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Bioorganic & Medicinal Chemistry 14 (2006) 7034–7043
Bioorganic & Medicinal Chemistry

Biochemical and functional characterization of an L-amino acid oxidase isolated from *Bothrops pirajai* snake venom

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Received 20 April 2006; revised 29 May 2006; accepted 8 June 2006
Available online 30 June 2006

Abstract—In this work we describe the isolation of a new L-amino acid oxidase (LAAO) referred to as BpirLAAO-I from *Bothrops pirajai* snake venom, which was highly purified using a combination of molecular exclusion, affinity, and hydrophobic chromatography steps. BpirLAAO-I homodimeric acid glycoprotein (approximate *Mr* and p*I* of 130,000 and 4.9, respectively) displays high specificity toward hydrophobic/aromatic amino acids, while deglycosylation does not alter its enzymatic activity. The N-terminal LAAO sequence of its first 49 amino acids presented a high similarity between a amino acid sequence with other LAAOs from: *Bothrops* spp., *Crotalus* spp., *Calloselasma rhodostoma*, *Agkistrodon* spp., *Trimeresurus* spp., *Pseudechis australis*, *Oxyuranus scutellatus*, and *Notechis scutatus*. BpirLAAO-I induces time-dependent platelet aggregation, mouse paw edema, cytotoxic activity against *Escherichia coli*, *Pseudomonas aeruginosa*, *Leishmania* sp., and tumor cells, and also a typical fago (M13mp18) DNA fragmentation. Platelet aggregation, leishmanicidal and antitumoral activities were reduced by catalase. Thus, BpirLAAO-I is a multifunctional protein with promising biotechnological and medical applications.

1. Introduction

Snake venoms are recognized as useful sources of bioactive substances showing a wide range of pharmacological activities. This complex cocktail of both toxic and non-toxic components includes several peptides and enzymes, such as L-amino acid oxidases (LAAO, EC 1.4.3.2) which may represent 1–9% of the total venom proteins.[1] L-Amino acid oxidases are flavoenzymes which catalyze the stereospecific oxidative deamination of an L-amino acid substrate to a corresponding α-ketoacid with hydrogen peroxide and ammonia production.[2] These enzymes, which are widely distributed in many different organisms, exhibit a marked affinity for hydrophobic amino acids, including phenylalanine, tryptophan, tyrosine, and leucine.[3]

Although the exact biological function of snake venom LAAOs is still unknown, these enzymes are postulated to be toxins that may be involved in the allergic inflammatory response and specifically associated with mammalian endothelial cells damage.[4,5] Before 1990, studies in the field of SV-LAAOs dealt mainly with their enzymatic and physiochemical properties.[6,7] However, in the last decade these enzymes have become an

Abbreviations: ATCC, American Type Culture Collection; BpirLAAO, *Bothrops pirajai* L-amino acid oxidase; FAD, flavin adenine dinucleotide; ip, intrapritoneal via; LAAO, L-amino acid oxidase; PBS, phosphate-buffered saline; PGNase, peptide *N*-glucosidase; RP-HPLC, reverse-phase high performance liquid chromatography; SDS–PAGE, sodium dodecyl sulfate polyacrylamide gel eletrophoresis; SV-LAAO, snake venom L-amino acid oxidase.

Keywords: Bactericidal and cytotoxic effects; *Bothrops pirajai*; L-amino-acid oxidase; Platelet aggregation; Snake venom; Structural analysis.

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doi:10.1016/j.bmc.2006.06.025

interesting subject for pharmacological, structural, and molecular characterizations. Briefly, structural and functional characterization of SV-LAAOs has shown: different molecular masses, a broad range of bactericidal activities, platelet aggregation effects, edema, apoptosis induction, and other activities.[1,8–16] The present work reports the isolation, biochemical and pharmacological characterization of a new L-amino acid oxidase isoform from *Bothrops pirajai* (BpirLAAO-I) snake venom, with special attention to its bactericidal, leishmanicidal, and cytotoxic effects, in addition to platelet aggregation and edema induction.

2. Results and discussion

2.1. Purification and biochemical characterization of BpirLAAO-I

In this work, the purification of BpirLAAO-I was successfully carried out by three chromatographic steps (Fig. 1). After the first chromatography on a Sephadex G-75 column, the active fraction referred to as PI was identified by means of enzymatic assays (Fig. 1A). PI fraction was concentrated and subsequently placed on a Benzamidine–Sepharose column (Fig. 1B). LAAO activity was detected in the second fraction, named PI-BII. The active pool (PI-BIIa) was resolved into other seven fractions on a Phenyl-Sepharose column (Fig. 1C). The active fraction named BpirLAAO-I isoform was still analyzed for purity by HPLC reversed-phase chromatography (Fig. 2A). BpirLAAO-I molecular mass was estimated to be approximately 66 kDa by SDS–PAGE (Fig. 2B), its p*I* being around 4.9. BpirLAAO-I deglycosylation was confirmed by PAGE, when the enzyme was treated with PGNase F and *O*-glycosidase (Fig. 2C). The BpirLAAO-I enzymatic activity was not modified after deglycosylation (Fig. 2D), suggesting that the sugar portion is not crucial for its activity.

SV-LAAOs are usually homodimeric, FAD-binding glycoproteins, with a molecular mass of around 110–150 kDa when measured by gel filtration under non-denaturing conditions. However, the molecular mass of SV-LAAOs is around 50–70 kDa when assayed by SDS–PAGE, both under reducing and non-reducing conditions. Thus, most of the SV-LAAOs are homodimeric glycoproteins associated by non-covalent bonds with an approximately p*I* of 4.4–8.2.[8,11,13,15–20]

BpirLAAO-I N-terminus sequencing demonstrates that the purified enzyme shows a high degree of purity (Fig. 2). Moreover, the first 49 N-terminal amino acid residues from BpirLAAO-I were accessed by Edman degradation in order to obtain additional evidence of enzyme purity, and performing structural comparisons between BpirLAAO-I and different SV-LAAOs (Fig. 3). Comparison of the N-terminal amino acid sequence between BpirLAAO-I and LAAO enzymes previously reported from other snake venoms revealed a close sequence similarity. In the N-terminal sequences, at least 32 amino acid residues were found to be fully conserved in all sequences, suggesting the presence of a highly conserved Glu rich motif. The structure of LAAO from *Calloselasma rhodostoma* revealed that residues 5–25 constituted one part of the substrate-binding domain. From the comparison of the N-terminal sequence of LAAOs at least 13 out of 24 amino acids were found highly conserved, suggesting that these conserved amino acids may play an important role in substrate binding. The cDNA-deduced amino acid sequence of snake venom LAAOs revealed that the N-terminal sequence of these proteins contains a highly conserved β–α–β-fold domain for the adenylate moiety of FAD binding.[1,3,5]

Pawelek et al.[3] showed a high-resolution X-ray crystallographic structure of *C. rhodostoma* LAAO which indicates that SV-LAAO is a dimer. Each subunit consists of three domains: a FAD-binding domain, a substrate-binding domain, and a helical domain. A deep groove is formed at the interface between the substrate-binding and the helical domains, providing the substrate access to the active site. According to our CD analysis BpirLAAO-I secondary structure is predicted to contain: 48% α-helix, 20% β-sheet, 12% β-turn, and 20% random coil structure.

For further biochemical characterization of BpirLAAO-I its affinity to different substrates was accessed. BpirLAAO-I showed higher affinity to hydrophobic and long side-chain amino acids, namely, Phe > Tyr > Trp > Leu > Met > Ile > Val > His. For other amino acids (Ala, Arg, Pro, Thr, Ser, Glu, Gly, Lys) the catalytic affinity was very low (Table 1). After deglycosylation the affinity picture did not change (results not shown). A previous study also reports affinity pattern for LAAOs isolated from other snake venoms;[1] this catalytic preference can be explained by differences of side-chain binding sites in the enzyme—responsible for substrate specificity.[17]

As expected when the BpirLAAO-I was submitted was exposed to very high or low extreme temperatures and pHs we could observe a strong reduction of its L-Phe and L-Leu catalytic activity (data not shown). Optimum pH and temperature for enzymatic activity of BpirLAAO-I were between 6.0–7.4 and 37 °C, respectively.

2.2. Functional characterization of BpirLAAO-I

As a kind of naturally existing protein library, snake venoms have been widely investigated for therapeutic purposes and other clinical uses.[21,22] Snake venom proteins from *Bothrops* genus affect the homeostasis prey in different ways promoting hemorrhages, hemolysis, edema, and other effects on blood circulation and nervous systems.[23] The most well-known venom components are the hemorrhagic metalloproteases, serine-proteases, and phospholipases A_2.[24–26] On the other hand, L-amino acid oxidases are widely expressed in snake venoms and catalyze the oxidative deamination of L-amino acids, producing the corresponding α-ketoacids, hydrogen peroxide, and ammonia.

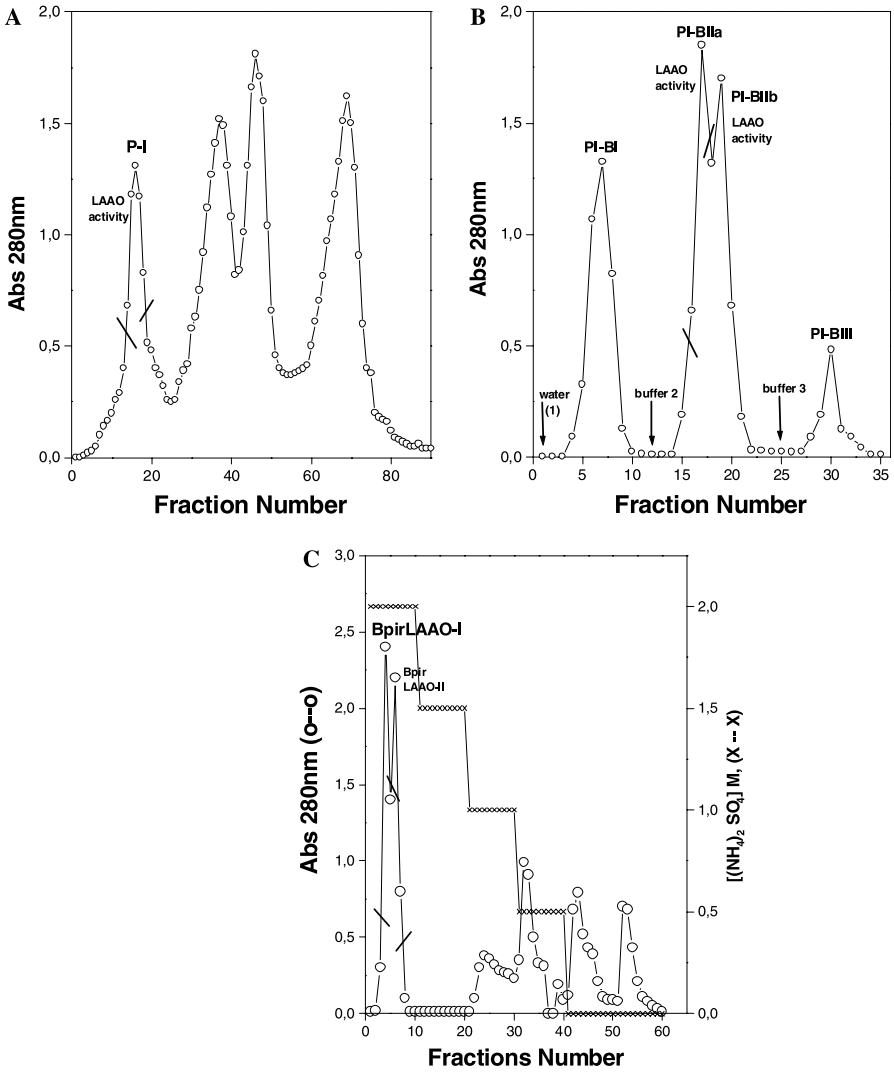

Figure 1. Isolation of BpirLAAO. (A) Gel filtration chromatography of *Bothrops pirajai* venom on a 4.0×90.0 cm column of Sephadex G-75 previously equilibrated with ammonium bicarbonate buffer (0.05 M, pH 7.8) and eluted at the flow rate of 30 mL/h with the same buffer. (B) PI fraction was chromatographed on a 1.8×4.5 cm column of Benzamidine–Sepharose previously equilibrated and eluted with Milli-Q water (1), followed by 0.02 M sodium phosphate (buffer 2) and glycine buffer (0.02 M, pH 3.2) (buffer 3) a flow rate of 5 mL/tube. (C) PI-BIIa was chromatographed on a 1.8×5.5 cm column of Phenyl-Sepharose previously equilibrated with Tris–HCl (0.01 M, pH 8.6), plus 2.0 M ammonium sulfate and eluted with a decreasing concentration gradient from 2.0 to 0.0 M of ammonium sulfate.

One of the most reported biological effects of SV-LAAOs is the induction or inhibition of platelet aggregation.[8,13,14,18,27–29] In our work, the BpirLAA O-I induced a dose-time-dependent platelet aggregation in platelet rich plasma (PRP), and catalase inhibited that activity (Fig. 4A). Du and Clemetson[1] suggested that the hydrogen peroxide production promoted a rapid increase of tromboxan A_2 synthesis and consequently the platelet aggregation. Indeed, Toyama et al.[13] showed that incubation of casca-LAAO from *Crotalus durissus cascavella* in the presence of catalase abolished its effects on platelet aggregation. Also, indomethacin and aspirin, which have been used as general inhibitors of cyclooxygenase enzyme, were able to inhibit platelet aggregation induced by casca-LAAO, suggesting that the hydrogen peroxide production involves a subsequent activation of the inflammatory response pathway.[13]

BpirLAAO-I was also able to induce a dose-time-dependent edema in the mouse paw. Even at low concentration (0.04 μg/μL), an extensive edema could be observed within 1 h after enzyme inoculation, followed by mouse physiological recovery (upon 3 h) and consequently edema activity decreasing (Fig. 4B), likewise observed in experiments previously described.[10,13] Once more, the inflammatory response could be recruited in order to explain this observation, particularly when considering the SV-LAAOs phospholipase A_2 activity.[13]

As shown in Figure 5 BpirLAAO-I inhibited the growth of approximately 4×10^5 CFU of *Pseudomonas aeruginosa* (ATCC-27853) and *Escherichia coli* (ATCC-25923) when compared with positive controls (Fig. 5A and B). Bactericidal effect against both Gram positive and Gram negative bacteria were previously reported for LAAOs from *Bothrops alternatus*, *Crotalus adamanteus*,

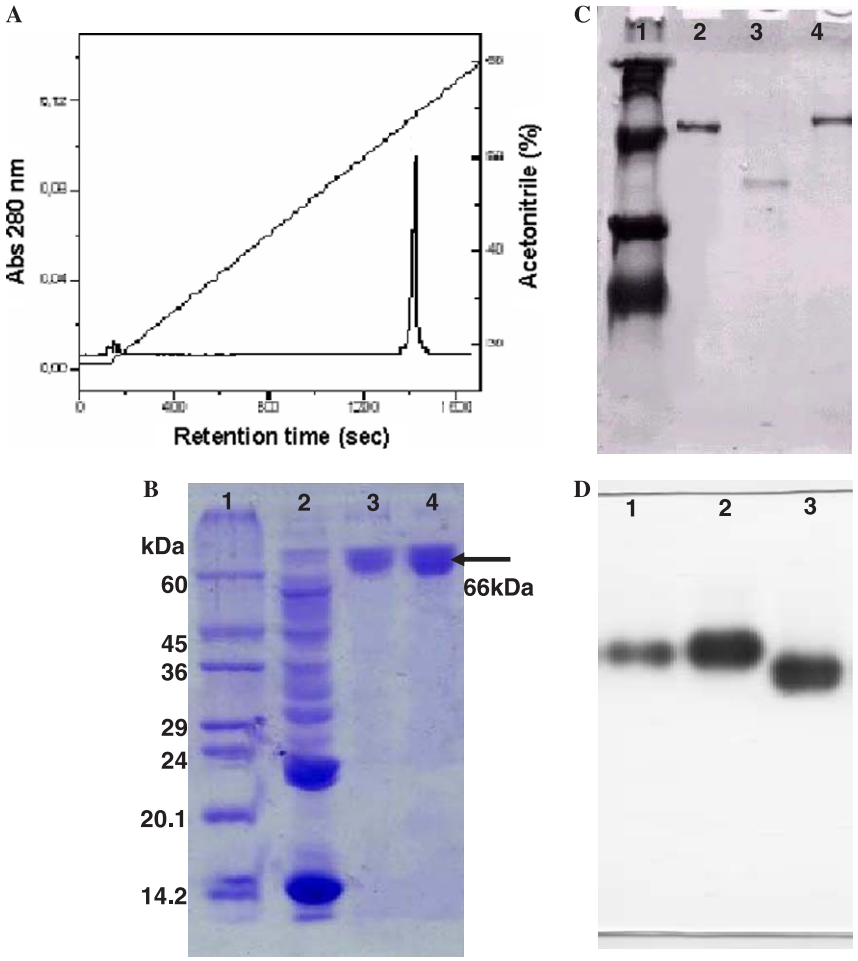

Figure 2. Purity analysis of BpirLAAO. (A) Chromatography profile by RP-HPLC of BpirLAAO-I. (B) SDS–PAGE at 12% (w/v) in Tris–glycine buffer, pH 8.4, for 120 min at 10 mA and 200 V. Lines: 1-Molecular weight markers; 2-*Bothrops pirajai* venom (30 μg); 3-BpirLAAO-I (30 μg); 4-BpirLAAO-II (40 μg). (C) 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-BpirLAAO-I; 3-BpirLAAO-I after treatment with PGNase F; 4-BpirLAAO-I after treatment with *O*-glycosidase. (D) Native PAGE (12%) of BpirLAAO-I stained by enzymatic activity. Lines: 1-BpirLAAO-I; 2-BpirLAAO-I after treatment with *O*-glycosidase; 3-BpirLAAO-I after treatment with PGNase F.

C. durissus cascavella, *Pseudechis australis*, and *Trimeresurus jerdonii* snake venoms.[8,13,28–30] According to experiments previously described, the SV-LAAOs bactericidal activity is dependent on hydrogen peroxide production and consequently cell damage, since in the presence of catalase this activity is abolished.[8,13]

Bothrops pirajai LAAO-I showed leishmanicidal activity (Fig. 5C). We also investigated the cellular viability of *L. amazonensis, L. braziliensis, L. donovani*, and *L. major* after incubation with BpirLAAO-I, which induced a dose-dependent mortality of promastigote forms of different *Leishmania* species. *L. braziliensis* was the most susceptible to the toxic effect of BpirLAAO-I. Briefly, the purified enzyme showed an EC_{50} of 1.46 mg/mL against *L. amazonensis*, 1.06 mg/mL against *L. braziliensis*, 1.26 mg/mL against *L. donovani*, and 1.20 mg/mL against *L. major*. In the presence of catalase, *L. donovani* species showed 100% survival (Fig. 5C) once again suggesting the involvement of hydrogen peroxide production in death mechanism. Recently, it was demonstrated that *B. moojeni* LAAO caused a high mortality of promastigote forms of different species of *Leishmania* in vitro, suggesting a promising therapeutic strategy against leishmaniasis and other intracellular parasitic infections.[9]

The cytotoxic effect was observed only for S180 tumor, human breast (SKBR-3), acute T cell leukemia (Jurkat) cancer, and Erlich ascitic tumor (EAT) cell lines. No significant cell death was observed for macrophages (Fig. 6). The addition of BpirLAAO-I directly to EAT cells resulted in dose-dependent tumor killing, which was inhibited by catalase. Furthermore, BpirLAAO-I was able to induce fago DNA fragmentation after incubation at 37 °C for 24 h. In comparison to the negative control, lower doses of BpirLAAO-I degraded around 300 ng DNA (Fig. 7).

As demonstrated by previous reports, SV-LAAOs have been extensively studied for their cytotoxic effects and apoptosis/necrosis induction.[15,18,19] As observed in bacteria, the dose-dependent effect of BpirLAAO-I on fago DNA and tumor cells could be also related to

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                                    1                                                51
BpirLAAO[Bothrops pirajai] ADDK-NPLEE-FRETNYEVFLEIAKNGLKATSNPKRVVIVGAGMAGLSAAY
BmooLAAO[B.moojeni]        ADDR-NPLEECFRETDYEEFLEIAKNGLSTTSNPKRVVIVGAGMSGLSAAY
BjussuLAAO[B.jararacussu]  ADDR-NPLEECFRETDYEEFLEIAKNGLSTTSNPKRVVIVGAGMSGLSAAY
Apoxin[Crotalus atrox]     AHDR-NPLEECFRETDYEEFLEIAKNGLTATSNPKRVVIVGAGMAGLSAAY
OXLA_CROAD[C.adamanteus]   AHDR-NPLEECFRETDYEEFLEIAKNGLTATSNPKRVVIVGAGMAGLSAAY
TSV-LAO[T.stejenegeri]     ADDR-NPLEECFRETDYEEFLEIARNGLKATSNPKHVVIVGAGMSGLSAAY
M-LAO[A.blomhoffi]         ADDR-NPLEECFRETDYEEFLEIARNGLKATSNPKHVVIVGAGMSGLSAAY
LAAO[Agkistrodon halys]    ANDR-NPLEECFRETDYEEFLEIARNGLKATSNPKHVVVVGAGMSGLSAAY
cascaLAAO[C.d.cascavella]  ADDR-NPLEQCFRETDYEEFLEIARNNLKATSNPKHVVIVGAGMAGLSAAY
LAAO[Calloselasma]         ADDR-NPLAECFQENDYEEFLEIARNGLKATSNPKHVVIVGAGMAGLSAAY
LAAO[Pseudechis]           ADDRRRPLEECFREADYEEFLEIAKNGLQRTSNPKRVVVVGAGMAGLSAAY
LAAO[Notechis]             ADDRRRPLEECFQEADYEEFLEIARNGLNETSNPKHVVVVGAGMAGLSAAY
LAAO[Oxyuranus]            ADVRRNPLEECFREADYEEFLEIARNGLKKTSNPKHVVVVGAGMAGLSAAY
consensus                  *. : .** : *:* :** *****:*.*  *****:**:*****:******
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Figure 3. Comparison of the N-terminal amino acid sequence of *Bothrops pirajai* BpirLAAO-I with those of other snake venom LAAOs. Completely conserved residues in all sequences are marked by asterisks. The gaps are inserted in sequences in order to attain maximum homology. Sequence analysis of the N-terminal region of BpirLAAO-I with LAAOs from other venoms, TSV-LAO from *Trimeresurus stejnegeri* (gi60729671); M-LAO from *Agkistrodon blomhoffi* (gi15887054); BmooLAAO from *Bothrops moojeni* (gi39841346); BjussuLAAO from *Bothrops jararacussu* (gi39841344); Apoxin-1 from *Crotalus atrox* (gi5565692); OXLA_CROAD from *Crotalus adamanteus* (gi6093636); LAAO from *Agkistrodon halys pallas* (gi48425312); LAAO from *Calloselasma rhodostoma* (gi6850960); LAAO from *Pseudechis australis* (gi68304020); LAAO from *Oxyuranus scutellatus* (gi68304016); LAAO from *Notechis scutatus* (gi68304018); cascaLAAO from *Crotalus d. cascavella*.

Table 1. Substrate specificity of BpirLAAO-I

Amino acid	Specific activity (U/mg)
Phe	765
Tyr	685
Trp	585
Leu	580
Met	440
Ile	435
Val	410
His	300
Ala	180
Arg	120
Pro	30
Thr	30
Ser	30
Glu	10
Gly	5
Lys	5

hydrogen peroxide production. Tempone et al.[9] showed that hydrogen peroxide was a strong inductor of apoptosis in promastigote forms of *Leishmania* ssp. In this case, cells submitted to the oxidative stress induced by hydrogen peroxide could activate heat shock proteins and initiate cell membrane/cytoplasmatic disorganization, DNA fragmentation, apoptosis, and therefore cell death.

The research for new therapeutic approaches is relevant in order to search for more efficient drugs to control tumor cells, bacterial and leishmanicidal infections. In this aspect, *Leishmania* causes a spectrum of diseases ranging from self-healing ulcers to disseminated and often fatal infections, depending on the parasite species involved and host's immune response. Adequate protective vaccines against infections are still being 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 develop parasitic resistance.[31] Snake venoms are complex protein cocktails that could be used as source for the development of new therapeutic drugs, since a broad range of pharmacological actions are described for their compounds. Finally SV-LAAOs that act mainly through hydrogen peroxide production are interesting multifunctional enzymes, not only for a better understanding of the ophidian envenomation mechanism, but also due to their biotechnological potential as model of therapeutic drugs.

3. Materials and methods

3.1. Reagents and venom

Bothrops pirajai snake venom, vacuum dried and stored at 4 °C, was kindly supplied by the biologist Luiz H. A. Pedrosa (FMRP-USP, Ribeirão Preto-SP, Brazil). Sephadex G-75, Benzamidine–Sepharose, and Phenyl-Sepharose Fast Flow were from Amersham Life Science Inc. All other reagents used for chemical and biological characterization were of analytical grade and purchased from Sigma Chem. Co. or Gibco BRL.

3.2. L-Amino acid oxidase purification and purity analysis

Bothrops pirajai crude venom (500 mg) was applied on a Sephadex G-75 column (4.0×90.0 cm), equilibrated

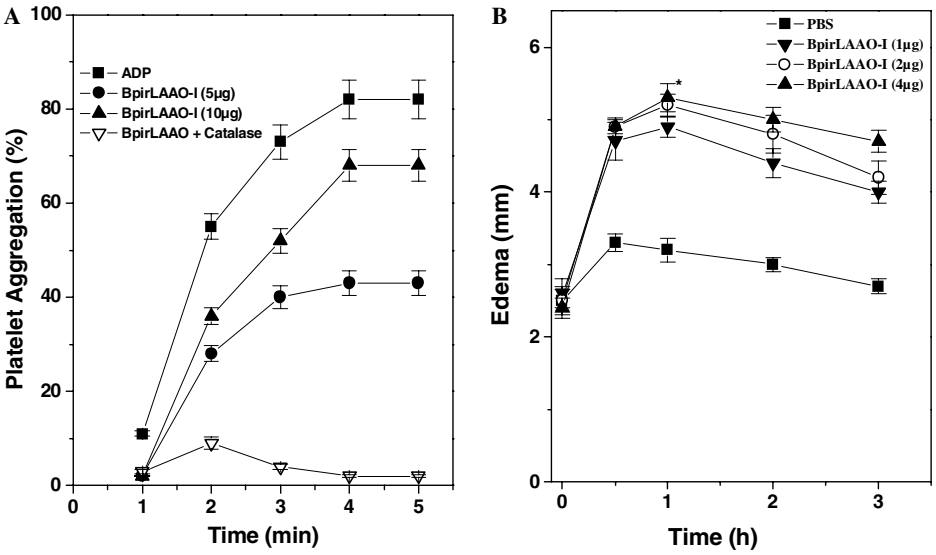

Figure 4. Pharmacological activity of BpirLAAO-I. (A) Time-dependent effect on platelet aggregation induced by BpirLAAO (5 and 10 μg/μL) in comparison with the control group (PRP + ADP, 10 μg/mL). This activity was reduced in the presence of catalase (100 μg/mL). (B) Edema-inducing activity of increasing concentrations of BpirLAAO-I in PBS (0.04, 0.08, and 0.16 μg/μL) after subplantar injection in mice (22–25 g). PBS was also used as a negative control. Each bar represents the means ± SD ($n = 4$). (*) Increase statistical significance relative to control.

with 0.05 M ammonium bicarbonate (ambic), pH 7.8. Elution was carried out using the same buffer at a flow rate of 30.0 mL/h, 10 mL/tube being collected. The resulting fractions were assayed for their enzymatic activity. The active fraction was concentrated by ultrafiltration (AMICON YM 30,000) and applied on a Benzamidine–Sepharose column (1.8 × 4.5 cm), previously equilibrated with Milli-Q water. The active fraction was eluted using Milli-Q water, 0.02 M sodium phosphate buffer, pH 7.8, and glycine buffer (0.02 M, pH 3.2) at a flow rate of 5.0 mL/tube. Subsequently this fraction was applied on a Phenyl-Sepharose Fast Flow column (1.8 × 5.5 cm), previously equilibrated with 2.0 M ammonium sulfate plus 0.01 M Tris–HCl (pH 8.6). In this step, the active fraction was eluted with an ammonium sulfate reverse gradient (2.0–0.0 M), at a flow rate of 2.5 mL/tube and stored at 4 °C for future assays.

For the purity assay, 100 μg of the LAAO fraction obtained from the Phenyl-Sepharose chromatography was applied onto a HPLC C4 reverse-phase column (0.46 × 15.0 cm) equilibrated with 0.1% (v/v) trifluoracetic acid (TFA), followed by an acetonitrile gradient from 28% to 60% (v/v) in 0.1% TFA for 32 min. All chromatographic procedures were performed at room temperature. The sample was also assayed for purity by 12% (w/v) SDS–PAGE, in Tris–glycine buffer, pH 8.4, for 120 min at 10 mA and 200 V.

3.3. Protein determination and L-amino acid oxidase assay

Protein concentration was determined by the method previously described.[32] LAAO activity was measured by an adaptation of the method previously described.[17] In this assay, the oxidative deamination of L-leucine produced hydrogen peroxide, which was reduced, in the presence of horseradish peroxidase, by *o*-phenylenediamine to produce a colored oxidized product, which was spectrophotometrically monitored at 490 nm. Briefly, 10 μL of enzyme sample (1 μg/μL) was incubated at 25 °C with 490 μL of a solution containing 200 μg of *o*-phenylenediamine, 20 mg of L-Leu, and 10 μg of horseradish peroxidase (HRP) in 10 mM Tris–HCl buffer at pH 7.2. The reaction was stopped by addition of 0.5 mL of 10% (m/v) citric acid. One unit (1 U) of enzyme activity was defined as the amount of enzyme able to produce 1 μmol of hydrogen peroxide/min, under the described conditions. In order to find out the affinity for different substrates, other amino acids (20 mg) were assayed, under the same conditions. To access the BpirLAAO-I stability under different ranges of temperatures and pH, 10 μg of enzyme was incubated for 2 h at pH 2.0, 4.0, 6.0, 7.4, 8.0, 10.0, and temperatures of −20, 0, 4, 25, 37, 60, and 100 °C. After incubation, the enzyme activity was measured for enzyme-coupled assay using L-Leu and L-Phe as substrates.

3.4. Biochemical characterization

The p*I* analysis was performed as previously described.[33] For determination of N-linked sugars, the enzyme was submitted to PGNase F treatment under denaturing or non-denaturing conditions. Briefly, a solution of BpirLAAO-I (0.75 μg/μL) in phosphate buffer (50 mM, pH 7.5) was treated with 1 μL PGNase F (0.08 U/mL) and incubated at 37 °C for 4 h. PAGE and enzymatic assays were subsequently carried out to evaluate deglycosylation and BpirLAAO-I activity.

3.5. N-terminal amino acid sequence

For N-terminal sequencing, 5 μg of the BpirLAAO-I sample was applied on a 12.5% SDS–PAGE gel and electroblotted onto a polyvinylidene difluoride (PVDF)

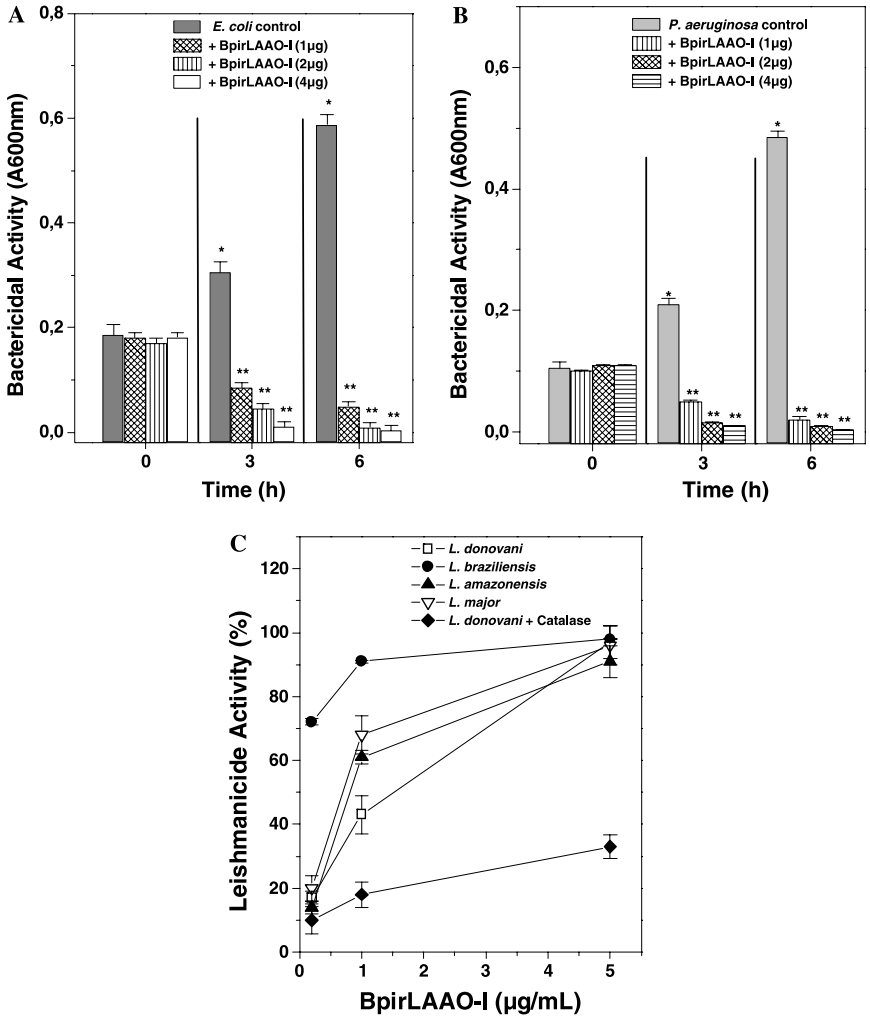

Figure 5. Microbicidal activity of BpirLAAO-I. Evaluation of BpirLAAO-I bactericidal activity *Escherichia coli* (A) and *Pseudomonas aeruginosa* (B). BpirLAAO-I (1, 2, and 4 μg/mL) was incubated with 4×10^5 CFU of each bacterium for 0, 3, and 6 h. After incubation, the bacterial growth was spectrophotometrically measured (Abs600 nm). (C) Leishmanicidal dose-dependent effect induced by the BpirLAAO-I upon *Leishmania* sp. parasite. This activity (BpirLAAO-I + *L. donovani*) was reduced in the presence of catalase (100 μg/mL). Data are expressed by means ± SD ($n = 3$). Control wells were cultured in the presence of culture medium alone. (*) Increase statistical significance relative to control. (**) Decrease statistical significance relative to time 0.

membrane (ProBlott™, Perkin-Elmer Applied Biosystems Division). After staining with Coomassie brilliant blue, the protein band of interest was cut out and submitted to Edman degradation in a gas-phase PPSQ-23A Shimadzu sequence equipment under the conditions recommended by the manufacturer.[8]

3.6. Circular dichroism (CD) analysis

CD analysis was performed using a Jasco J-810 instrument. Protein samples (0.1–1.0 mg/mL) were dissolved in 10.0 mM phosphate buffer, pH 7.0, at 20.0 °C. Each spectrum, used for further calculations, represented an average of three measurements in the range of 195–250 nm, collected at 0.2 nm intervals, with a spectral band width of 0.5 nm and 4 s integration time. The CD spectra were expressed as mean residual ellipticity. To estimate the proportions of the secondary structure of the BpirLAAO-I, CD spectra were evaluated by a computer program. Reference spectra were taken from myoglobin, lysozyme, ribonuclease A, and hemoglobin.

3.7. Pharmacological assays

3.7.1. Platelet aggregation. This assay was carried as previously described.[34] Platelet-rich plasma (PRP) was prepared from citrated human blood by centrifugation (360*g*/12 min) at room temperature. PRP samples obtained as above were centrifuged at 1370*g* for 20 min and the platelet pellets were suspended in a calcium-free Tyrode's solution containing 0.35% (w/v) bovine serum albumin (BSA) and 0.1 mM EGTA (final concentration), pH 6.5, and washed twice by centrifugation. The final pellet was then suspended in Tyrode–BSA, pH 7.5, without EGTA. The suspension volume was adjusted to give $3–4 \times 10^5$ platelets/μL. The platelet aggregation was measured for 5 min by turbidimetric assay using whole blood Lumi-Aggregometer (Chrono-Log Corporation). Assays were performed at 37 °C in siliconized glass cuvettes using 200 μL PRP, under stirring, and aggregation was triggered after pre-incubation for 2 min with aliquots of the purified enzyme. Control experiments were carried out using the platelet agonists

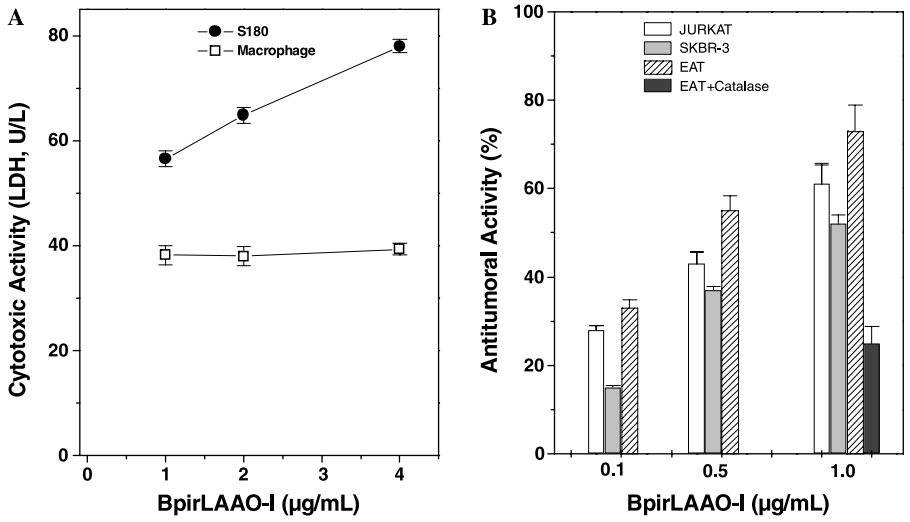

Figure 6. Cytotoxic activity of BpirLAAO-I. (A) For S180 tumor cells and macrophages, BpirLAAO-I at the same concentrations were incubated with 1×10^5 cells. After 3 h, cytotoxicity was measured by LDH dosage. (B) Antitumoral activity of BpirLAAO-I upon different cell lines. At different concentrations (0.1, 0.5, and 1.0 μg/mL) LAAO was incubated with human breast cancer cells (SKBR-3), acute T cell leukemia (JURKAT), and Erlich ascitic tumor (EAT) cell lines. The activity (BpirLAAO-I + EAT) was reduced in the presence of catalase (100 μg/mL). Data are expressed by means ± SD ($n = 03$). Control wells were cultivated in the presence of culture medium alone.

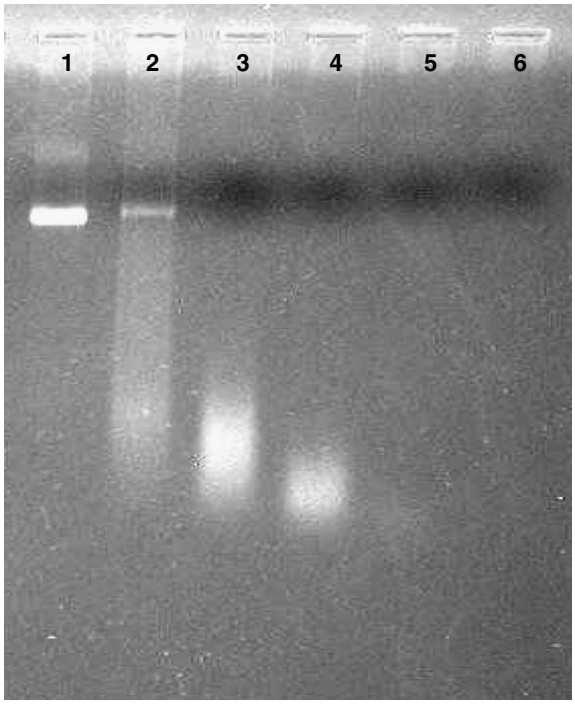

Figure 7. DNA fragmentation assay in fago DNA after treatment with BpirLAAO-I. DNA was incubated with increasing amounts of enzyme for 24 h at 37 °C. The fragmentation was analyzed in a 2% agarose gel. Lane (1): control (only fago DNA; 300 ηg); lanes (2), (3), (4), (5) and (6): DNA (300 ηg) incubated with 1, 2.5, 5, 10, and 20 μg of BpirLAAO-I, respectively.

alone (ADP, 10 μg/mL). BpirLAAO-I (10 μg/mL) was preincubated with catalase (100 μg/mL) for 30 min at room temperature and assayed for platelet aggregation.

3.7.2. Edema inducing activity. Groups of five Swiss male mice (20–25 g) were injected in the right subplantar area with different concentrations of BpirLAAO-I in saline (0.04, 0.08, and 0.16 μg/μL). The left subplantar area received 25 μL of saline, as a negative control. After 0.5, 1, 2, and 3 h, the paw edema was measured using a low-pressure spring caliper (Mitutoyo-Japan). Before inoculation, the paw volume of each mouse was measured as a basal control.

3.8. Cytotoxic assays

3.8.1. Bactericidal activity. Approximately 10^5 Colony-Forming Units (CFU) of *Escherichia coli* (ATCC-25923) and *Pseudomonas aeruginosa* (ATCC-27853) was incubated with 2 mL of Mueller–Hinton medium at 37 °C for 24 h. After growth, aliquots containing approximately 4×10^5 CFU of each bacterium were incubated with increasing concentrations of BpirLAAO-I (1, 2, and 4 μg/mL) and maintained at 37 °C. The growth of bacteria was spectrophotometrically monitored at 600 nm at times of 0, 3, and 6 h after innoculation. The bactericidal activity was measured by the decreasing of absorbance at 600 nm.

3.8.2. Cytotoxic effects of the BpirLAAO-I on *Leishmania* viability. *Leishmania strains* and culture conditions: Promastigote forms of all *Leishmania* species used in our experiments (*L. amazonensis*—MPRO/BR/72/M1841-LV-79; *L. braziliensis*—MHOM/BR/75/M2904; *L. major*—LV-39, clone 5-Rho-SU/59/P; and *L. donovani*—clone LV9-3 from MHOM/ET/67/HU3) were grown in M199 medium (Gibco) supplemented with 40 mM Hepes (pH 7.4), 0.1 mM adenine, 7.7 mM hemin, 10% (v/v) heat-inactivated fetal calf serum (FCS), 50 U/mL penicillin, and 50 μg/mL streptomycin. Cultures were incubated at 26 °C, and cells were kept at densities ranging between 5×10^5 and 3×10^7 parasites/mL. Viability strains were evaluated from motility, and cell density was determined using a hemocytometer.

Cytotoxic effect of the BpirLAAO-I on *Leishmania* viability: Briefly, parasites (3×10^6/well) were incubated in M199 medium supplemented with 10% heat-inactivated FCS in the presence or absence of BpirLAAO-I (0.2–5 μg/mL) for 4 h. Promastigotes of *L. donovani* were incubated with BpirLAAO-I (5 μg/mL) and catalase (0.1 mg/mL) for 12 h at 25 °C in a microplate assay, in order to abolish the action of hydrogen peroxide. Control groups without BpirLAAO-I, in the presence or absence of catalase (SIGMA), were also assayed. Parasites were then pulsed with 0.5 μCi/well [^{3}H]thymidine, and the incorporation of radioisotope by viable cells was accessed after 16 h in a β-counter.[35] Values of EC_{50} were obtained from a sigmoid dose–response curve using the GraphPad Prism Software.

3.8.3. Tumor cells and macrophages. These assays were performed as previously described.[36] S180 tumor cells, collected at the day of experiment, were washed twice, counted, and plated at a density of 1×10^5 cells into a 96-well plate. Peritoneal macrophages were obtained from Swiss male mice (25–30 g) that received ip injections of 4 mL of cold PBS buffer. Peritoneal exudates were collected with a sterile syringe. Cells were washed twice at 400*g*/15 min/4 °C, counted, suspended in complete RPMI medium, and plated as above described. The cells were incubated for 30 min at 37 °C and non-adherent cells removed, suspended in complete RPMI medium, and used in the experiments. Tumor cells or peritoneal macrophages were incubated with BpirLAAO-I at different concentrations (1, 2, and 4 μg/mL). Control was cultured in the absence of enzyme. After an incubation period of 3 h, the activity of lactic dehydrogenase (LDH) from normal or damaged cells was measured (Bioclin Kit).

3.8.4. Cancer cell lines culture and tumor cytotoxic activity. Human breast (SKBR-3) and acute T cell leukemia (Jurkat) cancer cell lines were maintained on RPMI 1640 medium supplemented with 2 mM L-glutamine, 1.5 g/l sodium bicarbonate, 4.5 g/l glucose, 10 mM Hepes, 1.0 mM sodium pyruvate, 10% fetal bovine serum, 100 U/mL penicillin, and 100 μg/mL streptomycin. All cell culture reagents were purchased from Gibco. All cell lines were maintained at 37 °C in 5% CO_2 and 95% air with more than 95% humidity.

Tumor cytotoxic activity of LAAO was assayed with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) staining as previously described.[37] Tumor cells cultivated in appropriate flasks and maintained in continuously exponential growth were detached with 0.05% trypsin plus 0.02% EDTA in calcium-free phosphate-buffered saline (PBS) and washed three times with RPMI medium at 500*g*/15 min/10 °C. Cells were disposed in 96-well plates at a density of 1×10^5 cells per well. After 24 h, the medium was removed and fresh medium, with or without different concentrations of LAAO (0.1, 0.5, and 1.0 μg/mL), was added to the wells and incubated for 24 h. Cytotoxic rate was calculated as follows: % of cytotoxicity of compounds = 1 − Abs drug treated/Abs control × l00.

In some experiments, cytotoxic activity was determined on Erlich ascitic tumor (EAT) cells grown in the peritoneal cavity of Swiss mice.[38] EAT cells were suspended in Tyrode–Ringer buffer (4×10^6 cells in the final volume of 1 mL) and incubated with several concentrations of LAAO (0.1, 0.5, and 1.0 μg/mL) for 60 min. One hundred microliters of Tryphan blue solution (1% in saline) was then added and the unviable stained cells, as well as unstained cells, were independently counted using a hemocytometer.

3.9. DNA fragmentation assay

DNA fragmentation assays were performed by incubation of 1, 2.5, 5, 10, and 20 μg of BpirLAAO-I with a solution containing fago DNA (300 ng M13mp18/μL) in 10 mM phosphate-buffered saline (PBS) at 37 °C for 24 h. For negative control, fago DNA was incubated in the absence of enzyme under the same conditions. Each sample was stained with ethidium bromide and analyzed by gel electrophoresis on 2% agarose, carried out at 100 V by approximately 1 h. Finally, the gel was photographed on a UV transilluminator for evaluation of DNA fragmentation.

3.10. Statistical analysis

Data are presented as mean values ± SD. For statistical significance data were analyzed by Student's unpaired *t*-test at 5% level.

Acknowledgments

This work was supported by Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP), Fundação de Amparo à Pesquisa de Minas Gerais (FAPEMIG), and Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Brazil. We are grateful to Prof. Dr. M. V. Souza (UnB, Brasília-DF) for amino acid sequence; Patrícia H. Ribeiro (DACTB-FCFRP-USP, Ribeirão Preto-SP) for antitumoral activity; John Pietro Nunes (Hemocentro Regional de Uberlândia-Fundação Hemominas-MG) and Danilo Menaldo (DACTB-FCFRP-USP, Ribeirão Preto-SP) for their helpful technical collaboration.

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