Biochemical and Biophysical Research Communications **280,** 620–624 (2001)
doi:10.1006/bbrc.2000.4175, available online at http://www.idealibrary.com on IDEAL®

Bothrops moojeni Venom Kills *Leishmania* spp. with Hydrogen Peroxide Generated by Its L-Amino Acid Oxidase

A. G. Tempone,* H. F. Andrade, Jr.,† P. J. Spencer,* C. O. Lourenço,* J. R. Rogero,* and N. Nascimento*

**Radiobiology Division, Instituto de Pesquisas Energéticas e Nucleares–IPEN/CNEN-SP, Travessa R 400, Cid Universitária, 05508-900 São Paulo, SP, Brazil; and †Protozoology Laboratory, Instituto de Medicina Tropical de São Paulo-FMUSP-Av. Dr. Enéas de Carvalho Aguiar 470, 05403-000 São Paulo, SP, Brazil*

Received December 21, 2000

Leishmaniasis is an endemic tropical disease in South America, with few therapeutic approaches. Snake venoms are complex protein mixtures with biological actions that could be used as tools for drug development. Here we show that *Bothrops moojeni* crude venom presented a killing effect *in vitro* against *Leishmania spp.* promastigotes, but not with amastigotes, as determined by a viability assay using the mitochondrial oxidative function. Purification of active fractions from crude venom was performed by molecular exclusion and ion exchange chromatography. Anti-Leishmania and L-amino acid oxidase (L-AAO, EC.1.4.3.2.) activities co-eluted in the same fractions. The molecular weight of the active enzyme was estimated to be 140 kDa by molecular exclusion chromatography, and 69 kDa by SDS–PAGE, with a 4.8 isoelectric point. Using substrate subtraction and catalase for scavenging, the action of L-AAO was demonstrated to be hydrogen-peroxide-dependent. © 2001 Academic Press

Key Words: **Leishmaniasis; parasites; *Bothrops moojeni;* snake venom; L-amino acid oxidase; *Leishmania amazonensis; Leishmania panamensis; Leishmania chagasi.***

Leishmaniasis includes a spectrum of human infectious disease ranging from self-healing cutaneous ulceration to a progressive and lethal visceral infection (1). The disease is estimated to affect about 12 million people, with 400,000 new cases per year occurring worldwide. Leishmaniasis is prevalent in 88 countries throughout the world, including 16 developed countries (WHO, 1990). The first-line drugs for the treatment of leishmaniasis have been the toxic pentavalent antimony (2), with clinical resistance and treatment failures discussed in several reports (2–5).

Snake venoms are complex mixtures of enzymes and biologically active peptides consisting of proteins and, to a lesser extent, of carbohydrates and lipids (6). Venoms of numerous land snakes posses cytotoxic or lytic effects on tumor cells *in vitro* (7, 8). Some of these venom proteins present a clear antibacterial action, as shown for *Pseudechis australis* venom with its L-amino acid oxidase fraction against Gram-negative and positive bacteria (9). Growth inhibition of *Leishmania spp.* and *T.cruzi* by snake venom has been reported (10), but no attempt was made to purify the active fraction. In the present study, we demonstrate that *Bothrops moojeni* crude venom presents anti-Leishmania activity, associated with L-amino acid oxidase activity and characterize its mode of action.

MATERIALS AND METHODS

Parasite and venom. Promastigotes of *Leishmania* (*L.*) *amazonensis* (LV-79 strain), *Leishmania* (*L.*) *chagasi* (M 6445 strain) and *Leishmania* (*V.*) *panamensis* (MI 3237 strain) were grown at 25°C in RPMI-PR$^-$ 1640 medium, supplemented with 20% fetal calf serum and gentamycin (30 μg/mL). The promastigotes used for all experiments, clearly presented the classical log growth and stationary phase reached at 1×10^7 parasites/mL. *L.*(*L.*) *amazonensis* amastigotes (LV-79 strain), isolated from infected BALB/c mice, were kindly furnished by Dr. Sílvia C. Alfieri, from ICB-Universidade de São Paulo. Amastigotes were kept in RPMI 1640 supplemented with 20% fetal bovine serum at 34°C. Crude dried *Bothrops moojeni* venom was kindly supplied by Instituto Butantan, and stored at −20°C.

Viability assay by MTT oxidation. The viability of parasites were assayed colorimetrically by the mitochondrial oxidation of MTT (3-[4,5-dimethylthiazol-2-y1]-2,5diphenyl-tetrazolium bromide). Briefly, MTT (2 mg/mL) was dissolved in the culture medium and sterilized by filtration (0.22 μm). Multiwell plates were seeded with cells (4×10^5 cells/well) in a final volume of 100 μL/well, and incubated for 18 h at 25°C. MTT (50 μL/well) was added and the plate incubated for 4 h at 25°C. Finally, 60 μL/well of 10% SDS in HCl 0.01 M (SDS-HCl) was added, followed by 18 h of incubation (11). The optical density (OD) was detected at 570 nm with a MULTISKAN MS microplate reader and

0006-291X/01 $35.00

correlated with the number of living promastigotes, adequately standardized for each plate (data not shown).

Effective concentration 50% of L-AAO. The EC_{50} of the samples was determined against promastigotes of different species and amastigotes *of L.*(*L.*) *amazonensis,* using the MTT assay. The protein concentrations ranged from 30 to 0.15 μg/mL, with 4×10^5 promastigotes/well added, and 300 to 0.244 μg/mL with amastigotes. Control wells without any sample were used as 100% growth. The death rate was calculated by the following equation: % killing = ((OD control well − OD sample well)/OD control well)*100. These transformed data were used to demonstrate the anti-Leishmania activity in venom fractionation data. The EC_{50} was calculated with a sigmoid dose-response model using the GraphPad Prism Software.

Venom fractionation. Molecular exclusion chromatography was performed using a Fast Protein Liquid Chromatography apparatus (FPLC-Pharmacia). Crude venom was dissolved in 1 mL 50 mM Tris-HCl, pH 8.5 and applied to a Superdex 200 (1.6 × 70 cm) prep grade column at a flow rate of 0.5 ml/minute, with OD 280 nm monitoring. Protein concentration was determined by the method of Bradford (12) using bovine serum albumin as standard. Pooled fractions presenting anti-*Leishmania* activity and L-amino acid oxidase (L-AAO) activities were applied to a Resource Q 5/5 column (Pharmacia) equilibrated with buffer A (50 mM Tris-HCl, pH 8.5). After an initial 10 ml wash with 15% buffer B (50 mM Tris-HCl, pH 8.5, and 1 M NaCl), the material was eluted using a linear gradient 15–40% with same buffer. After determination of anti-Leishmania and L-AAO activities, active samples were dialyzed in buffer A and concentrated by ultrafiltration (AMICON). A significant loss of the prosthetic group occurred due to dialysis, and additional FAD (1 μM) was added before storage (13). All samples were sterilized by filtration (0.22 μm) and stored at 4°C.

SDS–PAGE and isoelectric focusing (IEF). Protein composition and molecular weight determination were performed by SDS-PAGE (Mini-Protean-BioRad), by the method of Laemmli (14) using a denaturing system with or without 2-β-mercaptoethanol. Molecular weight standards were run to allow molecular weight extrapolation. The acrylamide concentration used for gel preparation was 10%. The slabs were stained with Coomassie Brilliant Blue G-250. The isoelectric point of the purified L-AAO was determined with a Multiphor II system (Pharmacia) using a 5% polyacrylamide gel with ampholines (3.5–9.5). The proteins were applied and the gel submitted to 1500 V for 90 min. The gel was stained with Coomassie Brilliant Blue (Sigma).

L-amino acid oxidase (L-AAO) activity. Crude venom and the chromatographic fractions were assayed for L-AAO activity using a colorimetric assay in a 96-well microplate. The assay was derived by an adaptation of a reported peroxidase test (15). The reaction mixture contains 100 mM Tris/HCl, pH 8.5, 3 mM L-leucine as substrate, horseradish peroxidase (10 IU/mL) and o-phenylenediamine (OPD), (0.1 mg/mL). Samples were incubated for 1 h at 25°C, and OD was determined at 414 nm at 5 min intervals on a microplate reader, for kinetics. Hydrogen peroxide standards were used and the linear regression data calculated with the GraphPad Prism Software. L-AAO activity was expressed as nmoles H_2O_2/min.

Characterization of L-AAO action on promastigotes. Promastigotes of *L.*(*L.*) *amazonensis* (4×10^5/well) were incubated with L-AAO (30 μg/mL) and catalase (0.3 mg/mL) in a final volume of 100 μL/well for 18 h at 25°C in a microplate assay, in order to abolish the action of H_2O_2. Control groups without L-AAO, with or without catalase, and with or without 5 mM hydrogen peroxide (SIGMA) were also tested. Promastigotes were incubated with L-AAO in Hanks Balanced Salt Solution (HBSS) without amino acids under the same conditions, for substrate suppression. The viability was determined by the MTT assay.

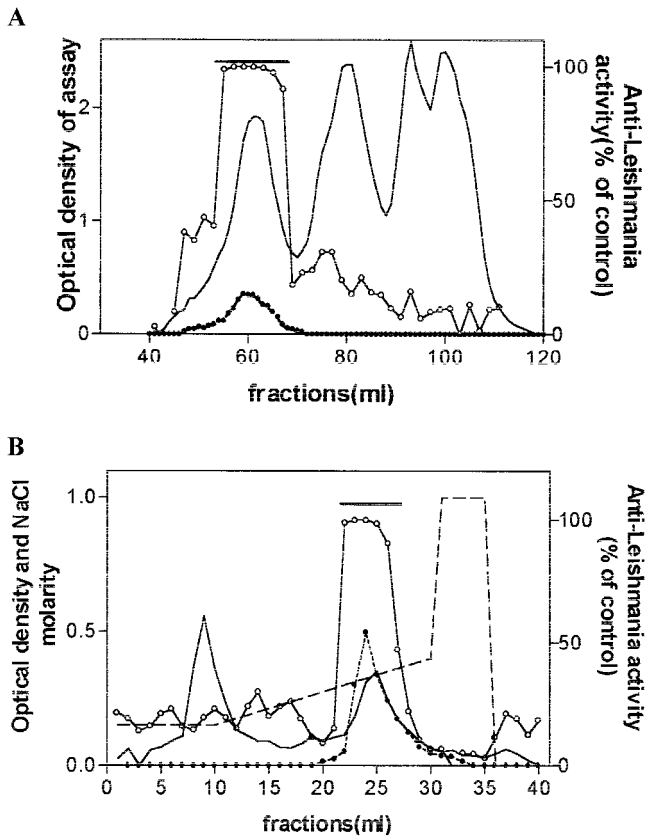

FIG. 1. (A) Molecular exclusion chromatography of *B. moojeni* venom on a Superdex 200 column. (B) Ion exchange chromatography of pooled fractions with L-AAO or anti-Leishmania activities. The activities were determined by the L-leucine assay and MTT assay, respectively. Solid line without symbols, optical density at 280 nm; dashed line with open circles (○- - -○), L-AAO activity; solid line with closed circles (●- - -●) anti-Leishmania activity; dashed line without symbols, NaCl ionic gradient. Bar represents pooled fractions for subsequent procedure.

RESULTS

Venom fractionation. The *Bothrops moojeni* venom, applied to a Superdex 200 column as described in Materials and Methods, yielded four protein peaks (Fig. 1A). L-AAO and anti-Leishmania activities of the fractions are also presented in Fig. 1A, showing that only the first peak presented both activities. The molecular weight of the active fraction was estimated to be around 140 kDa, as determined by comparison with standard proteins previously eluted from the same column. The fractions exhibiting L-AAO and anti-Leishmania activities were pooled and applied to an ion-exchange column, as described in Materials and Methods, yielding two main fractions (Fig. 1B): one unbound to the resin and the other eluted at 300 mM NaCl, which presented both L-AAO and anti-Leishmania activities. SDS-PAGE showed that the purified L-AAO migrated as a single band with molecular weight of approximately 69 kDa (Fig. 2A). The purified

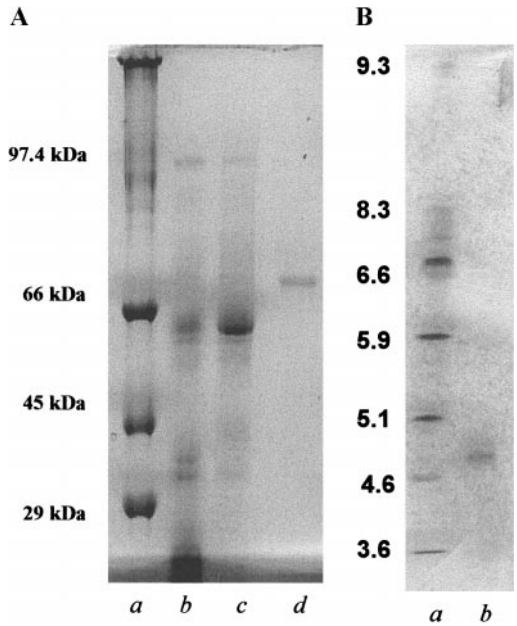

FIG. 2. (A) SDS–PAGE of the purified L-AAO. a, MW markers (numbers indicate kDa of standards); b, crude venom c, molecular exclusion chromatography pooled fractions; d, Ion exchange chromatography of the purified L-AAO fraction. (B) Isoelectric focusing of purified L-AAO (b) from *B. moojeni* venom, with corresponding isoelectric point standards (a). MW and isoelectric point of the unknown fraction estimated by linear regression model of standards using the GraphPad Prism Software. The gels were stained with Coomassie brilliant blue (Sigma).

fraction showed only one band by isoelectric focusing (Fig. 2B), with an isoelectric point of 4.8. The protein yield of this purification is shown in Table 1.

Effective concentration 50% (EC_{50}). By serial dilution of the purified L-AAO and crude venom, we were able to determine the EC_{50} using the MTT method. *B. moojeni* crude venom showed an EC_{50} of 7.56 μg/mL (±0.020) against *L.*(*L.*) *amazonensis* promastigotes. The purified L-AAO presented an EC_{50} of 1.44 μg/ml (±0.07) against *L.*(*L.*) *amazonensis,* 1.19 μg/ml (±0.008) against *L.*(*V.*) *panamensis* and 1.08 μg/ml (±0.002) against *L.*(*L.*) *chagasi* (Fig. 4 and Table 2).

TABLE 1

Quantitative Analysis of the Purification Schedule Used for L-AAO from *B. moojeni* Venom

Purification step	Total protein (Mg)	L-AAO activity (nmoles H_2O_2/min)	Specific activity (units/μg)
Crude venom	297, 0	0.0426	0.017
Molecular exclusion chromatography	40, 0	0.196	0.0784
Ion exchange chromatography	4.35	5.74	2.296

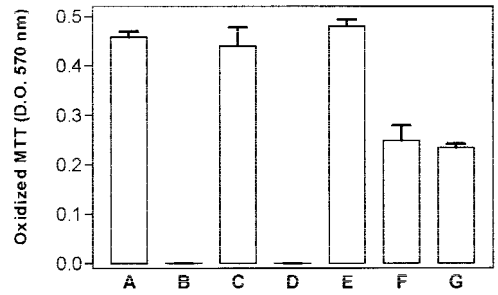

FIG. 3. Characterization of L-AAO activity against promastigotes of *L.*(*L.*) *amazonensis.* (A) Promastigotes in RPMI-1640 medium; (B) promastigotes in RPMI-1640 medium + 5 mM H_2O_2; (C) promastigotes in RPMI-1640 medium + 5 mM H_2O_2 + 0.3 mg/ml catalase; (D) promastigotes in RPMI 1640 medium + 30 μg/mL L-AAO; (E) promastigotes in RPMI 1640 medium + 30 μg/mL L-AAO + 0.3 mg/mL catalase; (F) promastigotes in HBSS; (G) promastigotes in HBSS + 30 μg/mL L-AAO. Promastigote viability is expressed as OD 57O nm of oxidized formazan from MTT. Bars represent SEM of replicates.

Using amastigotes of *L.*(*L.*) *amazonensis* (LV-79 strain), the EC_{50} of L-AAO could not be determined. Amastigotes were not affected with the initial concentration of 300 μg/mL, as observed by 100% viability found in the MTT assay (data not shown).

Characterization of L-AAO action in promastigotes. Promastigotes incubated with the purified L-AAO, in the presence of catalase, showed 100% survival (Fig. 3C). Promastigotes incubated in HBSS for substrate suppression, in the presence of L-AAO, also showed 100% survival (Fig. 3G). Promastigotes showed H_2O_2 susceptibility, as observed when the commercial product was added (100% death), as shown in Fig. 3B.

DISCUSSION

In the present study, we show that *B. moojeni* venom presents an anti-Leishmania activity, which appears to

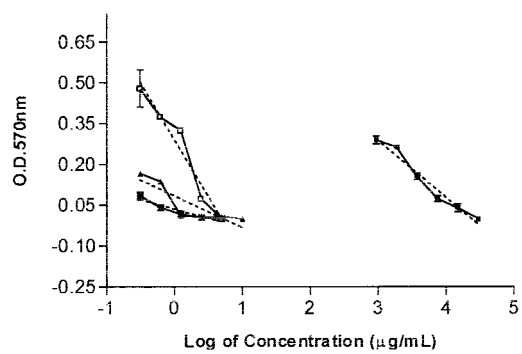

FIG. 4. Effective dose 50% (EC_{50}) of crude *B. moojeni* venom and its purified L-AAO against *Leishmania spp.* ▲—▲ L-AAO against *L.*(*L.*) *panamensis;* □—□ L-AAO *against L.*(*L.*) *amazonensis;* *—* L-AAO against *L.*(*L.*) *chagasi;* ■—■ Crude venom *against L.*(*L.*) *amazonensis.* The viability of parasites was determined by the MTT assay at O.D. 570 nm. Linear regression graphics were analyzed in GraphPad Prism.

TABLE 2

Effective Concentration 50% of Crude Venom and L-AAO against *Leishmania spp.*

Sample	*Leishmania spp.*	EC_{50} (μg/mL)	*r*
Crude venom	*L.*(*L.*) *amazonensis*	7.56 (±0.020)	0.9818
L-AAO	*L.*(*L.*) *amazonensis*	1.44 (±0.062)	0.9734
L-AAO	*L.*(*L.*) *panamensis*	1.19 (±0.0083)	0.8643
L-AAO	*L.*(*L.*) *chagasi*	1.08 (±0.0024)	0.9384

Note. EC_{50}, effective concentration 50%; *r*, linear correlation coefficient.

be related to the action of an enzyme, constituting 1.5% of crude venom, characterized as a L-amino acid oxidase. The crude venom was fractionated and the active component purified. The biochemical properties of the purified enzyme were consistent with findings obtained elsewhere for other snake L-amino acid oxidases (16). The molecular weight and the homodimeric structure of L-AAO, found in our experiments, was also described for many other L-amino acid oxidases, ranging from 60 kDa in the monomeric form to 150 kDa as a dimer (16, 17a, 18). The presence of multiple forms of this enzyme in some snake venoms was also reported (19, 20), but it was not found in our experiments using *B. moojeni* venom. The observed isoelectric point (pI = 4.8) of L-AAO is similar to those observed for the same enzyme from other snake venoms (13, 18, 21). By the enzymatic assay, the L-AAO presented 135-fold more activity than crude venom, suggesting a significant degree of purification.

L-AAO catalyzes the oxidative deamination of L-amino acids to produce the corresponding α-keto-acid, hydrogen peroxide and ammonia (17b, 22). In our experiments, promastigotes survived after incubation with L-AAO in a solution without amino acids (HBSS), showing the dependence of substrate for L-AAO activity. Using catalase as a scavenger for hydrogen peroxide (24), we obtained complete abolition of anti-Leishmania activity, suggesting that the action could be ascribed to H_2O_2 production, and not to a keto-acid generated by the enzyme, that would probably not be affected by catalase. The action of L-AAO from a snake venom on human gastric carcinoma cell lines, was also ascribed to hydrogen peroxide, leading to cell death (16).

Leishmania spp. had slightly different susceptibilities to L-AAO, as observed for the EC_{50} of the purified enzyme. This could be explained by different enzymatic patterns in each strain or species (23). Despite of the EC_{50} against *L.*(*L.*) *amazonensis* promastigotes for L-AAO was 5-fold higher than crude venom, kinetic parameters showed a 135-fold increase in L-AAO activity compared to the crude venom. This discrepancy could be ascribed to the restriction of substrate and other facts involving the culture media in the promastigote *in vitro* assay, that can lead to an inefficient action of the enzyme. Different substrate specificity for L-AAO was reported for *Calloselasma rhodostoma* venom (18).

Some authors reported apoptosis-induced cell death after incubation with L-amino acid oxidase (15, 25). Suhr and co-workers (25) showed that L-AAO, purified from *Agkistrodon halys* venom, induced the cell-apoptosis mechanism. 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 (26).

As reported by Fernandez-Gomes and co-workers (10), components ranging from 30 to 40 kDa present in *Cerastes cerastes* venom, caused growth inhibition of *Leishmania* (*L.*) *donovani* amastigotes. Our data showed no evidence of other proteins presenting detectable anti-Leishmania activity in *B. moojeni* venom. The results obtained with the incubation of L-AAO with amastigotes, suggest that these parasites have an efficient protective system against free radicals and hydrogen peroxide. Catalase, superoxide dismutase, and other enzymes were described in *Leishmania spp.*, as its antioxidant defense mechanism. Promastigotes were found to be deficient in catalase and glutathione peroxidase, with 80 to 95% promptly killed by macrophage hydrogen peroxide production during the infection (22). Murray and co-workers (27) demonstrated that *Leishmania donovani* amastigotes contained 3-fold more catalase and 14-fold more glutathione peroxidase and were four times more resistant to enzymatically generated hydrogen peroxide. Our results were consistent with these findings, in which promastigotes presented susceptibility to the purified enzyme. Unlike other venoms (9, 10), no other active fraction could be observed. This fact can be ascribed to major differences in the venom composition.

Snake venoms are commonly used as tools for the development of new therapeutic approaches, since a broad range of pharmacological actions are described for these substances (28–30). Considering the few drugs currently available for leishmaniasis, as well as their high toxicity and significant side effects, the development of new drugs appears as a necessity. The understanding of the L-AAO mode of action upon parasites may trigger the design of new drugs or therapeutical approaches. Indeed, if one was able to target a hydrogen peroxide generator, as *B. moojeni* L-AAO, towards the parasitophorous vacuole, this would represent a highly specific treatment not only for leishmaniasis, but also for other intracellular parasites.

ACKNOWLEDGMENTS

The authors are grateful to Dr. Bruno A. Cardi for advice and helpful criticism and to Cleusa F. Takakura for technical assistance.

The experimental work was supported by grants from FAPESP (No. 99/08491-4), IPEN/CNENSP and LIMHCFMUSP (49).

REFERENCES

1. Peters, W., and Killick-Kendrick, R. (1987) *in* The Leishmaniasis in Biology and Medicine, Vol. 1, Academic Press Inc., London.
2. Balaña-Fouce, R., Reguera, R. M., Cubría, C., and Ordóñez, D. (1998) The pharmacology of leishmaniasis. *Gen. Pharmac.* **30,** 435–443.
3. Berman, J. D., Chulay, J. D., Hendricks, L. D., and Oster, C. N. (1982) Susceptibility of clinically sensitive and resistant *Leishmania* to pentavalent antimony *in vitro*. *Am. J. Trop. Med. Hyg.* **31,** 459–465.
4. Ibrahim, M. E., Hag-Ali, M., El-Hassan, A. M., Theander, T. G., and Kharazmi, A. (1994) *Leishmania* resistant to sodium stibogluconate: Drug associated macrophage-dependent killing. *Parasitol. Res.* **80,** 569–574.
5. Paul, M., Durand, R., Fessi, H., Rivollet, D., Houin, R., Astier, A., and Deniau, M. (1997) Activity of a new liposomal formulation of Amphotericin B against two strains of *Leishmania infantum* in a murine model. *Antimicrob. Agents Chemother.* **41,** 1731–1734.
6. Devi, A. (1968) *in* The Protein and Nonprotein Constituents of Snake Venoms in Venomous Animals and Their Venoms (Bucherl, W., Buckley, E., and Deulofen, Eds.), V, Academic Press, NY.
7. Chaim-Mathyas, A. and Ovadia, M. (1987) Cytotoxic activity of various snake venoms on melanoma, B16/F10 and chondrosarcoma. *Life Sci.* **40,** 1601–1607.
8. Ovadia, M. (1987) Isolation and characterization of three hemorragic factors from the venom of Vipera palestinae. *Toxicon* **16,** 479–487.
9. Stiles, B. G., Sexton, F. W., and Weinstein, S. A. (1991) Antibacterial effects of different snake venoms: Purification and characterization of antibacterial proteins from *Pseudechis australis* (Australian king brown or mulga snake) venom. *Toxicon* **29,** 1129–1141.
10. Fernandez-Gomes, R., Zerrouk, H., Sebti, F., Loyens, M., Benslimane, A., and Ouaissi, M. A. (1994) Growth inhibition of *Trypanosoma cruzi* and *Leishmania donovani infantum* by different snake venoms: Preliminary identification of proteins from *Cerastes cetrastes* venom which interact with the parasites. *Toxicon* **32**(8), 875–882.
11. Tada, H., Shiho, O., Kuroshima, K., Koyama, M., and Tsukamoto. (1986) An improved colorimetric assay for interleukin 2. *J. Immunol. Methods.* **93,** 157–165.
12. Bradford, M. M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem* **72,** 248–253.
13. Sánchez, E. O. F., and Magalhães, A. (1991) Purification and partial characterization of an L-amino acid oxidase from bushmaster snake (surucucu pico de jaca) *Lachesis muta muta* venom. *Brazilian J. Med. Biol. Res.* **24,** 249–260.
14. Laemmli, U. K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227,** 680–685.
15. Torii, S., Naito, M., and Tsuruo, T. (1997) Apoxin I, a novel apoptosis-inducing factor with L-amino acid oxidase activity purified from western diamondback rattlesnake venom. *J. Biol. Chem.* **272,** 9539–9542.
16. Ahn, M. Y., Lee, B. M., and Kim, Y. S. (1997) Characterization and cytotoxicity of L-amino acid oxidase from the venom of king cobra (*Ophiophagus hannah*). *Int. J. Biochem. Cell. Biol.* **29,** 911–919.

17a. Tan, N. T., and Saifuddin, M. N. (1991) Substrate specificity of King Cobra (Ophiophagus hannah) venom L-amino acid oxidase. *Int. J. Biochem.* **23**(3), 323–327.

17b. Tan, N.-H., and Swaminathan, S. (1992) Purification and properties of L-amino acid oxidase from monocellate cobra (*Naja naja kaouthia*) venom. *Int. J. Biochem.* **24,** 967–973.

18. Ponnudurai, G., Chung, M. C. M., and Tan, N.-H. (1994) Purification and properties of L-amino acid oxidase from Malayan pit viper (*Calloselasma rhodostoma*) venom. *Arch. Biochem. Biophys.* **313,** 373–378, doi:10.1006/abbi.1994.1401.
19. Nakano, K., Inamas, Y., Hagihara, S., and Obo, F. (1972) Isolation and properties of L-amino acid oxidase in habu snake (*Trimeresurus flavoviridis*) venom. *Acta. Med. Univ.Kagoshima.* **14,** 229–239.
20. Shaham, N., and Bdolah, A. (1973) L-amino acid oxidase from *Vipera palaestinae* venom: Purification and assay. *Com. Biochem. Physiol.* **46,** 691–698.
21. Ueda, M., Chang, C. C., and Ohno, M. (1988) Purification and characterization of L-amino acid oxidase from the venom of Trimeresus mucrosquamatus (Taiwan Habu Snake). *Toxicon* **26**(8), 695–706.
22. Pessatti, M. L., Fontana, J. D., Furtado, M. F. D., Guimarães, M. F., Zanette, L. R. S., Costa, W. T., and Baron, M. (1995) Screening of *Bothrops* snake venoms for L-amino acid oxidase activity. *Appl. Biochem. Biotechnol.* **51,** 197–210.
23. Mehlotra, R. K. (1996) Antioxidant defense in parasitic protozoa. *Crit. Rev. Microbiol.* **22**(4), 295–314.
24. Nabi, Z. F., and Rabinovitch, M. (1984) Inhibition by superoxide dismutase and catalase of the damage of isolated *Leishmania mexicana amazonensis* by phenazine methosulfate. *Mol. Biol. Parasitol.* **10,** 297–303.
25. Suhr, S. M., and Kim, D. S. (1996) Identification of the snake venom substance that induces apoptosis. *Biochem. Biophys. Res. Commun.* **224,** 134–139, doi:10.1006/bbrc.1996.0996.
26. Krobitsch, S., Brandau, S., Hoyer, C., Schmetz, C., Hubel, A., and Clos, J. (1998) *Leishmania donovani* heat shock protein 100. Characterization and function in amastigote stage differentiation. *J. Biol. Chem.* **13,** 6488–6494.
27. Murray, H. W. (1981). Susceptibility of Leishmania with a macrophage cell-line: Correlation between intracellular killing and the generation of oxygen intermediates. *J. Exp. Med.* **153,** 1690.
28. Marlas, G., and Bon, C. (1982) Relationship between the pharmacological action of crotoxin and its phospholipase activity. *Eur. J. Biochem.* Jun **15;**125(1):157–165.
29. Kamata, K., Arai, Y., Sakamoto, A., Kasuya, Y., and Samejima, Y. (1989) Pharmacological action of the phospholipase A2 from the venom of *Trimeresurus flavoviridis* on the smooth muscle of the rat stomach. *Life Sci.* **44**(2), 137–142.
30. Chen, S. Z., Gopalakrishnakone, P., and Gwee, M. C. (1994) Pharmacological effects and pathological changes induced by the venom of *Pseudechis australis* in isolated skeletal muscle preparations. *Toxicon* **32**(3), 303–315.