0273-2289/95/5152—0197$06.80

Screening of *Bothrops* Snake Venoms for L-Amino Acid Oxidase Activity

MARCOS L. PESSATTI,[1] JOSÉ D. FONTANA,[1] MARIA F. D. FURTADO,[2] MANOEL F. GUIMÃRAES,[1] LORENZO R. S. ZANETTE,[1] WALLIANA T. COSTA,[1] AND MADALENA BARON*,[1]

[1]LQBB—Biomass Chemo/Biotechnology Laboratory, Department of Biochemistry, UFPR—Federal University of Parana PO Box 19046 (81531-970), Curitiba—PR, Brazil; and [2]LH—Herpetology Laboratory, IB—Butantan Institute, São Paulo—SP, Brazil

ABSTRACT

Toxins, enzymes, and biologically active peptides are the main components of snake venoms from the genus *Bothrops*. Following the venom inoculation, the local effects are hemorrhage, edema, and myonecrosis.

Nineteen different species of Brazilian *Bothrops* were screened for protein content and L-amino acid oxidase activity. *B. cotiara*, formerly found in the South of Brazil, is now threatened with extinction. Its venom contains a highly hemorrhagic fraction and, as expected from the deep yellow color of the corresponding lyophilized powder, a high L-amino acid oxidase (LAO) activity was also characterized. Flavin adenine dinucleotide (FAD) is its associate coenzyme. *B. cotiara* venom LAO catalyzed the oxidative deamination of several L-amino acids, and the best substrates were methionine, leucine, tryptophan, and phenylalanine, hence, its potential application for the use in biosensors for aspartame determination and for the removal of amino acids from plasma. High levels for LAO were also found in other species than *B. cotiara*. In addition, the technique of isoelectric focusing (IEF) was employed as a powerful tool to study the iso- or multienzyme distribution for LAO activity in the *B. cotiara* snake venom.

Index Entries: *Bothrops cotiara* venom; LAO/L-amino acid oxidase; hemorrhagic activity; LAO isoelectrofocusing; LAO zymogram.

*Author to whom all correspondence and reprint requests should be addressed.

INTRODUCTION

Bothrops is a genus of poisonous snakes found in Central and South America (*1*). The clinical symptoms provoked by the venoms, after inoculation, are characterized by local lesions, that is, hemorrhage, edema, and myonecrosis, as well as cardiovascular alterations, coagulation disorders, and renal damages (*2*). These pathologies are attributed to a complex mixture of bioactive components, like enzymes, hemorrhagic factors, and myotoxins (*3*). Some enzymatic activities have been investigated in the bothropic venoms, including studies about protease (*4*), phospholipase A2 (*5,6*), and thrombin-like enzyme (*7*).

The purpose of this investigation was to examine the L-amino acid oxidase (LAO) activity in venoms obtained from 19 species of the genus *Bothrops*, very widespread in Brazil. There are a few reports about the occurrence of LAO in bothropic venoms, but most studies were reported for other genera, such as *Crotalus* (*8*) and *Pseudechis* (*9*).

LAO is being currently used for the removal of free L-amino acids from human plasma in order to improve the quantitative estimation of δ-aminolaevulinic acid (ALA) in cases of congenital and AIDS-provoked porphyrias, saturnism, and tyrosinemia (*10,11*).

Another application refers to a biosensor system combining an immobilized pronase column and the LAO electrode connected in series. Such an instrument is being developed for the determination of aspartame (L-aspartyl-L-phenylalanine methyl ester) in dietary food products (*12*).

Under this focus, the preparation of LAO from Brazilian *Bothrops* species can be envisaged as a valid biotechnological prospect.

MATERIALS AND METHODS

Materials

Brazilian bothropic snake venoms were supplied by the Butantan Institute, Sao Paulo as lyophilized powders. Commercial snake venom preparations were purchased from Sigma Chemical Co. (St. Louis, MO). All samples were maintained under refrigeration. Bovine albumin (fraction V), Coomassie brilliant blue G and R-250, peroxidase (40 and 80 U/mg) from horseradish, L-amino acids, and flavin adenine dinucleotide (FAD) were obtained from Sigma. Other reagents were of analytical grade.

Protein Assay

Venom solutions were assayed for protein concentration according to the method of Bradford (*13*). The triplicate tests with venom samples (50 μg in a volume of 0.1 mL of 100 m*M*, pH 7.5, Tris-HCl buffer) were carried

out using 5 mL of protein reagent, vortexing the content in the tube, and measuring the absorbance at 595 nm with Varian DMS-80 spectrophotometer (Intralab, Sao Paulo, Brazil), after 20 min. The standard curve of bovine albumin was also made in the presence of the same buffer, in the 10–100 μg protein range.

L-Amino Acid Oxidase Activity

Venom solutions were analyzed by measuring the hydrogen peroxide generated during the oxidation of L-amino acids as described by Aisaka and Terada (*14*). The reaction mixture contained 2 m*M* of L-leucine (or other indicated L-amino acid), 16 m*M*, pH 7.5, Tris-HCl, 0.04 m*M* of 4-aminoantipyrine, 0.7 m*M* of phenol, 5 U of peroxidase, and enzyme (5 μg of venom in a volume of 10 μL with the same buffer) in a total volume of 3.0 mL. After incubation at 37°C for 20 min, the absorbance of the quinoneimine dye formed was measured at 500 nm. One unit of enzyme was defined as the amount of enzyme that produces 1 nmol of hydrogen peroxide/min. The standard curve of hydrogen peroxide was calculated using solutions containing 8.3–166.0 nmol of H_2O_2.

Coenzyme Spectral Analysis

Flavin adenine dinucleotide (4.2 m*M* in 0.1*M*, pH 7.0, phosphate buffer) spectrum was recorded taking advantage of its multiband feature at 263, 375, and 450 nm (*15*). A solution of *B. cotiara* snake venum (10 mg/mL) was analyzed in the same conditions with spectral scanning at 100 nm/min and chart speed of 2 cm/min.

Analytical Isoelectric Focusing

A *B. cotiara* snake venom solution (0.8 mg in 40 μL of 0.09% sodium chloride) was applied in the middle position of an Ampholine precast polyacrylamide gel (PAG) plate (80 × 55 × 1 mm), pH range from 3.5 to 9.5 (Pharmacia, Uppsala, Sweden). The electrode solutions were 1*M* phosphoric acid for the anode and 1*M* sodium hydroxide for the cathode, and the initial running conditions were 150 V, 8 mA and 1 W. After 3.5 h, the voltage was increased to 450–500 V until the final drop of the current to <0.3 mA. One-half of the gel was stained with coomassie, and the other half was dipped in the reaction system for the LAO activity for 10–20 min at room temperature. The coomassie staining solutions were 11.6% trichloroacetic acid and 3.4% sulphosalicylic acid for fixation, 0.25% coomassie brilliant blue R-250 in methanol:acetic acid:water (50:7:43) for staining, and 1-propanol:methanol:acetic acid:water 50:50:50:100) for destaining. For the LAO reaction in this technique *o*-dianisidine was used as coupling agent (*16*) instead of 4-aminoantipyrine.

High-Performance Liquid Chromatography Analysis

The bimodular SC 600E/WISP 172 machine (Waters/Millipore, Milford, MA) was equipped with a 484 tunable absorbance detector at 280 nm and sensitivity 1. A sample of *B. cotiara* snake venom (150 μg in 15 μL of 50 m*M*, pH 7.5, Tris-HCl buffer) was eluted with the same buffer (flow rate = 1 mL/min; 420 psi) through a Protein Pak DEAE-5PW column (7.5 mm × 7.5 cm) and the gradient operated from 0 to 100% with 50 m*M* NaCl.

Metal Analysis

A venom sample of *B. cotiara* (1.0 mg/mL in 0.1*M*, pH 7.5, phosphate buffer) was analyzed for zinc content on a CG atomic absorption spectrophotometer.

Hemorrhagic Activity of Determination

The assay for the determination of hemorrhagic activity (*17*) in *B. cotiara* venom was made on Wistar mice weighing between 18 and 22 g. Samples containing 1, 5, 15, 30, and 60 μg of venom (sample volume of 100 μL in 0.09% NaCl) were injected id to a depilated area in each different animal. After 2 h, the intensity of the skin responses was estimated from the inside face. The cross diameters of each hemorrhagic spot were measured in millimeters (mm), and the area of each spot was taken as indicator for the intensity of the response. The negative control test was carried out inoculating only 0.09% NaCl.

RESULTS AND DISCUSSION

Considering the animal kingdom as a rich source of bioactive and/or poisonous compounds, the Viperidae family, including the three main genera *Bothrops, Crotalus,* and *Lachesis,* contributes venoms of high toxicity. A feature that characterizes this snake family is the presence of solenoglyphic fangs, which facilitates the venom inoculation (*18*).

Most of the snake bites in Latin America, from Mexico to Argentina, are caused by species of the genus *Bothrops.* Venoms from these snakes induce a pathophysiological picture characterized by local damage, as hemorrhage, edema, and myonecrosis, and systemic effects, involving cardiovascular, coagulation, and renal alterations. The antivenom administration, rapidly after venom inoculation, is able to neutralize the systemic disorders, but it is not totally efficacious in the neutralization of local tissue damage (2). Owing to the relevance of local effects induced by *Bothrops* snakes, it becomes necessary to look for a better understanding of the inflammatory events (*19*) that follow envenomation, in order to develop better therapeutic strategies complementary to the conventional serotherapy. Moreover, the isolation, fractionation, and characterization

of the bioactive compounds in snake venoms are an important task to make possible their use as powerful tools in the study of basic inflammatory mechanisms. These procedures could also allow a more rational basis for selecting the venoms to be used in the production of antivenoms.

Toxins, enzymes, and biologically active peptides are found in varying quantities in snake venoms, depending on the species. In a comparative study of enzymatic activities using several animal vemons obtained from snake, lizard, toad, scorpion, spider, bee, wasp, and ant, it was observed that snake venom is the richest source of degradative enzymes (*20*). One of them is LAO, originally studied in the Australian mulga snake from the *Pseudechis* genus (*9*). Since most of Brazilian *Bothrops* venoms display a marked yellow color and LAO is a flavin adenine dinucleotide (FAD)-containing enzyme, we decided to investigate the occurrence of such a kind of enzyme in 19 samples arising from different species. The interest was further extended to hemorrhagic activity, and our main research focus was on *Bothrops cotiara,* a snake formerly found in the South of Brazil (States of Parana and Santa Catarina).

LAO catalyzes the oxidative deamination of L-amino acids (*21*) according to the reaction that follows:

$$\text{L-amino acid} + H_2O + O_2 \rightarrow \text{keto-acid} + NH_3 + H_2O_2$$

The 19 species of *Bothrops* displayed a quite variable profile when LAO activity was assayed with 4-aminoantipyrine as chromogen (Table 1). It may be observed that six venoms presented SA above 1000 U/mg, three venoms below 100 U/mg, and the 10 remaining between these two values. The sp. *pradoi* venom showed the highest value, whereas the sp. *itapetiningae* venom was almost devoid of LAO activity. The venom of *B. cotiara* exhibited a high level for LAO. The protein contents of the examined samples were around 0.60–1.16 mg/mg of lyophilized venom, when the protein determination was carried out by the method of Bradford (*13*). A value of 0.78 mg of protein/mg of venom was found for sp. *cotiara* in accordance with the result obtained by Furtado et al. (*23*). Tris interference was higher when protein estimation was carried out by the method of Lowry.

Since two commercial snake venom preparations were available, their LAO specific activities were also assayed, as shown in the Table 2, and the results were smaller than that of *B. cotiara.* A interesting feature of the Brazilian bothropic venoms showing a high level of LAO was the deep yellow color that could be associated with the presence of the usual FAD prosthetic group of LAOs. In order to confirm this, a sample of *B. cotiara* venom was spectrally scanned from 700 to 200 nm (Fig. 1A). Two major absorption bands (465 and 390 nm) effectively corresponded to those observed for the LAO prepared from the *Crotalus adamanteus* venom (*23*). Furthermore, the pure coenyzme assayed under the same conditions displayed the same two bands and one additional around 270 nm (Fig. 1B). In fact, these are the routine bands that characterize FAD (*15*).

Table 1
L-Amino Acid Oxidase Activity
and Protein Concentration in Genus *Bothrops* Snake Venoms

Snake	Habitat	Protein mg protein/ mg venom	LAO activity,[a] U/mL	Specific activity,[b] U/mg
Bothrops				
alternatus	—	1.00	314.95	629.90
atrox	Amazonia	0.80	478.29	1195.73
billineatus	Amazonia	0.68	512.49	1507.32
brazili	Amazonia	0.82	194.39	474.12
castelnaudi	—	0.76	197.00	518.42
cotiara	PR, SC	0.78	386.74	991.64
erythromelas	—	1.00	12.38	24.76
fonsecai	PB, SP, RJ, MG	0.80	273.92	684.80
hyoprorus	Amazonia (N)	0.60	19.94	66.47
insularis	Queimada Grande Island	0.86	355.15	825.93
itapetiningae	SP, MT, GO	0.96	2.13	4.44
jararaca	(SE)	0.72	314.95	874.86
jararacussu	(SE)	1.16	391.87	675.64
leucurus	—	0.60	320.08	1066.93
marajoensis	Marajo Island	0.68	214.91	632.09
mojeni	GO, MT, TO, MG SP, PR, (NE)	0.78	530.33	1359.82
neuwiedii	All states, except Amazonia	1.00	655.97	1311.94
pirajai	BA(S)	0.64	112.29	350.91
pradoi	ES	0.88	666.42	1514.59

[a] L-Leucine was used as substrate.
[b] nmol $H_2O_2 \cdot min^{-1} \cdot mg^{-1}$ protein.
LAO = L-Amino acid oxidase.
Regions: SE = southeast, NE = northeast, S = south, N = north.
States: AC = Acre, AM = Amazonas, AP = Amapa, BA = Bahia, ES = Espirito Santo, GO = Goias, MA = Maranhao, MG = Minas Gerais, MT = Mato Grosso, PA = Para, PB = Paraiba, PR = Paraná, RJ = Rio de Janeiro, RO = Rondonia, RR = Roraima, SC = Santa Catarina, SP = Sao Paulo, TO = Tocantins.
Amazonia: AM, PA, MA, AC, AP, RO, RR.

We also adopted the usual substrates L-leucine and L-phenylalanine to assay the LAO activity against increasing concentrations of *B. cotiara* venom. The curves in the Fig. 2A and B showed that the relationship between microgram of venom and absorbance, at 500 nm, is not linear for amounts of venom up to 15 μg. In all instances, the LAO activity measurements, using leucine, were higher than those obtained with phenylalanine.

Table 2
L-Amino Acid Oxidase Activity[a]
in Commercial Snake Venoms

Commercial snake venom	Specific activity[b] U/mg
Bothrops atrox LAO (Sigma A-4257)	690.42
Crotalus atrox LAO (Sigma A-5147)	682.71

[a]L-Leucine was used as substrate.
[b]nmol $H_2O_2 \cdot min^{-1} \cdot mg^{-1}$ protein.

Table 3 summarizes the specific LAO activity of *B. cotiara* venom evaluated with these usual, as well as several unusual substrates. Among the latter, some of them underwent a small degree of oxidative deamination by the enzyme. The decreasing order of activity was cystine, histidine, tyrosine, isoleucine, and cystein. The highest activities were obtained for methionine, leucine, tryptophan, and phenylalanine. Oxidative deamination of other amino acids by *Bothrops cotiara* may be neglected.

Our study of the isoenzymes distribution for LAO in *B. cotiara* venom was previously carried out by conventional electrophoresis using twin cellulose acetate strips and 50 m*M*, pH 8, Tris-HCl as running buffer. One strip was stained with Coomassie, and the other revealed for LAO activity using leucine/*o*-dianisidine. Coomassie stain showed eight bands, but its counterpart zymogram only three. Protein profile had a large and strong band with low pI, which lacks LAO activity. In order to improve the resolution of the proteic components from *B. cotiara* venom, it was submitted to isoelectric focusing (IEF) zymogram in the pH range of 3.5–9.0 using a polyacrylamide gel plate, as shown in Fig. 3. The number of bands visualized by coomassie corresponded to 30, whereas the active bands for LAO (using leucine as substrate) were 19. Moreover, confirming the preliminary cellogel result, strong coomassie-positive bands in the acidic region were not detected with the enzymatic reaction. The reason for this could be enzyme partial inactivation at the lower pH zone. Repeating the zymogram using other substrates (e.g., phenylalanine), the electrophoretic profiles were similar to that obtained with leucine, but different intensities of color for the bands were noted, depending on the chemical nature of L-amino acid. Enzyme activity with methionine was the strongest, whereas activity with serine was negative in accordance with the results obtained in the spectrophometric assay. In this way, it is apparent that the LAO zymogram, using *o*-dianisidine as chromogen, is a powerful tool to screen LAO activity using several L-amino acids as substrates. As one example, the zymogram with *B. atrox* venom led to a quite different zymogram profile that contained only six LAO-positive bands (results not shown).

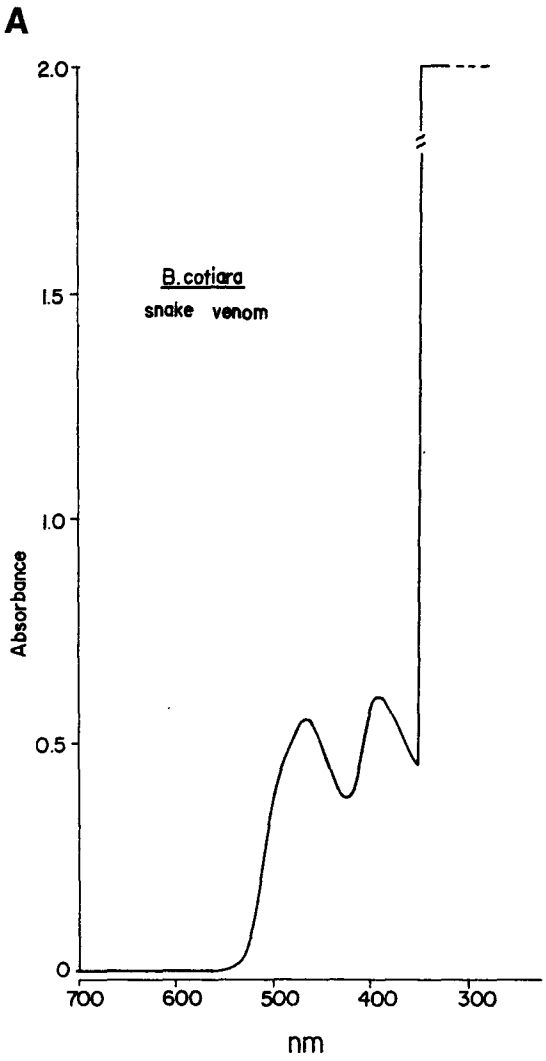

Fig. 1. UV-visible spectral analyses. A= *Bothrops cotiara* snake venom.

Since the IEF analysis was able to provide a consistent, but only qualitative profile of the active protein components present in *cotiara* venom, we decided to submit this venom to high-performance liquid chromatography (HPLC) on a Protein Pak DEAE-5PW column in order to obtain quantitative information at least of the protein distribution in the sample. The chromatographic pattern observed in Fig. 4 shows 21 protein peaks for *cotiara* venom. Four of them were prominent and appeared well separated in the salt gradient. This result was coincident with that reported by Furtado et al. (3), who analyzed the venom from the same species using a Mono-Q column, thus obtaining 19 peaks. Since charge difference was the protein property explored in both IEF and HPLC techniques, the respective profiles agreed.

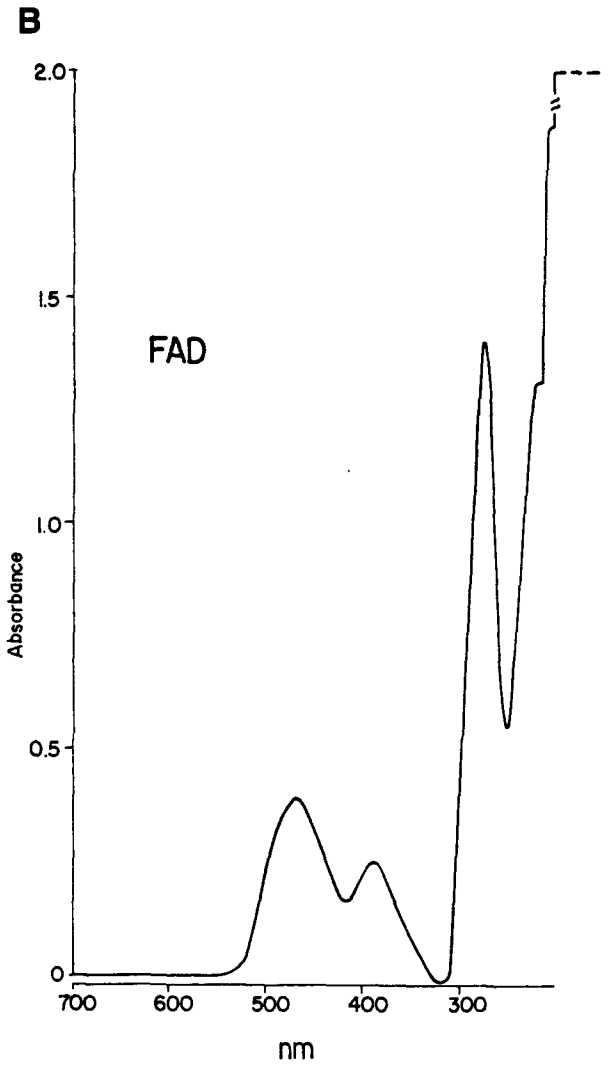

Fig. 1. (*continued*). B = Flavine adenine dinucleotide (FAD).

The occurrence of LAO in almost all venoms from Brazilian *Bothrops* snakes could indicate that this particular enzyme activity is involved with the toxic action of these venoms. The redox catalysis they bring about could also have a complementary action to the other activities already demonstrated in snake venoms, as for example, the hemorrhagic activity, that is also very characteristic for bothropic venoms (2). Amino acid oxidative deamination, in fact, may complement the action of any extensively proteolytic enzyme.

When assaying *B. cotiara* venom for hemorrhagic activity (Fig. 5), it was noted, after 2 h, that a defined hemorrhagic spot could be observed even when the venom protein amount was limited to 1 μg. Increase by 5 to 60× the crude venom dose produced an increase in the intensity of the

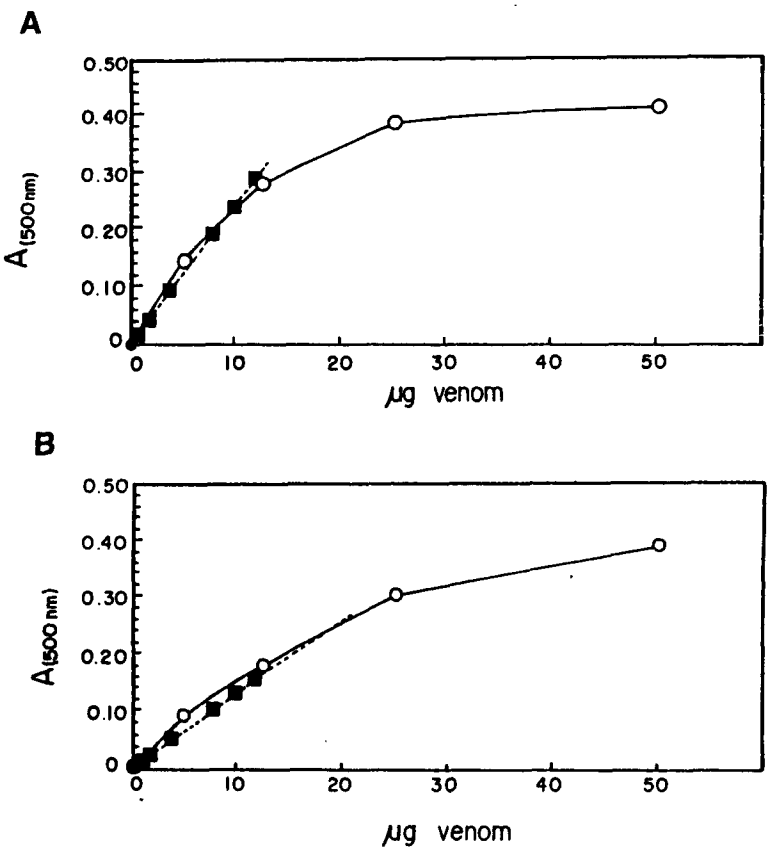

Fig. 2. *B. cotiara* venom LAO activity.* A = L-leucine substrate. B = L-phenylalanine substrate. (---○---○--- = assay range: 5–50 µg of venom). (-■-■-= assay range: 1–12 µg of venom). (*) 4-aminoantipyrine as chromogen.

Table 3
L-Amino Acid Oxidase Activity[a]
of *Bothrops cotiara* Snake Venom Using Various Substrates

Amino acid	Specific activity[b] U/mg
L-Methionine	917.58
L-Leucine	883.84
L-Tryptophan	717.74
L-Phenylalanine	582.79
L-Cystine	46.61
L-Histidine	40.39
L-Tyrosine	25.89
L-Isoleucine	18.12
L-Cysteine	7.77

[a] 2 m*M* amino acids as substrates.
[b] nmol $H_2O_2 \cdot min^{-1} \cdot mg^{-1}$ protein.

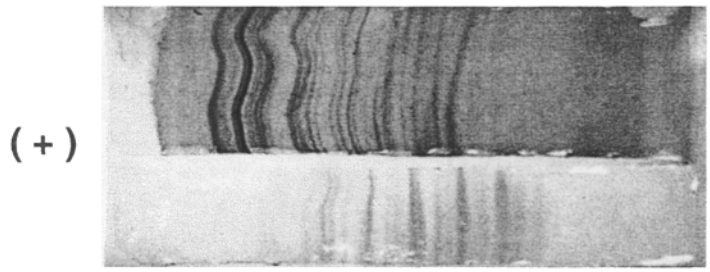

Fig. 3. PAGE isoelectric focusing-based zymogram for *B. cotiara* venom. Bottom: Coomassie staining. Top: LAO reaction with *o*-dianisidine as chromogen.

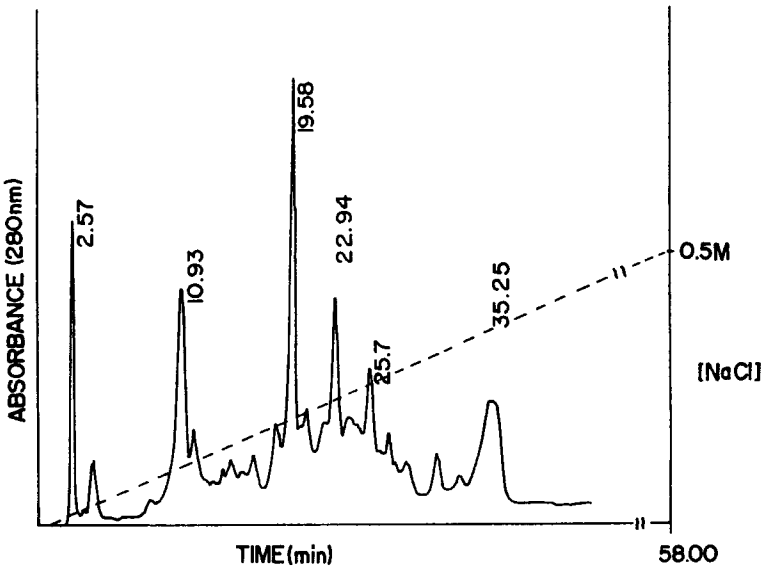

Fig. 4. HPLC analysis of *B. cotiara* venom (details in Materials and Methods).

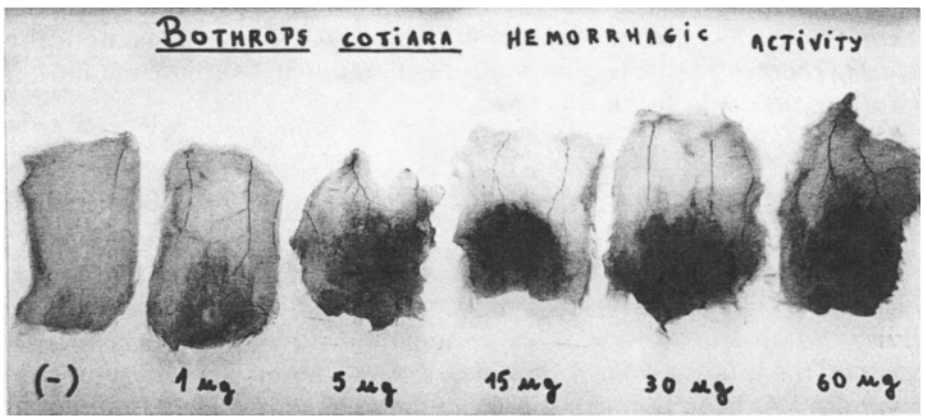

Fig. 5. Hemorrhagic activity assay of *B. cotiara* venom.

Table 4
Dose-Response Correlation for Hemorrhage Following *B. cotiara* Venom id Injection in Mice

Venom, μg	Area, mm^2
1	212
5	270
15	311
30	354
60	477

localized hemorrhagic spot, but not in proportion to the dose (Table 4). Since many hemorrhagic components isolated from snake venoms are Zn^{2+}-containing proteases (2), *B. cotiara* venom was analyzed by atomic absorption, and the content of 990 μg zinc/g venom corresponded to the values generally reported for venoms from different snake species (24). Thus, it is supposed that the hemorrhagic toxins in the *B. cotiara* also depend on the presence of Zn^{2+} to exhibit their activities.

Of the analyses investigated up to now, it can then be suggested that the *B. cotiara* venom LAO and hemorrhagic activities play some concomitant or at least sequential role in tissue lesion following the snake bite.

Moreover, LAO activity was detected in almost all venoms from Brazilian bothropic snakes (Table 1), indicating that it is an ordinary enzymatic activity within *Bothrops* genus. The practical application to be envisaged for the preparation of LAO from Brazilian bothropic species is the possibility to design a sensitive LAO electrode through enzyme immobilization in order to detect L-amino acid (25). This type of biosensor, combining pronase, is useful in the food industries for aspartame determination in dietary food products (12). Another application for LAO is its use for the removal of free L-amino acids from human plasma to improve the quantitative estimation of δ-aminolaevulinic, a precursor of porphyrins (10,11). In addition to the cases of congenital porphyrias, other pathological conditions, like saturnism, tyrosinemia, and AIDS-provoked porphyrias, also require a reliable quantitative evaluation of δ-aminolaevulinic. *B. cotiara* venom could fulfill this need.

CONCLUSIONS

The majority of the venoms obtained from Brazilian bothropic snakes, which are widely spread in that country, showed variable levels of LAO activity, when leucine was used as substrate. Enzyme-associated FAD explained the intense yellow color of *B. cotiara* venom. The IEF zymogram for venom LAO reaction using polyacrylamide gel revealed a number of

enzyme bands. Using different substrates, the reaction for LAO proceeded with different rates and final intensities, the strongest reaction being with methionine, a very important amino acid when dealing with feeder and fodder (e.g., single-cell protein quality evaluation). Therefore, the venoms of *Bothrops cotiara* and more closely related Brazilian species deserve future biotechnological applications. The venom of *B. cotiara* also presented intense hemorrhagic activity when injected intradermically on mice.

ACKNOWLEDGMENTS

The authors wish to thank CNPq and CAPES (funding agencies of Brazil). We are very grateful to Edmir Goncalves from Laboratório de Análises Clinicas Frischmann-Aisengart and Sérgio A. A. Morato from Museu de História Natural de Curitiba. The help of Nancy P. C. Albuquerque and José A. Florëncio is greatly appreciated.

REFERENCES

1. Kamiguti, A. S. and Cardoso, J. L. C. (1989), *Toxicon* **27(9),** 955–963.
2. Gutierrez, J. M. and Lomonte, B. (1989), *Mem. Inst. Butantan* **51(4),** 211–223.
3. Leite, L. C. C., Furtado, M. F. D., Correa, T. C., and Raw, I. (1992), *Comp. Biochem. Physiol.* **102B(3),** 515–520.
4. Lomonte, B. and Gutierrez, J. M. (1983), *Rev. Biol. Trop.* **31(1),** 37–40.
5. Reichl, A. P., Serrano, S. M. T., Assakura, M. T., and Mandelbaum, F. R. (1989), *Mem. Inst. Butantan* **51(4),** 225–237.
6. Bultron, E., Thelestam, M., and Gutierrez, J. M. (1993), *Biochim. Biophys. Acta* **1179,** 253–259.
7. Tanaka, N., Nakada, H., Itoh, N., Mizuno, Y., Takanishi, M., Kawasaki, T., Tate, S., Inagaki, F., and Yamashina, I. (1992), *J. Biochem.* **112,** 68–74.
8. Wellner, D. and Meister, A. (1960), *J. Biol. Chem.* **235(7),** 2013–2018.
9. Stiles, B. G., Sexton, F. W., and Weinstein, S. A. (1991), *Toxicon* **29(9),** 1129–1141.
10. Hermes-Lima, M. (1990), *Ciëncia Hoje* **13(75),** 16, 199.
11. Bechara, E. J. H. (1993), personal communication.
12. Male, K. B., Luong, J. H. T., Gibbs, B., and Konishi, Y. (1993), *Appl. Biochem. Biotechnol.* **38(3),** 189–201.
13. Bradford, M. M. (1976), *Anal. Biochem.* **72,** 248–254.
14. Aisaka, K. and Terada, O. (1981), *Agric. Biol. Chem.* **45(10),** 2311–2316.
15. Dawson, R. M. C., Elliott, D. C., Elliott, W. H., and Jones, K. M. (eds.) (1991), in *Data for Biochemical Research.* Clarendon Press, Oxford, pp. 124–125.
16. Huggett, A. S. G. and Nixon, D. A. (1957), *J. Biochem.* **66,** 12.
17. Kondo, H., Kondo, S., Ikezawa, H., and Murata, R. (1960), *Jpn. J. M. Sci. Biol.* **13,** 43–51.
18. Jared, C. and Furtado, M. F. D. (1988), *Ciëna Hoje* **7(42),** 27–32.

19. Lomonte, B., Tarkowski, A., and Hanson, L. A. (1993), *Inflammation* **17(2),** 93–105.
20. Tan, N. and Ponnudurai, G. (1992), *Comp. Biochem. Physiol.* **1003C(2),** 299–302.
21. Whelan, W. (ed.) (1974), *The Enzyme Commission,* Academic, pp. 176–177.
22. Furtado, M. F. D., Colletto, G. M. D. D., and Silva, W. D. (1991), *Mem. Inst. Butantan* **53(2),** 149–159.
23. Curti, B., Massey, V., and Zmudka, M. (1968), *J. Biol. Chem.* **243(9),** 2306–2314.
24. Friedrich, C. and Tu, A. T. (1971), *Biochem. Pharmacol.* **20,** 1549–1556.
25. Radu, G. L. and Coulet, P. R. (1993), *Analusis* **21,** 101–105.