed to H_2O_2. Induction of aggregation is also explained by H_2O_2 formation and thromboxane A_2 synthesis but independent of ADP [3]. However, several lines of evidence have revealed that the biological actions of ophidian LAAOs are only partly dependent on H_2O_2, suggesting that there are specific receptors or targets on the cells [5,39]. Yet, we have not found any direct interaction of *Bl*-LAAO with the cell surface of endothelial cells. Notwithstanding any direct cell interaction, in the present study we demonstrated that *Bl*-LAAO showed cytotoxicity towards a variety of cultured human cells including gastric carcinoma MKN-45, RKO, duodenal HUTU tumor cells and fibroblasts. The enzyme was able to induce apoptosis in these cells as examined by the two color fluorescence cell viability assay. A number of LAAOs from snake venoms were reported to show cytotoxicity by inducing cell apoptosis or necrosis [10–12,26,40]. Apoptosis induced by these enzymes is mainly attributed to the H_2O_2 generated upon oxidation of Leu/Met or other appropriate substrates, however, the specificity varies from one enzyme to another. It has been reported that the depletion of an essential amino acid substrate can trigger apoptosis by some members of the LAAO family using several cell lines. The enzyme from *C. rhodostoma* degrades L-amino acids (Tyr, Phe, Val, Leu) very efficiently within 4 h [40]. The apoptosis-inducing-protein (AIP) from parasite infected fish, depleted only Lys from mammalian cell cultured medium [41]. Studies conducted by Murakawa et al. [41] have demonstrated that AIP induced apoptosis in cells by two different mechanisms with different time courses; one fast and dependent on H_2O_2, the other slow and independent of H_2O_2. Similar results were reported in the characterization of the mechanism of apoptosis induced by the enzyme from *C. rhodostoma* [11,40]. The apoptosis-inducing activity of *Bl*-LAAO is caused by the enzymatic reaction product H_2O_2 as *Bl*-LAAO at 50 μg/ml was able to produce approximately 60 μM H_2O_2 within 4 h in HAMF12 medium. This dose is enough to induce apoptosis in MKN-45 cells.

We showed that *Bl*-LAAO presents an anti-Leishmania activity *in vitro*. Promastigotes of *L .braziliensis* and *L. chagasi* exhibited similar susceptibility to the venom enzyme. Very low EC_{50} values (0.07 and 0.08 μM) of the purified enzyme was obtained against the two *Leishmania* species. Removal of H_2O_2 by addition of catalase to the medium abolished approximately 90% of the leishmanicidal effect, suggesting a mechanistic role of H_2O_2. In this regard, catalase and superoxide dismutase, were reported in amastigotes of *Leishmania* sp., as its antioxidant defense mechanism [37]. Promastigotes were found to be deficient in catalase and glutathione peroxidase, with 80 to 90% promptly killed by macrophage H_2O_2 production during the infection. Our data are consistent with these findings, in which promastigotes of *Leishmania* sp. showed high susceptibility to *Bl*-LAAO.

In conclusion, *Bl*-LAAO, a major component of *B. leucurus* venom, is a cytotoxin acting primarily via the generation of high amounts of H_2O_2 which kill the cells. The functional mechanism and corresponding structural basis of snake venom flavoprotein activity need to be investigated in detail. Obviously, better knowledge of the mechanism of action of animal toxins allow us to consider the use of LAAOs as anticancer agents, as tools in biochemical studies to investigate cellular processes, and potentially give a better understanding of the envenomation mechanism.

Conflict of interest

The authors have declared no conflict of interest.

Acknowledgments

References

[1] P. Macheroux, O. Seth, C. Bollschweiler, et al., L-amino acid oxidase from Malayan pit viper *Calloselasma rhodostoma*. Comparative sequence analysis and characterization of active and inactive forms of the enzyme, Eur J Biochem 268 (2001) 1679–1686.

[2] A.E. Zeller, Snake venom action: are enzymes involved in it? Experientia 33 (1977) 143–150.

[3] X.-Y. Du, K.J. Clemetson, Snake venom L-amino acid oxidases, Toxicon 40 (2002) 659–665.

[4] Y.H. Sakurai, A. Takatsuka, A. Yoshioka, et al., Inhibition of human platelet aggregation by L-amino acid oxidase purified from *Naja naja kaouthia* venom, Toxicon 39 (2001) 1829–1833.

[5] R. Li, S. Zhu, J. Wu, W. Wang, Q. Lu, K.J. Clemetson, L-amino acid oxidase from *Naja atra* activates and binds to human platelets, Acta Biochim Biophys Sin 40 (2008) 19–26.

[6] R.G. Stabeli, S. Marcussi, G.B. Carlos, et al., Platelet aggregation and antibacterial effects of an L-amino acid oxidase purified from *Bothrops alternatus* snake venom, Bioorg Med Chem 12 (2004) 2881–2886.

[7] T. Ehara, S. Kitajima, N. Kansawa, T. Tamiya, T. Tsuchiya, Antimicrobial action of achacin is mediated by L-amino acid oxidase, FEBS Lett 531 (2002) 509–512.

[8] Y.J. Zhang, J.H. Wang, W.H. Lee, et al., Molecular characterization of *Trimeresurus stejnegeri* venom L-amino acid oxidase with potential anti-HIV activity, Biochem Biophys Res Commun 309 (2003) 598–604.

[9] P. Ciscotto, R.A. Machado de Avila, A.E.F. Coelho, et al., Antigenic, microbicidal and antiparasitic properties of an L-amino acid oxidase isolated from *Bothrops jararaca* snake venom, Toxicon 55 (2009) 330–341.

[10] S. Torii, K. Yamane, T. Mashima, et al., Molecular cloning and functional analysis of apoxin I, a snake venom-derived apoptosis-inducing factor with L-amino acid oxidase activity, Biochemistry 39 (2000) 3197–3205.

[11] S.R. Ande, P.R. Kommoju, S. Draxl, et al., Mechanism of cell death induction by L-amino acid oxidase, a major component of ophidian venom, Apoptosis 11 (2006) 1439–1451.

[12] S.M. Suhr, D.S. Kim, Comparison of the apoptotic pathways induced by L-amino acid oxidase and hydrogen peroxide, J Biochem Tokyo 125 (1999) 305–309.

[13] S.C. França, S. Kashima, P.G. Roberto, et al., Molecular approches for structural characterization of *Bothrops* L-amino acid oxidases with antiprotozoal activity: cDNA cloning, comparative sequence analysis, and molecular modeling, Biochem Biophys Res Commun 355 (2007) 302–306.

[14] E.F. Sanchez, T.V. Freitas, D. Ferreira-Alves, et al., Biological activities of venoms from South American snakes, Toxicon 30 (1992) 95–103.

[15] C.A. Bello, A.L. Hermogenes, A. Magalhaes, et al., Isolation and biochemical characterization of a fibrinolytic proteinase from *Bothrops leucurus* (white-tailed-jararaca) snake venom, Biochimie 88 (2006) 189–200.

[16] E.F. Sanchez, L.M. Gabriel, S. Gontijo, et al., Structural and functional characterization of a P-III metalloproteinase, leucurolysin-B, from *Bothrops leucurus* venom, Arch Biochem Biophys 468 (2007) 193–204.

[17] E.F. Sanchez, J.A. Eble, P-III metalloproteinase (Leucurolysin-B) from *Bothrops leucurus* venom: isolation and possible inhibition, in: C.T. Supuran, J.Y. Winum (Eds.), Drug Design of Zinc-Enzyme Inhibitors, 1st Edn., John Wiley & Sons, New Jersey, 2009, pp. 789–812.

[18] A. Magalhaes, H.P.B. Magalhaes, M. Richardson, et al., Purification and properties of a coagulant thrombin-like enzyme from the venom of *Bothrops leucurus*, Comp Biochem Physiol Mol Integrative Physiol 146A (2007) 565–575.

[19] D.A. Higuchi, C.M.V. Barbosa, C. Bincoleto, et al., Purification and partial characterization of two phospholipases A$_2$ from *Bothrops leucurus* (white-tailed-jararaca) snake venom, Biochimie 89 (2007) 319–328.

[20] U.K. Laemmli, Cleavage of structural proteins during the assembly of the head of bacteriophage T4, Nature London 227 (1970) 680–685.

[21] C.-J.S. Edgell, C.C. McDonald, J.B. Graham, Permanent cell line expressing human factor VIII-related antigen established by hybridization, Proc Natl Acad Sci 80 (1983) 3734.

[22] J.A. Eble, S. Neiland, T. Brancht, M. Mormann, J. Peter-Katalinic, G.S. Pohlentz, The alpha2beta1 integrin-specific antagonist rhodocetin is a cruciform, heterodimeric molecule, FASEB J 23 (2009) 2917–2927.

[23] E.F. Sanchez, S.F. Schneider, A. Yarleque, et al., The novel metalloproteinase atroxlysin-I from Peruvian *Bothrops atrox* (Jergón) snake venom acts both on blood vessel ECM and platelets, Arch Biochem Biophys 496 (2010) 9–20.

[24] L.F.M. Izidoro, M.C. Ribeiro, G.R.L. Souza, et al., Biochemical and functional characterization of an L-amino acid oxidase isolated from *Bothrops pirajai* snake venom, Bioorg Med Chem 14 (2006) 7034–7043.

[25] S.A. Ali, S. Stoeva, A. Abbasi, et al., Isolation, structural, and functional characterization of an apoptosis-inducing L-amino acid oxidase from leaf-nosed viper (*Eristocophis macmahoni*) snake venom, Arch Biochem Biophys 384 (2000) 216–226.

[26] M. Samel, H. Vija, G. Rönnholm, J. Siigur, N. Kalkkinen, E. Siigur, Isolation and characterization of an apoptotic and platelet aggregation inhibiting L-amino acid oxidase from *Vipera berus berus* (common viper) venom, Biochim Biophys Acta 1764 (2006) 707–714.

[27] M. Samel, K. Tönismagi, G. Rönnholm, et al., L-amino acid oxidase from *Naja naja oxiana* venom, Comp Biochem Physiol 149B (2008) 572–580.

[28] M.B. Hayes, D. Wellner, Microheterogeneity of L-amino acid oxidase. Separation of multiple components by polyacrylamide gel electrofocusing, J Biol Chem 244 (1969) 6636–6644.

[29] J.F. Wei, H.W. Yang, X.L. Wei, L.Y. Qiao, W.Y. Wang, S.H. He, Purification, characterization and biological activities of the amino acid oxidase from *Bungarus fasciatus* snake venom, Toxicon 54 (2009) 262–271.

[30] B.G. Stiles, F.W. Sexton, S.A. Weinstein, Antibacterial effects of different snake venoms: purification and characterization of antibacterial proteins from *Pseudoechis australis* (Australian king brown or mugla snake), Toxicon 29 (1991) 1129–1141.

[31] P.D. Pawelek, J. Cheah, R. Coulombe, P. Macheroux, S. Ghisla, A. Vrielink, The structure of L-amino acid oxidase reveals the substrate trajectory into an enantiomerically conserved active site, EMBO J 19 (2000) 4202–4215.

[32] E.F. Sanchez, A. Magalhaes, Purification and partial characterization of an L-amino acid oxidase from bushmaster snake (surucucu pico de jaca) *Lachesis muta muta* venom, Brazilian J Med Biol Res 24 (1991) 249–260.

[33] K. Tönismägi, M. Samel, K. Trummal, et al., L-amino acid oxidase from *Vipera lebetine* venom: isolation, characterization, effects on platelets and bacteria, Toxicon 48 (2006) 227–237.

[34] Y. Jin, W.H. Lee, Y. Zhang, Molecular characterization of L-amino acid oxidase from king cobra venoms, Toxicon 50 (2007) 479–489.

[35] X.L. Wei, J.F. Wei, L.Y. Qiao, Y.L. Liu, T. Huang, S.H. He, Purification, characterization and potent lung lesion activity of an L-amino acid oxidase from *Agkistrodon blomhoffii ussurensis* snake venom, Toxicon 50 (2007) 1126–1131.

[36] I.M. Moustafa, S. Foster, A.Y. Lyubimov, A. Vrielink, Crystal structure of LAAO from *Calloselasma rhodostoma* with an L-phenylalanine substrate insights into structure and mechanism, J Mol Biol 364 (2006) 991–1002.

[37] A.G. Tempone, H.F. Andrade Jr., P.J. Spencer, C.O. Lourenco, J.R. Rogero, N. Nascimento, *Bothrops moogeni* venom kills *Leishmania spp.* with hidrogen peroxide generated by its L-amino acid oxidase, Biochem Biophys Res Commun 280 (2001) 620–624.

[38] H. Takatsuka, Y. Sakurai, A. Yoshioka, et al., Molecular characterization of L-amino acid oxidase from *Agkistrodon halys blomhoffii* with special reference to platelet aggregation, Biochim Biophys Acta 1544 (2001) 267–277.

[39] H. Zhang, Q. Yang, M. Sun, M. Teng, L. Niu, Hydrogen peroxide produced by two amino acid oxidases mediates antibacterial actions, J Microbiol 42 (2004) 336–339.

[40] S.R. Ande, H. Fussi, H. Knauer, et al., Induction of apoptosis in yeast by L-amino acid oxidase from the Malayan pit viper *Calloselasma rhodostoma*, Yeast 25 (2008) 349–357.

[41] M. Murakawa, S.K. Jung, K. Iijima, S. Yonehara, Apoptosis-inducing protein, AIP, from parasite-infected fish induces apoptosis in mammalian cells by two different molecular mechanisms, Cell Death Differ 8 (2001) 298–307.