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Antiviral and antiparasite properties of an L-amino acid oxidase from the Snake *Bothrops jararaca*: Cloning and identification of a complete cDNA sequence

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ABSTRACT

L-Amino acid oxidases (LAAOs, EC 1.4.3.2) are flavoenzymes that catalyze the stereospecific oxidative deamination of an L-amino acid substrate to the corresponding α-ketoacid with hydrogen peroxide and ammonia production. The present work describes the first report on the antiviral (Dengue virus) and antiprotozoal (trypanocidal and leishmanicide) activities of *Bothrops jararaca* L-amino acid oxidase (BjarLAAO-I) and identify its cDNA sequence. Antiparasite effects were inhibited by catalase, suggesting that they are mediated by H_2O_2 production. Cells infected with DENV-3 virus previously treated with BjarLAAO-I, showed a decrease in viral titer (13–83-fold) when compared with cells infected with untreated viruses. Untreated and treated promastigotes (*T. cruzi* and *L. amazonensis*) were observed by transmission electron microscopy with different degrees of damage. Its complete cDNA sequence, with 1452 bp, encoded an open reading frame of 484 amino acid residues with a theoretical molecular weight and p*I* of 54,771.8 and 5.7, respectively. The cDNA-deduced amino acid sequence of BjarLAAO shows high identity to LAAOs from other snake venoms. Further investigations will be focused on the related molecular and functional correlation of these enzymes. Such a study should provide valuable information for the therapeutic development of new generations of microbicidal drugs.

1. Introduction

Snake venom components have been widely used in medicine as diagnostic or therapeutic tools and also as models in the studies of processes in cell biology. Snake venom proteins have been considered responsible for the killing of *Leishmania* spp. [1–4], HIV virus [5] and *Plasmodium falciparum* [6]. Recent studies revealed that the crude venom of South American *Bothrops* snakes inhibited growth of *Leishmania major* and *Trypanosoma cruzi* [1] and induced programmed cell death in *T.*

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cruzi [7]. However, isolation and characterization of the active component have yet to be carried out. In the last few years, L-amino acid oxidases (LAAOs, EC 1.4.3.2), enzymes from the class of oxidoreductases, have become an attractive object for the studies of enzymology, structural biology and pharmacology [8].

L-amino acid oxidases are flavoenzymes that catalyze the stereospecific oxidative deamination of an L-amino acid substrate to the corresponding α-ketoacid with the production of hydrogen peroxide and ammonia, with the reduction of FAD, via an imino acid intermediate [8]. The liberated hydrogen peroxide has been thought to contribute for most of the toxic effects of LAAOs. Snake venom LAAOs are usually homodimeric FAD-(flavine adenine dinucleotide) or FMN-(flavine mononucleotide) binding glycoproteins with a molecular mass around 110–150 kDa, when measured by gel filtration under non-denaturing conditions, and p*I* ranging from 4.4 to 8.12. These enzymes have been isolated from different venoms and are thought to contribute to their toxicity. LAAO effects on platelets and on induction of apoptosis, as well as its hemorrhagic, antibacterial and antiparasite effects have been shown to vary widely [8].

The present work describes the first report on the antiviral (Dengue virus) and antiprotozoal (trypanocidal and leishmanicide) activities of an L-amino acid oxidase isolated from *Bothrops jararaca* snake venom. Also, a cDNA sequence coding for this enzyme has been identified and compared with other snake venom LAAOs.

2. Materials and methods

2.1. Materials

A specimen of *B. jararaca* snake was supplied by the serpentarium of Universidade de São Paulo, Ribeirão Preto-SP. The venom was collected, vacuum desiccated and stored at 4 °C. All animal care was in accordance with the guidelines of the Brazilian College for Animal Experimentation (COBEA) and was approved by the Committee for Ethics in Animal Utilization of the USP (Proc. No. 05.1.1410.53.7) and IBAMA (Proc. No. 11781-1). *Leishmania* species used in the study were *L. amazonensis* (MPRO/BR/72/M1841-V-79), *L. braziliensis* (MHOM/BR/75/M2904) and *L. major* (MHOM/SU/59/P-39 clone 5ASKH). All other reagents needed for chemical and biological characterization were acquired from Amersham Life Science Inc., Sigma Chem. Co., BioLab, GIBCO BRL or Mediatech.

2.2. Biochemical characterization

For the purification of BjarLAAO-I, three chromatographic steps (Sephadex G-75, Benzamidine-Sepharose and Phenyl-Sepharose) were carried out as previously described [9]. For the enzyme purity assay, about 1% of the active sample was applied on a HPLC C18 reverse phase column (0.46 cm × 15 cm) equilibrated with 0.1% (v/v) trifluoracetic acid (TFA) and chromatographed on acetonitrile using a concentration gradient from 28 to 60% (v/v) in 0.1% (v/v) TFA for 32 min, at a flow rate of 1 ml/min, in addition to a 12% (w/v) SDS-PAGE and isoelectric focusing gel [9]. Treatment with peptide-N4-(acetyl-β-glucosaminyl)-asparagine amidase (PNGase F) under denaturing or nondenaturing conditions: a sample of 20 μg of purified enzyme was dissolved in 20 μl of 50 mM phosphate buffer, pH 7.5, treated with 1 μl of PNGase F (0.08 U/ml) and incubated at 37 °C for 4 h. PAGE and enzymatic assays were subsequently carried out to monitor deglycosylation and activity [9]. Amino acid sequence analysis was performed by a protein microsequencing system. For N-terminal sequencing, 20 μg of the BjarLAAO sample was applied on a 12% SDS-PAGE and electroblotted onto a polyvinylidene difluoride (PVDF) membrane. After staining with Coomassie blue the protein band of interest was cut and submitted to Edman degradation and also the internal peptide amino acid sequence was obtained from LAAO previously digested with trypsin and tryptic peptides analysed by ESI-CID-MS/MS [9].

2.3. Antiviral activity

2.3.1. Cytotoxic study

To measure the cytotoxicity of BjarLAAO-I on cell culture, a standard assay with C6/36 cells (*Aedes albopictus*) was used. Briefly, C6/36 cells in L15 medium plus 10% FBS were seeded and incubated at 28 °C for 24 h. Different amounts of enzyme (1.0; 2.5.0 and 5.0 μg/well) were added to C6/36 cell cultures and, after 4 days of incubation at 28 °C, the supernatants were removed and the remaining living cells were assessed, staining with 0.1% trypan blue solution. The percentage of stained cells was then determined.

2.3.2. Treatment of cells with DENV-3 and/or BjarLAAO-I + DENV-3

C6/36 cells were plated at 1×10^6 cells/ml/well with L15 medium plus 10% FBS in 12-well plates and then incubated for 24 h at 28 °C. 3.0 μg of BjarLAAO-I plus different amounts to DENV-3 (50, 250 and 500 PFU) diluted in PBS were pre-incubated at room temperature for 60 min (final volume = 50 μl). These solutions were then added to C6/36 cells and the plates were incubated for 60 min at 28 °C with intermittent mild agitation every 15 min. After this time, L15 medium plus 2% FBS to complete the assay volume were added. After 4 days at 28 °C, the cell culture supernatants were analyzed and quantified by RT-PCR system [10]. The controls used were C6/36 cells treated or untreated with different amounts of DENV-3, without BjarLAAO-I, under the same conditions described before.

2.4. Cytotoxic effect of LAAO on Leishmania viability

The direct cytotoxic effect of the BjarLAAO-I on *Leishmania* species was measured. Briefly, parasites (3×10^6 well^{-1}) were incubated in M199 medium supplemented with 10% heat-inactivated fetal calf serum (FCS) in the presence or absence of LAAO (0.2–5 μg/ml) for 4 h. Promastigotes of *L. braziliensis* were incubated with LAAO (10 μg/ml) and catalase (0.5 mg/ml) for 12 h at 25 °C in a microplate assay, in order to abolish the action of H_2O_2. Control groups without LAAO, with or without catalase, and with or without 6 mM hydrogen peroxide

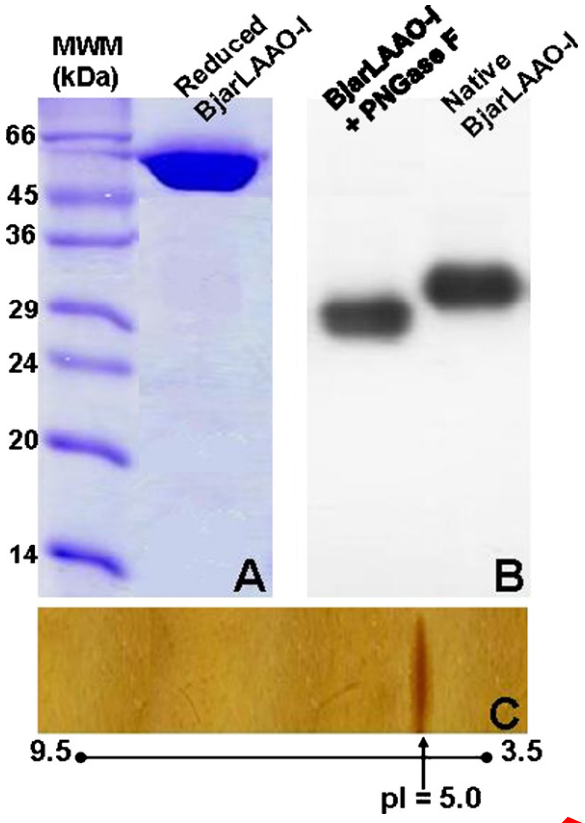

Fig. 1 – Purity analysis of BjarLAAO-I. (A) SDS-PAGE at 12% (w/v) in Tris–glycine buffer, pH 8.4 for 120 min at 10 mA and 200 V. Lines: 1, molecular weight markers; 2, *Bothrops jararaca* LAAO (30 μg). (B) Native PAGE (12%) BjarLAAO-I stained by enzymatic activity (see Section 2). Lines: 1, BjarLAAO-I after treatment with PNGase F (30 μg); 2, BjarLAAO-I native (30 μg); (C) Isoelectric focusing gel. Line: 1, BjarLAAO-I native (20 μg).

(SIGMA) were also tested. Parasites were then pulsed with 0.5 μCi/well [^{3}H] thymidine, and the incorporation of radioactivity by viable parasites was determined after 16 h in a β-counter [1,2].

2.5. Trypanocidal activity

LAAO was tested in vitro against *Trypanosoma cruzi* Y strain. The bioassays were carried out using infected blood of Swiss mice, which was collected on the parasitemic peak by cardiac puncture (7th day after infection with Y strain). The infected blood was diluted with non infected mice blood to achieve a concentration of 2×10^6 trypomastigotes forms/ml. BjarLAAO-I was added to the infected mouse blood to provide concentrations of 0.5, 2.0, and 8.0 μg/ml [1,2]. Plates were incubated at 4 °C for 24 h and trypanocidal activity was evaluated by the trypomastigote forms of the parasite that remained, according to Brener [11]. The bioassays were performed in triplicate on titration microplates (96 wells) containing 200 μl of mixture/well. Negative and positive controls containing either PBS or gentian violet (250 μg/ml) were run in parallel.

2.6. Electron microscopy

Promastigote forms were incubated in the absence or presence of LAAO (5 and 10 μg/ml) for 24 h. The parasites were washed twice in Ringer's solution (0.9% NaCl, 5.0% KCl, 5.0% $CaCl_2$) and fixed in a solution containing 2.5% glutaraldehyde, 4% formaldehyde and 3.7% sucrose in 0.1 M phosphate buffer (pH 7.2) for 1 h at room temperature. The parasites were then washed in 0.1 M cacodylate buffer (pH 7.2) and then gently scraped off with a rubber policeman and postfixed in a solution containing 1% OsO4, 0.8% potassium ferricyanide, and 5 mM calcium chloride in 0.1 M cacodylate buffer (pH 7.2) for 1 h at room temperature in the dark. They were then rinsed in cacodylate buffer, dehydrated in acetone and embedded in Epon. Ultrathin sections were stained with uranyl acetate and examined under a transmission electron microscope (900; Carl Zeiss, Oberkochen, Germany).

2.7. cDNA sequence of the L-amino acid oxidase

Total RNA was prepared from the *B. jararaca* venomous gland with RNeasy Mini Kit according to the manufacture's protocol. First strand cDNA was generated from total RNA using a tagged-oligo(dT) primer (5′-ggccacgcgtcgactagtac(t)-3′) with Super-Script II Reverse transcriptase (Invitrogen). The full length sequence of BjarLAAO was obtained by PCR using specific primers LAAO forward (atgaatgtcttctt-tatgttcte), LAAO internal forward (ggaaatctgagtcctggagc), LAAO reverse (ctcagaagcacgattcacatc), LAAO internal reverse (cgctttctttggcggaaggg) [1]. Terminator cycle sequencing ready reaction kit was used according to manufacturer's instructions (Applied Biosystems). PCR was carried out in a final volume of 60 μl containing 200 pmol of each primer, using 0.6 μl RT reaction mixture as DNA template. After denaturation at 94 °C for 5 min, Taq polymerase was added, followed by 31 cycles (94 °C for 30 s, 50 °C for 30 s, 72 °C for 2 min), and ended at 72 °C for 10 min. The electropherograms were analyzed by Sequencing Analysis software version 3.3 (Applied Biosystems). Basic local alignment search tools tblast-n and tblast-x were performed to identify the BjarLAAO-I (NCBI, Bethesda, MD, USA). Multiple alignments of the BjarLAAO-I sequence and sequences available in the GenBank and Swiss-Prot databases related to snakes were obtained using CLUSTALX program.

2.8. Statistical analysis

Data are presented as mean values ±S.D. obtained with recorded number of assays. For statistical significance, they were analyzed by Student's unpaired *t*-test at 5% level and performed using one-way ANOVA with differences considered significant if $p < 0.05$.

3. Results and discussion

Snake venom LAAOs (svLAAOs) represent interesting bioactive models for enzymology, structural biology and pharmacology. Recently, several svLAAOs have been purified and

characterized, showing distinct M_r, substrate preferences, platelet interactions and effects on hemorrhage induction and apoptosis [1,2,4,8,12–14]. Only four LAAOs have been isolated and characterized to date from *Bothrops* species, including those from *B. moojeni* with antitumor and leishmanicidal activity [2,3], *B. pirajai* and *B. alternatus* with antibacterial activity [12,13] and *B. insularis* with apoptotic and necrotic activities on renal system [15]. In a previous work, we have described the isolation of an LAAO from *B. jararaca* and characterized its antitumor activity [9]. In the present study, we show that this enzyme also possesses antiviral activity, as well as activity against *Leishmania* and *Trypanosoma*.

Under reducing SDS-PAGE conditions, the purified BjarLAAO-I showed a single band corresponding to an apparent molecular mass of 60 kDa (Fig. 1A), with pI of approximately 5.0 (Fig. 1C). Treatment of BjarLAAO-I with PNGase F (Fig. 1B) caused a change in the electrophoretic mobility in PAGE, indicating that the native enzyme is glycosylated. The enzymatic activity was not modified after deglycosylation (results not shown), suggesting that the sugar portion is not crucial for its activity. The biochemical properties of the purified BjarLAAO-I enzyme were consistent with those reported for other snake L-amino acid oxidases [8].

C6/36 cells treated with up to 3 μg of BjarLAAO-I did not show significant difference in the percentage of dead cells when compared with untreated cells (results not shown); therefore, this amount was chosen for antiviral tests. Cells infected with 500, 250 and 50 PFU of DENV-3, and previously treated with BjarLAAO-I, showed a decrease in viral titer (8-, 76-, and 13-fold, respectively) when compared with cells infected with untreated viruses (Table 1). Dengue viruses (DENV) are serious human pathogens that occur throughout the tropics and affect up to 100 million people each year. DENV belonging to genus *Flavivirus*, family *Flaviviridae*, are classified into four antigenically related serotypes (DENV-1 to DENV-4). Mosquitoes from genus *Aedes* (*A. aegypti*, *A. albopictus* and *A. polynesiensis*) play an important part in dengue transmission. The clinical spectrum of DENV infection can vary from an asymptomatic form, flu-like syndrome with rash (dengue fever [DF]), to dengue hemorrhagic fever or dengue shock syndrome (DHF/DSS). At the present time, there are no specific interventions for treatment or prevention of DF or DHF/DSS. Therefore, the development of new compounds for the treatment of patients infected with DENV is very important.

Table 1 – Viral titer in the supernatant of C6/36 cell culture infected with DENV-3 previously treated/untreated with BjarLAAO-I

Initial inoculums DENV-3 (PFU/well)	DENV-3 (PFU/ml) (after 4 days)	DENV-3 + BjarLAAO-I (PFU/ml) (after 4 days)
500	313	3.7
250	144	1.9
50	100	7.6

Viral titer was determined by RT-PCR.

The cellular viability of *Trypanosoma cruzi* and *Leishmania* sp. was investigated after treatment with LAAO. The addition of BjarLAAO-I directly to *T. cruzi* as well as to promastigotes of different *Leishmania* species resulted in a dose-dependent parasite killing (Fig. 2). This effect was almost completely abolished by the addition of catalase, suggesting that the release of H_2O_2 is directly involved with the parasiticidal effect of the enzyme. *Leishmania* species were more sensitive to the action of this LAAO than *T. cruzi*. Among the *Leishmania* species, *L. braziliensis* was by far the most sensitive to BjarLAAO-I, showing an almost complete cell death at the lowest dose tested. Amastigotes were not affected with an initial LAAO concentration of 200 μg/ml, as observed by the viability found after treatment (results not shown).

Leishmaniasis is an endemic tropical disease in South America with few therapeutic approaches. *Leishmania* causes a spectrum of diseases ranging from self-healing ulcers to disseminated and often fatal infections, depending on the

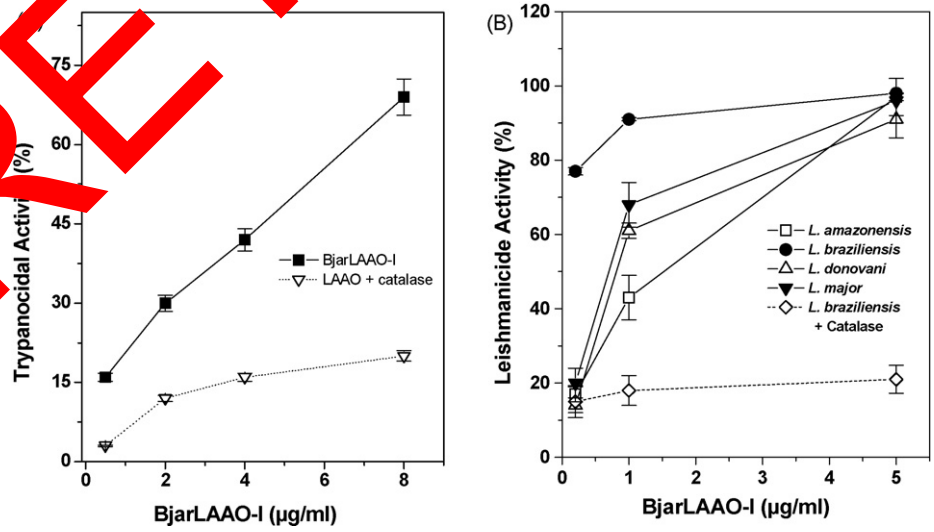

Fig. 2 – *Bothrops jararaca* LAAO parasiticide effects. Trypanocidal dose-dependent effect induced by the BjarLAAO-I enzyme on *Trypanosoma cruzi* parasite (A). Leishmanicide dose-dependent effect induced by the BjarLAAO-I enzymes on *Leishmania* spp. parasite (B). Data are expressed as the mean ± S.D. (*n* = 03).

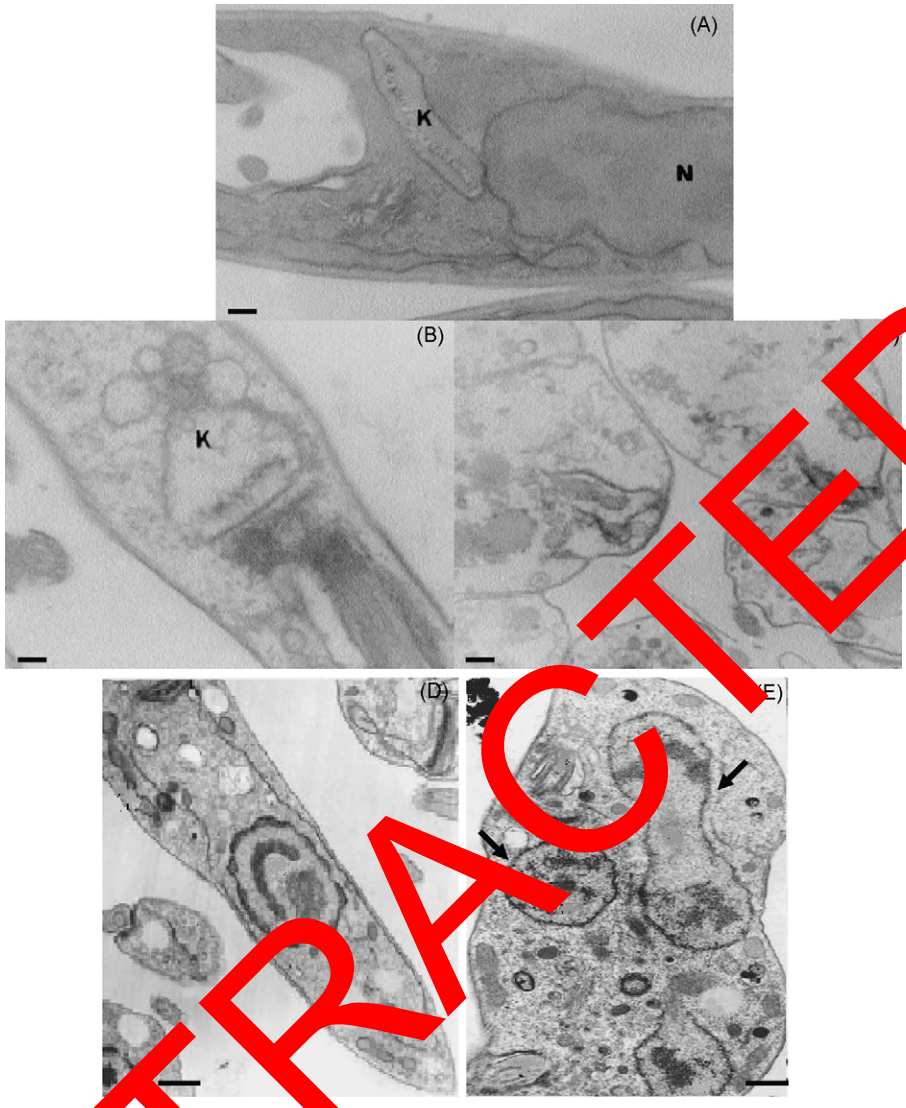

Fig. 3 – Transmission electron micro[illegible]pe of parasites treated with *Bothrops jararaca* LAAO. *Trypanosoma cruzi* epimastigote forms were incubated f[illegible] 24 h with 5 a[illegible] 15 μg/ml of BjarLAAO-I. (A) untreated parasite showing kinetoplast (*k*) and nucleus (*n*); (B) treat[illegible] parasite with 5 μg/[illegible] of BjarLAAO-I exhibiting kinetoplast disorganization. Note the gross alterations in the [illegible]ganizati[illegible] of the nuclear and kinetoplast chromatins. (C) Parasites completely destroyed after treatment with 15 [illegible]/ml[illegible] BjarLAAO-I. Transmission electron microscopy of *Leishmania amazonensis* promastigotes cultivated in [illegible]ntreate[illegible]) and tr[illegible]ed medium with BjarLAAO-I (E). Promastigotes treated for 24 h with enzyme (5 μg/ml) showing [illegible]terat[illegible]ns in t[illegible] fla[illegible]a or nucleus (arrows). Bars = 0.5–1.0 μm. These data are representative of three experi[illegible]ents.

species inv[illegible]d and host's immune response. Adequate protective vacc[illegible]es against trypanosomatid infections have yet to be developed, and drugs currently available for chemotherapeutic intervention are mostly unsatisfactory mainly because of their lack of specificity, toxicity to humans, and, in many cases, to developed parasite resistance [16]. Thus, one of the priorities in tropical medicine research has been the development of efficient drugs for treatment. The understanding of LAAO mode of action on parasites may trigger the design of new drugs or therapeutical approaches. Indeed, if one was able to target a hydrogen peroxide generator, as *B. jararaca* LAAO, towards the parasitophorous vacuole, this would represent a highly specific treatment not only for leishmaniasis, but also for other intracellular parasites.

Untreated and treated promastigotes (L. amazonensis) and epimastigotes (T. cruzi) were observed by transmission electron microscopy. Photomicrographs of the promastigotes with different degrees of damage are shown in Fig. 3. For treated *T. cruzi* with BjarLAAO-I, disruption of flagellar membranes, mitochondrial swelling and gross alterations in the organization of the nuclear and kinetoplast chromatins were detected. After 24 h in the presence of 15 μg of BjarLAAO-I, the parasites were completely destroyed (Fig. 3A–C). Mitochondrial swelling and important alterations in the organization of the nuclear and kinetoplast chromatins were

Fig. 4 – [illegible] and of deduced amino acid residues from BjarLAAO-I. Amino acid residues directly sequenced from the p[illegible]ein (underlined).

Table 2 – Peptide mass fingerprint of BjarLAAO obtained from tryptic peptides by MALDI-TOF-MS

m/z submitted	MH^+ matched	Delta (Da)	Start	End	Sequence
1165.830	1165.690	0.14	308	317	(R)IKFEPPLPPK (K)
1293.750	1293.645	0.15	86	96	(K)EGWYANLGPMR (L)
1293.730	1293.622	0.13	229	238	(K)HDDIFAYEKR (F)
1388.850	1388.693	0.15	340	351	(K)KFWEDDGIHGGK (S)
1486.830	1486.648	0.14	19	30	(R)ETDYEEFLEIAR (N)
1777.090	1776.838	0.22	86	100	(K)EGWYANLGPMRLPEK(H)[a]

[a] Met was modified by oxidation.

Fig. 5 – Comparison of *B. jararaca* BjarLAAO-I amino acid sequence with other L-amino acid oxidases from different snake venoms. Snake venom LAAOs from *Agkistrodon halys* AHP-LAAO (gi:82088273); *A. halys* M-LAO (gi:75570145); *Bothrops moojeni* BmooLAAO (gi:82127389); *B. jararacussu* BjussuLAAO (gi:82127391); *Calosellasma rhodostoma* (gi:20141785); *Crotalus adamanteus* (gi:6093636); *C. atrox* (gi:124106294); *Daboia russellii siamensis* (gi:70797645); *Notechis scutatus* (gi:123913796); *Oxyuranus scutellatus* (gi:123916680); *Pseudechis australis* (gi:123916679); *Trimeresurus stejnegeri* (gi:33355627 and gi:82090465). Multiple sequence alignment: (*) indicates positions with fully conserved residue; (:) indicates that one of the following high-scoring groups is conserved: K/R/Q/H, S/A, K/N/D, F/L/V/I, E/D/N/Q, T/S/A, I/M/L; (.) indicates that one of the following 'weaker' scoring groups is conserved: N/R/G, G/D/N, A/V/T, Q/K/E/R, S/K/G/A, D/K/H, C/S, T/P.

Fig. 5. (Continued)

observed by electron microscopy when *L. amazonensis* parasites were treated with 5.0 μg/ml of BjarLAAO-I (Fig. 3D and E). *B. jararaca* LAAO did not induce apoptosis in the macrophages cells, at concentrations of 1–25 μg/ml (results not shown).

Some authors have reported apoptosis-induced cell death after incubation with L-amino acid oxidase [8,14,17–20]. The oxidative stress induced by hydrogen peroxide could activate heat shock proteins described in *Leishmania* spp., inducing proteolytic activity inside the cell and also affecting mitochondrial function due to increased calcium concentrations [21].

A cDNA of 1452 bp was obtained, codifying a mature BjarLAAO-I with 484 amino acid residues (Fig. 4) correspondent to an estimated isoelectric point and molecular weight of 5.7 and 54,771.8, respectively. The N-terminal amino acid sequence and internal tryptic peptide sequences (Table 2), detected and characterized by mass spectrometry, suggested that this cDNA encodes the same enzyme purified from the venom (Fig. 4). Fig. 5 shows the amino acid alignment of *B. jararaca* LAAO and other LAAOs, indicating highly conserved amino acid residues. The structure of LAAO from *Calloselasma*

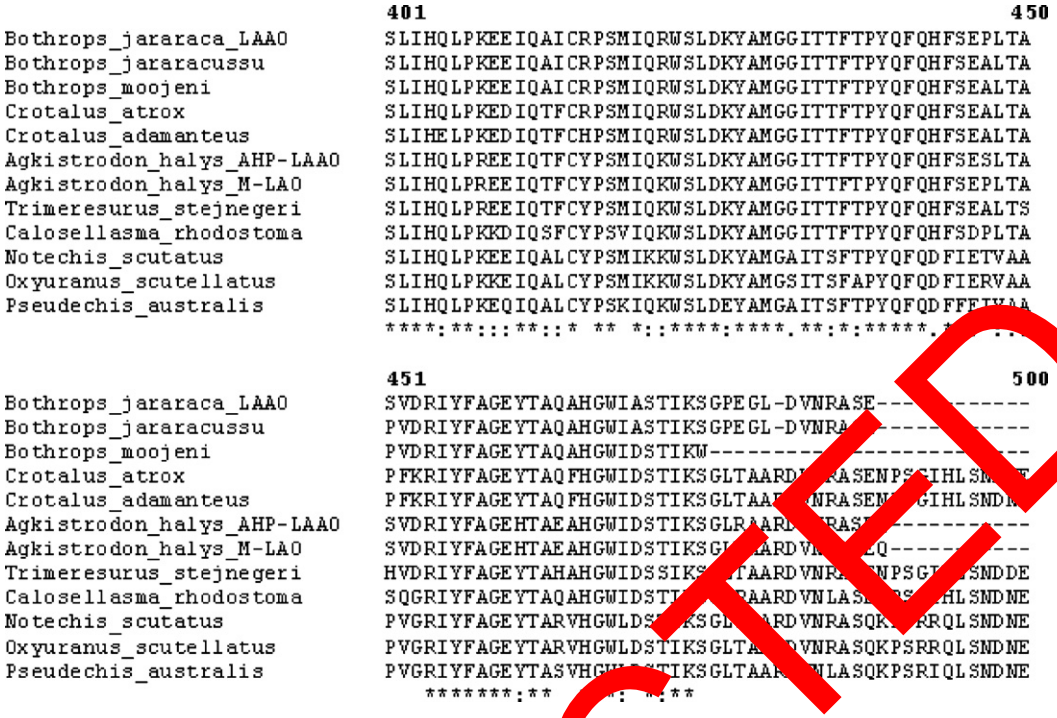

Fig. 5. (Continued).

rhodostoma revealed that residues 5–25 constitute one p… the substrate-binding domain. From the comparison of th… N-terminal sequence of LAAOs, at least 13 out of 24 amino ac… are highly conserved, suggesting that these c… d amin… acids may play an important role in the sub… ate bin… ng. The… cDNA-deduced amino acid sequence of… ake ven… LAAOs revealed that the N-terminal sequ… nce… f th… e prote… contains a highly conserved beta… pha-beta… d domain for the adenylate moiety of FAD … ding [1]. Paw… k and co-workers [22] showed a high… solut… X-ray cryst… ographic structure of *C. rhodostom…* LAAO, indic… ng it to be a dimer. Each subunit consist… of three doma… a FAD-binding domain, a substra… binding … omain and a helical domain. A deep groove … forme… at the interface between the substrate-binding a… e helical … omains, providing the substrate … to the … tive si… According to the circular dichroi… analy… s, BjarL… C secondary structure is predicte… to conta… 49% α-helix, 19% β-sheet, 12% β-turn and 20% ra… om … l stru… (results not shown).

Pawel… et al. [22] identified in the *C. rhodostoma* LAAO structure so… important residues involved in the stabilization of the FAD … olecule and in the orientation of an inhibitor to the active site of this enzyme. The side chains of residues Glu63, Arg71 and Glu457 make interactions with the FAD molecule, while the cofactor dimethylbenzene ring is surrounded by the hydrophobic residues Ile374, Trp420 and Ile430. According to these authors, another potentially essential residue of the *C. rhodostoma* LAAO structure is Lys326, which coordinates a water molecule that may be important in the hydrolytic attack on the imino intermediate. All these residues are conserved in the *B. jararaca* LAAO sequence, demonstrating the functional similarity between BjarLAAO-I and *C. rhodostoma* LAAO structures.

Snake … om LAAOs (svLAAOs) share a high degree of … ence homology among them, suggesting that additional LAAOs from other snake venoms might also exhibit antiviral, leishmanicide and trypanocidal activities. Further investigations will be focused on the related molecular and functional correlation of these enzymes. Such a study would provide valuable information on the therapeutic development of new generations of microbicidal drugs [23]. svLAAOs are therefore interesting multifunctional enzymes, not only for a better understanding of the ophidian envenomation mechanism, but also as models for potentially novel therapeutic agents.

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