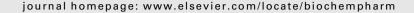


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# Antiviral and antiparasite properties of an L-amino acid oxidase from the Snake Bothrops jararaca: Cloning and identification of a complete cDNA sequence

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#### ABSTRACT

L-Amino acid o S, EC 1.4.3.2) are flavoenzymes that catalyze the stereospecific ino acid substrate to the corresponding  $\alpha$ -ketoacid with oxidative deamii ammonia production. The present work describes the first report on peroxide virus) and antiprotozoal (trypanocidal and leishmanicide) activities of antivi (Dengu Bothrops nino acid oxidase (BjarLAAO-I) and identify its cDNA sequence. araraca 1 vere inhibited by catalase, suggesting that they are mediated by roduction. Cells infected with DENV-3 virus previously treated with BjarLAAO-I, a decrease in viral titer (13-83-fold) when compared with cells infected with viruses. Untreated and treated promastigotes (T. cruzi and L. amazonensis) were observed by transmission electron microscopy with different degrees of damage. Its comlete cDNA sequence, with 1452 bp, encoded an open reading frame of 484 amino acid dues with a theoretical molecular weight and pI 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.

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# 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, Lamino 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  $\alpha$ -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 pI 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 described snake venom LAAOs.

# 2. Materials and methods

## 2.1. Materials

was suppl A specimen of B. jararaca sa by the serpentarium of Universide d São Paulo, ibeirão Preto-SP. The venom was collected, cuum desiccated and stored at 4 °C. All amal care was it ccordance with the guidelines of the Brazilia College for Animal Experimentation (COBEA and was proved by the Committee for ation of the USP (Proc. No. Ethics in Animal 05.1.1410.52 (Prog No. 11781-1). Leishmania d IBA species 1 ∠d in tÌ . amazonensis (MPRO/BR/72/ study oraziliensis (MHOM/BR/75/M2904) and L. √√-79), *I* M1844 o-SU/59/P). All other reagents major ` demical and biological characterization were Amersham Life Science Inc., Sigma Chem. acquired from Co., BioLab, GIB 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  $\times$  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  $20 \mu l$  of 50 mM phosphate buffer, pH 7.5, treated with  $1 \mu l$  of PNGase F (0.08 U/ml) and incubated at 37  $^{\circ}$ 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 80. system. For N-terminal sequencing, 24 sample was applied on a 12% SDS-T GE and electron a polyvinylidene difluoride VDF) membra roblotted . After staining with Coomassie blued the presin band of interest was cut and submitted to I man degration are also the internal peptide amino Ad sequence wa ained from LAAO previously diges with psin and tryptic peptides analysed by ESI-CV MS/M

## 2.3. Antival tivity

# 2.3.1. Cytotoxic study

To peasure the cytotoxic of BjarLAAO-I on cell culture, a standard assay with C6/36 cells (Aedes albopictus) was used. By affly, C6/36 cell oin L15 medium plus 10% FBS were seeded an incubated at 10 °C for 24 h. Different amounts of enzyme (1.0; 1.5.0 and 1.0  $\mu$ g/well) were added to C6/36 cell cultures and, after anys of incubation at 28 °C, the supernatants were considered and the remaining living cells were assessed, taining 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  $1\times10^6$  cells/ml/well with L15 medium plus 10% FBS in 12-well plates and then incubated for 24 h at 28 °C. 3.0  $\mu$ g of BjarLAAO-I plus different amounts to DENV-3 (50, 250 and 500 PFU) diluted in PBS were preincubated at room temperature for 60 min (final volume = 50  $\mu$ l). 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  $\times$  10<sup>6</sup> well<sup>-1</sup>) were incubated in M199 medium supplemented with 10% heatinactivated fetal calf serum (FCS) in the presence or absence of LAAO (0.2–5  $\mu g/ml$ ) for 4 h. Promastigotes of *L. braziliensis* were incubated with LAAO (10  $\mu g/ml$ ) 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

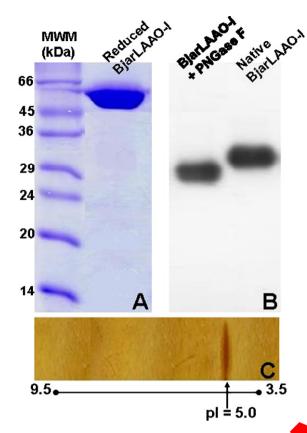


Fig. 1 – Purity analysis of BjarLAAO-I. (A) SDS-PAGE at 27% (w/v) in Tris-glycine buffer, pH 8.4 for 120 min at 10 mA and 200 V. Lines: 1, molecular weight markers; 2, Bothrop jararaca LAAO (30 μg). (B) Native PAGE (12%) — β. AAO-I stained by enzymatic activity (see Section 2). Line 1, BjarLAAO-I after treatment with PNGa F (30 μg); (B) BjarLAAO-I native (30 μg); (C) Isoelectric for sing gel. Line. 1, BjarLAAO-I native (20 μg).

(SIGMA) were also tested. Parasites were then pulsed with 0.5  $\mu$ Ci/well [ $^{3}$ H] the ridine, and the incorporation of radioactivity by viable, trasites was determined after 16 h in a  $\beta$ -counter [1,2].

# 2.5. ypano lal activ

LAAO 🗾 gainst Trypanosoma cruzi Y strain. The bioas s were carried out using infected blood of Swiss s collected on the parasitemic peak by cardiac puncture (7th 🕽 after infection with Y strain). The infected blood was diluted with non infected mice blood to achieve a concentration of  $2 \times 10^6$  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  $\mu$ g/ml) for 24 h. The parasites were washed twice in Ringer's solution (0.9% NaCl, 5.0% KCl, 5.0% CaCl<sub>2</sub>) 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% ferricyanide, and 5 mM calcium chloride in 0.1 M .codylate b er (pH 7.2) for 1 h at room temperature in the k. They were en rinsed in cacodylate buffer, dehydrated in a one and en edded in urany<sup>1</sup> Epon. Ultrathin sections was stained w. zetate and sion ele examined under a trans ron m ope (900; Carl any). Zeiss, Oberkochen, Ge

# 2.7. cDNA quence of the amin acid oxidase

Total RNA was pared from the B. jararaca venomous gland with RNeasy M. Kit according to the manufacture's col. First strand class was generated from total RNA ng a tagged-oligo(dT) primer (5'-ggccacgcgtcgactagtac(t)with Super-ript II Reverse transcriptase (Invitrogen). full length quence of BjarLAAO was obtained by PCR specific primers LAAO forward (atgaatgtcttctttatgttcte, AO internal forward (ggaaatctgagtcctggagc), reverse (ctcagaagcacgattcacatc), LAAO internal revers (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  $\pm$ 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<sub>r</sub>, 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 Bjar-LAAO-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 Bjar-LAAO-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 Bjar-LAAO-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 previo treated with BjarLAAO-I, showed a decrease in viral titer (8 76-, and 13-fold, respectively) when compared with cell infected with untreated viruses (Table 1). D (DENV) are serious human pathogens that cur thr ghout the tropics and affect up to 100 million eople e n vear. DENV belonging to genus Flavivirus, amı. (DENV-1 to classified into four antigenically rel ed seroty DENV-4). Mosquitoes from g Aedes (A. gypti, A. part in albopictus and A. polynesiene play n importan dengue transmission. The linical spec um of DENV infec-

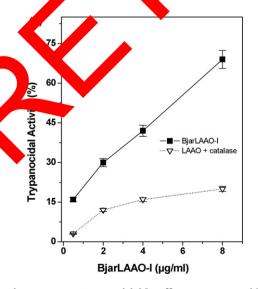
Table 1 – Viral titer in the supernatant of C6/36 cell culture infected with DENV-3 previously treated/untreated with BjarLAAO-I

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.					

tion can vary from an assympt atic n, flu-like s with rash (dengue fever [DFL]) dengue h orrha dengue shock syndrom (DHF/DS). At there are no specific in wention for treat sent time, for treatment or prevenre, the d S. T tion of DF or DHF/ elopment of new Spatier treatmen compounds for infected with DENV is very impor

The cellular viable of Trypanosoma cruzi and Leishmania sp. was investigated after atment with LAAO. The addition of no-I directly to T. is as well as to promastigotes of rent Leishmania species resulted in a dose-dependent asite killing 📜 g. 2). This effect was almost completely pa shed by the ddition of catalase, suggesting that the of H<sub>2</sub>O<sub>2</sub> directly involved with the parasiticidal effect Leishmania species were more sensitive to the of this LAAO than T. cruzi. Among the Leishmania pecies, 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



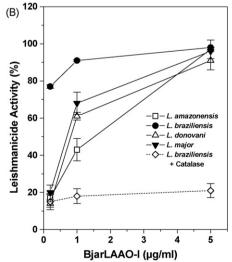


Fig. 2 – Bothrops jararaca LAAO parasiticide effects. Trypanocidal dose-dependent effect induced by the BjarLAAO-I enzyme on Trypanosoma cruzi parasite (A). Leishmanicide dose-dependent effect induced by the BjarLAAO-I enzymes on Leishmania spp. parasite (B). Data are expressed as the mean  $\pm$  S.D.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



Fig. 3 – Transmission electron microscope of parasites treated with Bothrops jararaca LAAO. Trypanosoma cruzi epimastigote forms were incubated ( $^{\prime}$  24 h with 5 and 15  $\mu$ g/ml of BjarLAAO-I. (A) untreated parasite showing kinetoplast ( $^{\prime}$ ) and nucleus ( $^{\prime}$ ); (B) treated parasite with 5  $\mu$ g/ml of BjarLAAO-I exhibiting kinetoplast disorganization. Note the gross alterations in the aganization of the nuclear and kinetoplast chromatins. (C) Parasites completely destroyed after treatment with 15  $\mu$ ml of BjarLAAO-I. Transmission electron microscopy of Leishmania amazonensis promastigotes cultivated in untreated ( $^{\prime}$ ) and tracked medium with BjarLAAO-I (E). Promastigotes treated for 24 h with enzyme (5  $\mu$ g/ml) showing atterations in the flag at a or nucleus (arrows). Bars = 0.5–1.0  $\mu$ m. These data are representative of three expertments.

species involved and host's immune response. Adequate protective vaccines 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

gcagatgacaaaaaccccttagaggaatgcttccgagaaactgactatqaqqaatttcta D D K N P L E E C F R E T D Y E E F qaqatcqccaqaaatqqtctqaaqqcaacatcaaacccqaaacqtqttqtqattqtaqqt <u>G L K A</u> T S N P K R V V I V gcaggaatgtctgggcttagtgcagcctatgttcttgcaaatgctggacatcaggtgaca A G M S G L S A A Y V L A N A G H Q V E R A G G Q K T Y R N E K qqctqqtatqccaatctcqqqcccatqcqtttacctqaqaaacacaqqattqtccqqqaa G P MRLP EKHRI tatatcagaaagtttggtctgcagttgaatgaattttctcaggaaaatgagaatgcatgg Y I R K F G L Q L N E F S Q E N E N A W tattttatcaaaaacatcaggaagagagtaggggaagtcaataaagaccctggcgtttt YFIKNIRKR V N K D P G E gactatcccgtgaagccttcagaagtaggcaaaagtgctggacagttatatgaagag D Y P V K P S E V G K S A G Q L Y E ctccaaaaggctgtagaagaattaagaaggactaactgcagctacatgctag LQKAVEELRRTNCSYM N K gacacctactcaacgaaggagtatctacttaaagaaggaaatctgagt cggagg DTYSTKEYLLKEGNL ctttta gatatgattggagacttactgaatgaagattctggctattatgt gaaago S F DMIGDLLNEDSGYY ctgaaacatgatgatatctttgcttatgaaaaaagatttg tgttggtg V G G M gataagttgcctacatccatgtatcgagccattcaggeeaaggtgcat raatqcccaa DKLPTSMYRAI K V H gtaatcaagatacagaagaatgctaaggaagtc agtgacatatcaaacctcagaaaag V T 1 VIKIQKNAKEV QTSEK atgcactac caagggccgcccgt gagacgttatctgtgacagctgattatgtcatt ETLSVTADYVI Т SRAAR cgcatcaagtttgaaccaccccttc ~aaagaaa tttqcqgtctgtccac KAHALRSVH P KFE tacagaagtggcaccaagatcttcctd ctto gaaattttgggaggatgatggc TKKFWEDD attcatggtgggaagtcg tgatc catcccgattcatctactaccctaaccat Т PSRFIYYPN H D L G G K aactttcctaatgq ttgggg tattata ctatggcattggtgatgatgccaattac N F P N AYGIGDDANY c'gatte 4 D F ggactgtggtgatattgtcattaatgacctttcattgatc tttgaagetc D C GDIVINDLSL raaggaagay ccaggccatctgtcgtccctcaatgattcaaagatgg gaagag ceaggeeacecgcegceeceaacgaccaaagacg Q L vctatgggtggtataaccaccttcactccctaccagtttcaacat MGGITTFTPYQF L D K accteteactgeaagegtagaeagaatetaetttgeaggggagtataeagee EPLTASVDRIYFAGEYTA ggattgccagcacaattaagtcaggtccagaggggctcgatgtgaat tcatqq' A H W I A S T I K S G P E G L D V N gag

and of deduced amino acid residues from BjarLAAO-I. Amino acid residues directly sequenced ein (underlined). from the

m/z submitted	MH <sup>+</sup> 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)

	1 50	Identity (%)
Bothrops_jararaca_LAA0	ADDK-NPLEECFRETDYEEFLEIARNGLKATSNPKRVVIVGAGMSGLSAA	100
Bothrops_jararacussu	ADDR-NPLEE CFRETD YEE FLEI AKNGL STTSNPKRVVI VGAGMSGLSAA	97 96
Bothrops_moojeni	ADDR-NPLEE CFRETDYEE FLEI AKNGL STTSNPKRVVI VGAGMSGLSAA	96 85
Crotalus_atrox Crotalus adamanteus	AHDR-NPLEECFRETDYEEFLEIAKNGLTATSNPKRVVIVGAGMAGLSAA AHDR-NPLEECFRETDYEEFLEIAKNGLTATSNPKRVVIVGAGMAGLSAA	85
Agkistrodon halys AHP-LAAO	ANDR-NPLEE CFRETDYEE FLEI ARNGLKATSNPKHVVVVGAGMAGLSAA	89
Agkistrodon halys M-LAO	ADDR-NPLEE CFRETDYEE FLEI ARNGLKATSNPKHVVI VGAGNSGLSAA	90
Trimeresurus stejnegeri	ADDR-NPLEE CFRETD YEE FLEI ARNGLKATSNPKHVVI VGAGNSGLSAA	87
Calosellasma rhodostoma	ADDR-NPLAECFQENDYEEFLEIARNGLKATSNPKHVVIVGAGMAGLSAA	85
Notechis scutatus	ADDRRRPLEE CFOE AD YEE FLEI ARNGINE TSNPKHVVVVGAGMAGISAA	78
Oxyuranus scutellatus	ADVRRNPLEECFRE AD YEE FLEI ARNGLKKTSNPKHVVVVGAGMAGLSAA	79
Pseudechis australis	ADDRRRPLEECFREADYEEFLEIAKNGLORTSNPKRVVVVGAGMAGLSAA	78
	* : .** ***: * ******* *** ****: **: *****	
	51 00	
Bothrops_jararaca_LAAO	YVLANAGHQVTVLEASERAGGQVKTYRNEKEGWYANLGPMRLPEKHRI	
Bothrops jararacussu	YVLANAGHOVTVLEASERAGGOVKTYRNEKEGWYANLGPMRLP HRIVR	
Bothrops moojeni	YVLANAGHQVTVLEASERAGGRVKTYRNEKEGWYANLGPMR/ KHRIVR	
Crotalus atrox	YVLAGAGHQVTVLEASERVGGRVRTYRKKDWYANLGPY PTKHRLYR	
Crotalus_adamanteus	YVLAGAGHQVTVLEASERVGGRVRTYRKKDWYANLC RLPTKHV R	
Agkistrodon halys AHP-LAAO	YVLSGAGHQVTVLEASERAGGRVRTYRNDKEDWYANLGA LPEV (IVR	<b>*</b>
Agkistrodon_halys_M-LAO	YVLSGAGHQVTVLEASERAGGRVRTYRNDKEGWY LGPMA .ARIVR	
Trimeresurus_stejnegeri	YVLAGAGHEVTVLEASERAGGRVRTYRNDEEG AN LGPMRL HRIVR	
Calosellasma_rhodostoma	YVLAGAGHQVTVLEASERPGGRVRTYRNEE ANLGPMRLPEL IV	
Notechis_scutatus	YVLAGAGHNVTLLEASERVGGRVNTYRNY ZGWY VGPMRLPERH. K	
Oxyuranus_scutellatus	YVLAGAGHKVTLLEASERVGGRVHTYRNEKEGWYVN. PMRLPERHRÏIR	
Pseudechis_australis	YVLAGAGHQVTLLEASERVGGRVNTVDMEKDGWYVNLU RLPERHRIIR	
	***:.***:**:****** **;	
	101 150	
Bothrops_jararaca_LAA0	EYIRKFGLQLNEFSQENENAW KNIRKRVGF NKDPGVLDYPVKPSEV	
Bothrops_jararacussu	EYIRKFGLQLNEFSQENENAWYI VIRKRV VNKDPGVLDYPVKPSEV	
Bothrops_moojeni	EYIRKFOLQLNE JAWYFIK. GEVNKDPGVLEYPVKPSEV	
Crotalus_atrox	EYIKK FOLKLINE QENE. FIKNIRKRVREVKNIPGLLEY PVKPSEE	
Crotalus_adamanteus	EYIKK FOLKLNE F. ENEM AWYF IKWIRKRVEEVKNNPGLLEY PVKPSEE EYIRK EGLOLNE FS WE AWYF IKWIRKRVGEVKKDPGVLKY PVKPSEE	
Agkistrodon_halys_AHP-LAAO Agkistrodon_halys_M-LAO	EYIP NEFSO NAWYFIKNIRKRVGEVKKDPGVLKYPVKPSEE	
Trimeresurus stejnegeri	EY KKFNL WEFSQE NAWHFVKNIRKTVGEVKKDPGVLKYPVKPSEE	
Calosellasma rhodostoma	IRKFOL NE FSQEN JAWYFIKNIRKKVGEVKKDPGLLKYPVKPSEA	
Notechis scutatus	VIRKER	
Oxyuranus scutellatus	E OV AKLNETT ANENAWYFIRNIRKRVWEVKKDPGVFKYPVKPSEE	
Pseudechis australis	EYN FGLELNEFIQENDNAWYFIKNIRKRVSEVKKDPGVFKYPVKPSEE	
	***: * . * * * * * * * * * * * * * * * *	
	151 200	
Bothrops_jararace_LAA0	CKS AG QLYEE SLQKAVEELRRTN CSYMLNKYD TY STKEY LLKE GN LSP GA	
Bothrops_jarar_assu	SAGQLYEE SLQKAVEELRRTNCSYMLNKYDTY STKEYLLKE GNLSP GA	
Bothrops_moo_hi	GR. AG QLYEE SLQKAVEELRRTN CSYMLNKYD TY STKEY LLKE GN LSP GA	
Crotalus_a	GKSAAQLYVE SLRKVVKELKRTNCKYILDKYDTY STKEYLLKE GNLSP GA	
Crotalus_ad ante	GKSAAQLYVE SLRKVVEELRSTNCKYILDKYDTY STKEYLLKE GNLSP GA	
Agkistrodon A AHP-LA/	GKSAGQLYEESLGKVVEELKRTNCSYILNKYDTYSTKEYLLKEGNLSPGA	
Aykı II II di II - LA	GKSAGQLYEESLGKVVEELKRTNCSYILNKYDTYSTKEYLLKEGNLSPGA	
Tri resu s_ste_ ge	GKSAEQLYEESLRKVEKELKRTNCSYILNKYDTYSTKEYLIKEGNLSPGA	
Cosella a_rhodo ma	GKSAGQLYEESLGKVVEELKRTNCSYILNKYDTYSTKEYLIKEGDLSPGA	
techis outstus	GKSASQLYRESLEKVIEELKRTNCSYILNKYDTYSTKEYLIKEGNLSRGA	
0. Ta s_seuc. Atus	GKSASQLYRESLKKVIEELKRTNCSYILNKYDTYSTKEYLIKEGNLSRGA	
Pse Chis_australis	GKSASQLYRESLQKVIEELKRTNCSYILNKYDTYSTKEYLIKEGNLSPGA	
_	anan nan mun ng gung mungugunannannannanngan ng an ma	

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.

\*\*\*\* \*\*\* \*\*\* \*. :\*\*: \*\*\*.\*:\*:\*\*\*\*\*\*\*\*

\*\*: \*\*: \*\*: \*

\* :

Bothrops\_jararaca\_LAAO Bothrops\_jararacussu Bothrops\_moojeni Crotalus atrox Crotalus\_adamanteus Agkistrodon\_halys\_AHP-LAA0 Agkistrodon halys M-LAO Trimeresurus\_stejnegeri Calosellasma rhodostoma Notechis scutatus 0xyuranus\_scutellatus Pseudechis australis

Bothrops jararaca LAAO Bothrops jararacussu Bothrops\_moojeni Crotalus atrox Crotalus adamanteus Agkistrodon\_halys\_AHP-LAAO Agkistrodon halys M-LAO Trimeresurus\_stejnegeri Calosellasma\_rhodostoma Notechis\_scutatus Oxyuranus\_scutellatus Pseudechis australis

Bothrops\_jararaca\_LAA0 Bothrops\_jararacussu Bothrops\_moojeni Crotalus atrox Crotalus adamanteus Agkistrodon\_halys\_AHP-LAAO Agkistrodon halys M-LAO Trimeresurus\_stejnegeri Calosellasma\_rhodostoma Notechis\_scutatus 0xyuranus\_scutellatus Pseudechis australi

Bothrops\_jara/aca\_LA Bothrops aracussu Bothrop oojeni atrox Crotal teus us\_adam Crot alys\_AHP-LAAO Agkis dor \_halys LAO akistr egeri rest ste Cal ellası ostoma Not his scut Lus cutellatus eudechis\_australis

VDMIGDL LNEDSGYYVSFIESLKHDDIFAYEKRFDEIVGGMDKLPTSMYR

VDMIGDLLNEDSGYYVSFIESLKHDDIFAYEKRFDEIVGGMDKLPTSMYQ VDMIGDLLNEDSGYYVSFIESLKHDDIFAYEKREDEIVGGMDKLPTSMYO VDMIGDLLNEDSGYYVSFIESLKHDDIFGYEKRFDEIVGGMDQLPTSMYE VDMIGDLLNEDSGYYVSFIESLKHDDIFGYEKRFDEIVGGMD0LPTSMYE VDMIGDLMNEDSGYYVSFPESLRHDDIFAYEKRFDEIVGGMDKLPTSMYR VDMIGDLMNEDSGYYVSFPESLRHDDIFAYEKRFDEIVGGMDKLPTSMYR VOM TGDI, MNEDA GV VV SETESMEHDDT FAVEER FDE TVD GMDE I. PTSM VR VDMIGDLLNEDSGYYVSFIESLKHDDIFAYEKRFDEIVDGMDKLPTAMYR VDMIGKLPNEDSSYYLSFIESLKSDDLFSYEKRFDEIVGGFDQLPISMYQ VDMIGDLLNEDSSYYLSFIESLKSDDLFSYEKRFDEIVGGFDQLPISMYQ VDMIGDLLNEDSSYYLSFIESLKSDDIFSYEKRFDEIVGGFDQLPRSMYO 

251 300 AIQEK--VHLNAQVIKIQKNAKEVTVTYQTSEKETLSVTADY CTTSR AIQEK--VHLNARVIKIQQDVKEVTVTYQTSEKETLSY TTSR TADYVIV AIQEK--VHLNARVIKIQQDVKEVTVTYQTSEKET SVTADYVIVCT AIKEKVQVHFNARVIEIQQNDREATVTYQTSANF MSSVTAD AIKEKVQVHFNARVIEIQQNDREATVTYQTSA IVCTTS YVIVCTTSR MASVT AIEEK--VHLNAQVIKIQKNAEKVTVVYQTPA PAKE ADYVIVC AIEEK--VHLNAQVIKIQKNAEKVTVVY TADYVIV AT PEKD TS TSR. AIEEK--VHFNAQVIKIQKNAEEVTV ETLSKETPS. CTTSR DIQDK--VHFNAQVIKIQQNDQKV AIAEM--VHLNAQVIKIQHNAEM AV. CPAKTLSYVTA IVCSTSR KTLSYVTAD YVIVCSSSR AIAEM--VHLNAQVIKIQHNAE.VRVAYQ AIAEK--VHLNAQVIKIQQNAEDVRVTYQT **LSYVIADYVIVCSTSR** 

301 HALRSVHYRSO AARRIKFEPPLPPK KI FLTCTKKFWEDDG IHGGKST AARRIKFEPPLPPKK LRSVHYRS KIFLTCTKKFWEDDGIHGGKST AARRIK PPLPPKKAN SVHV TKI FLTCTKK FWEDDG IHGGKST PKKAHALR RSGTKI FLTCKKKFWEDDG IRGGKST AARRIR AARRIK MALRSVHYRSGTKI FLTCTKKFWEDDG IHGGKST ♥VHYRSGTKI FLTCTKKFWEDEGIHGGKST ATRRIKE TRRIKFE KKAHALRSVHYRSGTKI FLTCTKKFWEDEG IHGGKST RIKFEP PLKKAHALRSVHYRSGTKI FLTCTKKFREDEG IHGGKST AV IKFNPP PKKAHALRSVHYRSGTKI FLTCTTKFWEDDG IHGGKST KKAHALRS IHYR SGTKI FLTCTRKFWEADG IHGGKST AA RIYFEPPI PPKKAHALRS IHYKSGTKI FLTC SKKFWEADG IHGGKST AARRIHFEPP LPPKKAHALRSIHYRSSTKI FLTCSQKFWEADGIHGGKST

\*\*\*\*\*\*\*

350

TDL PSRFIYY PNHN FPNGV GVII AY GIGDD AN YFEALD FED CGDIVINDL TDL PSRFIYY PNHN FPNGV GVII AY GIGDD AN YFEALD FED CGDIVINDL TDL PSRFIYYPNHNFPNGVGVII AYGIGDD ANYFQALDFED CGDIVINDL TDL PSRFIYY PNHN FTSGV GVII AY GIGDD AN FFQALD FKD CADIVINDL TDL PSRFIYY PNHN FTSGV GV II AY GIGDD AN FFOALD FKD CADIVINDL TDL PSRFIYYPNHNFTSGVGVII AYGIGDD ANFFQALDFKD CADIVINDL TDLPSRFIYYPNHNFTSGVGVIIAYGIGDDANFFOALDFKDCADIVINDL TDL PSRFIYYPNHNFTSGVGVII AYGIGDD AN FFQALDLKD CGDIVINDL TDL PSRFIYY PNHN FTNGV GVII AY GIGDD AN FFQALD FKD CADIVFNDL TDL PSRFIYYPNHNFTSDVGVIVAYTLADD AD FFQALDIKTSADIVINDL TDL PSRFIYYPNHNFTSGVGVIVAYTISDD AD FFQSLDIKTSADIVINDL TDL PSRFIYYPNHSFTSGIGVIVAYTLADDTDFFQALDIETSADIVINDL \*

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

Bothrops\_jararaca\_LAA0
Bothrops\_jararacussu
Bothrops\_moojeni
Crotalus\_atrox
Crotalus\_adamanteus
Agkistrodon\_halys\_AHP-LAA0
Agkistrodon\_halys\_M-LA0
Trimeresurus\_stejnegeri
Calosellasma\_rhodostoma
Notechis\_scutatus
Oxyuranus\_scutellatus
Pseudechis\_australis

Bothrops\_jararaca\_LAA0
Bothrops\_jararacussu
Bothrops\_moojeni
Crotalus\_atrox
Crotalus\_adamanteus
Agkistrodon\_halys\_AHP-LAA0
Agkistrodon\_halys\_M-LA0
Trimeresurus\_stejnegeri
Calosellasma\_rhodostoma
Notechis\_scutatus
Oxyuranus\_scutellatus
Pseudechis\_australis

SLIHQLPKEEIQAICRPSMIQRWSLDKYAMGGITTFTPYQFQHFSEPLTA
SLIHQLPKEEIQAICRPSMIQRWSLDKYAMGGITTFTPYQFQHFSEALTA
SLIHQLPKEEIQAICRPSMIQRWSLDKYAMGGITTFTPYQFQHFSEALTA
SLIHQLPKEDIQTFCRPSMIQRWSLDKYAMGGITTFTPYQFQHFSEALTA
SLIHELPKEDIQTFCYPSMIQRWSLDKYAMGGITTFTPYQFQHFSEALTA
SLIHQLPREEIQTFCYPSMIQKWSLDKYAMGGITTFTPYQFQHFSESLTA
SLIHQLPREEIQTFCYPSMIQKWSLDKYAMGGITTFTPYQFQHFSEPLTA
SLIHQLPKEEIQTFCYPSMIQKWSLDKYAMGGITTFTPYQFQHFSEPLTA
SLIHQLPKKEIQALCYPSWIKKWSLDKYAMGGITTFTPYQFQHFSEPLTA
SLIHQLPKEEIQALCYPSMIKKWSLDKYAMGSITSFTPYQFQDFIETVAA
SLIHQLPKKEIQALCYPSMIKKWSLDKYAMGSITSFAPYQFQDFIETVAA
SLIHQLPKKEIQALCYPSKIQKWSLDEYAMGAITSFTPYQFQDFIETVAA

500 SVDRIYFAGEYTAQAHGWIASTIKSGPEGL-DVNRASE-PVDRIYFAGEYTAOAHGWIASTIKSGPEGL-DVNRA PVDRIVEAGEVTAGAHGMIDSTIKM-----PFKRIYFAGEYTAQFHGWIDSTIKSGLTAARD KASENPS SIHLSI PFKRIYFAGEYTAQFHGWIDSTIKSGLTAA NRASEN GIHLSND SVDRIYFAGEHTAEAHGWIDSTIKSGLRAAR **Z**Q----SVDRIYFAGEHTAEAHGWIDSTIKSG ARDV NPSG TAARD VNR. SNDDE HVDRIYFAGEYTAHAHGWIDSSIK 00 AL SND NE SQGRIYFAGEYTAQAHGWIDST RAARD VN LAS RRQLSNDNE KS GL PVGRIYFAGEYTARVHGWLDS RDVNRASQK PVGRIYFAGEYTARVHGWLDSTIKSGLTA NVNRASQKPSRRQLSNDNE PVGRIYFAGEYTASVHG TIKSGLTAAR NLASQKPSRIQLSNDNE \*\*\*\*\*\*

Fig. 5. (Continued).

rhodostoma revealed that residues 5-25 constitute one p the substrate-binding domain. From the comparison of th terminal sequence of LAAOs, at least 13 out of 24 amino ac are highly conserved, suggesting that these car d amin acids may play an important role in the sub ate bin ng. The cDNA-deduced amino acid sequence of ake ven revealed that the N-terminal sequ nce ha-beta ار d domain for contains a highly conserved beta ling [1]. Paw the adenylate moiety of FAD k and coworkers [22] showed a high solut X-ray crysta ographic structure of C. rhodostom LAAO, indic ing it to be a dimer. Each subunit consist of three domai a FAD-binding domain, a substrati oinding omain and a helical domain. forme at the interface between the A deep groove e helical mains, providing the substrate-binding a tive si to the According to the circular substrate dichroig s, Bjarl. secondary structure is preanaly  $\frac{49\%}{\alpha}$  α-helix, 19% β-sheet, 12% β-turn and contai results not shown). 20% ra

Pawelt et al. [22] identified in the *C. rhodostoma* LAAO structure sold important residues involved in the stabilization of the FAD colecule 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.

Snaw Laom LAAOs (svLAAOs) share a high degree of sence homology among them, suggesting that additional LAAO. 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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  Lania donovani heat shock protein. Characterization and function in amastigote stage differentiation. J Biol Chem 1998;13:6488–94.
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