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PHARMACOLOGICAL EFFECTS OF SNAKE VENOM L- AMINO ACID OXIDASES

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ABSTRACT
L-Amino acid oxidases are flavoenzymes which catalyze the stereospecific oxidative deamination of an L-amino acid substrate to a corresponding a-ketoacid with hydrogen peroxide and ammonia production. These enzymes, which are widely distributed in many different organisms, exhibit a marked affinity for hydrophobic amino acids, including phenylalanine, tryptophan, tyrosine, and leucine. Snake venom LAAO induces platelet aggregation and cytotoxicity in various cancer cell lines. The enzyme has antibacterial activity inhibiting the growth of Gram-positive (Bacillus subtilis) and Gram-negative (Escherichia coli) bacteria. Specific substrates for the isolated protein are L-phenylalanine, L-tryptophan, L-methionine and L-leucine. The enzyme is stable at low temperatures (−20 ºC, −70 ºC) and loses its activity by heating at 70 ºC. These enzymes are postulated to be toxins that may be involved in the allergic inflammatory response and specifically associated with mammalian endothelial cells damage. However, in the last decade these enzymes have become an interesting subject for pharmacological, structural and molecular characterizations. Structural and functional investigations of these enzymes can contribute to the advancement of toxinology and to the elaboration of novel therapeutic agents.
KEYWORDS: L-Amino acid oxidases, cytotoxicity, platelet aggregation, enzymes, therapeutic agents.

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INTRODUCTION

Snake venoms are recognized as useful sources of bioactive substances showing a wide range of pharmacological activities. Snake venoms cause a variety of different biological effects as they are a mixture of simple and complex substances, such as biologically active peptides and proteins but their biochemical characteristics change according to the snake species studied. 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 a-ketoacid with hydrogen peroxide and ammonia production[2].

L-amino acid + H_2O + O_2 ⟶ a 2-oxo acid + NH_3 + H_2O_2

This enzyme belongs to the family of oxidoreductases, specifically those acting on the CH-NH2 group of donors with oxygen as acceptor. The systematic name of this enzyme class is L-amino-acid: oxygen oxidoreductase (deaminating)[3]. This enzyme is also called ophio-amino-acid oxidase. These enzymes, which are widely distributed in many different organisms, exhibit a marked affinity for hydrophobic amino acids, including phenylalanine, tryptophan, tyrosine, and leucine[4]. The enzyme has been widely used in biochemical studies for identification of optical isomers of amino acids, preparation of α-keto acids, and production of thyroxin from 3, 5-di-iodotyrosine[5]. It is responsible for the yellow colour in snake venoms containing flavin as the prosthetic group. LAAO has been found to contribute to the toxicity of the venom due to the production of hydrogen peroxide during the oxidation reaction. In the last 10–15 years LAAO has gained interest due to its effects on various microorganisms[6-8], blood components (platelets) [6, 9-11], bacteria[6, 7, 12, 13] and cells, including cancer cells[14, 15].

L-Amino acid oxidases (LAAOs) are widely distributed in the venomous snake families Viperidae, Crotalidae and Elapidae[16]. Besides those from snake venoms, different LAAOs have been isolated from sea animals, including achacin, from the mucus of the giant snail, Achatina fulica Fe´ russac, escapin[17], from the ink of the

sea hare Aplysia californica, and APIT from Aplysia punctata, as well as from bacteria, fungi and plants[1]. They are usually homodimeric FAD-binding glycoproteins with molecular mass around 110–150 kDa. They are responsible for the yellow color of snake venoms, containing flavin as the prosthetic group. LAAOs are thought to contribute to the toxicity of the venom due to the production of hydrogen peroxide during the oxidation reaction[13]. Some of the snake species having LAAOs in the venom are given in **Table1**.

The tumoricidal effect of SV- LAAO's is completely abrogated in the absence of the amino acids L-lysine and L-arginine. The enzyme is stable at temperatures from 0 to 58ºC. Similar to other FAD-binding enzymes, it is resistant against tryptic digest. Even digest with proteinase K fails to degrade the enzyme. The individual LAAOs differ in their substrate specificity: snake venom L-amino acid oxidases (SV-LAAOs) which constitute up to 30% (by weight) of the crude venom[24], possess a clear preference for hydrophobic amino acids. A fish capsule LAAO termed AIP (Apoptosis-Inducing Protein) which is induced by larval nematode infection of Scomber japonicus is highly specific for L-lysine[18]. Achacin, mucus LAAO from the African snail Achatina fulica, metabolizes a very broad range of substrates, including hydrophobic amino acids along with L-lysine, L-arginine, L-cysteine, L-asparagine, and L-tyrosine[17]. Similar to AIP and achacin, apoxin 1, the sv-LAAO of the western diamondback rattlesnake has been shown to induce apoptosis in cultured tumor cells within 24 h by an H2O2 dependent mechanism[19]. APIT, the A. punctata ink toxin is a weakly glycosylated L-amino acid oxidase with high substrate specificity for the essential amino acids L-lysine and L-arginine. A functional difference between APIT and many other LAAOs is the mode of cell death they induce in tumor cells. Whereas Korean snake venom LAAO, AIP, achacin, and apoxin 1 induce apoptosis in tumor cells[14,19] incubation with APIT leads to a necrosis-like oxidative damage of the cells within 6–8 h. This H2O2 induced cell death is abrogated completely in the presence of catalase without the induction of apoptosis. A similar phenotype of cell death has been described for the treatment with dolabellanin A. Although several LAAOs have been identified so far, their biological role is still unclear. Some LAAOs from venoms and secretions like achacin and dolabellanin A, as well as potential LAAOs like the aplysianins, display a bactericidal and anti-fungal activity[17] which might be involved in the protection of body cavities from bacterial and fungal infections. The hypothesis that LAAOs are part of chemical defense mechanisms is further supported by studies of the apoptosis inducing protein (AIP) from Chub mackerel showing that this LAAO is induced upon infection with larval nematodes[18].

In recent years, many scientists concentrated their efforts on molecular cloning and expression of SV-LAAOs as well as structure characterization of SV-LAAOs[4, 20]. As far as we know, LAAOs from bacterial, fungal and plant species are involved in the utilization of nitrogen sources. But the physiological role of SV-LAAOs in snake envenomation is not well understood. Although more and more SV-LAAOs have been isolated and well-studied, the mechanisms of effects on platelet and bacteria are inconsistent.

The pharmacological activities of the enzymes are connected with the production of hydrogen peroxide, but the real mechanisms of action need further and deeper investigation. Better knowledge of the mechanisms allows us to consider the use of SV-LAAOs as models for therapeutic agents, as tools in biochemical research to investigate cellular processes, and gives better understanding of the envenomation mechanism.

Purification and Biochemical Characterisation of L-Amino Acid Oxidases

Snake venom LAAOs (SV-LAAOs) represents an interesting bioactive model for enzymology, structural biology, and pharmacology. Recently, several SV-LAAOs have been purified and characterized, showing distinct Mr, substrate preferences, platelet interactions, hemorrhage induction, and apoptosis[1].Crude venom can be purified for Amino Acid oxidases. Purification can be carried out by chromatography followed by centrifugation and ultra filtration. The active fraction, named LAAO, can be analyzed for purity by reverse-phase HPLC chromatography. The purified LAAO can be subjected for SDS PAGE method of Lamaeli 1970[21] to determine the molecular weight and the native LAAO can be subjected to Size Exclusion Chromatography for determination of molecular mass. A more accurate molecular mass of LAAO can be obtained using a MALDI orthogonal time of flight mass spectrometer (MALDI-TOF). Isoelectric point of LAAO can be determined by isoelectric focusing. Glycoprotein nature of LAAO was demonstrated with the Schiff reagent on SDS-PAGE. Thus, most of the SV-LAAOs are homodimeric glycoproteins associated by non-covalent bonds with an approximately pI of 4.4–8.2. Protein concentration of LAAO could be quantified by BCA method, using bovine serum albumin (BSA) as standard[1] or by the method of Bradford (1976).The amino acid sequence of SV–LAAOs can be determined by Edman Degradation method (**Table 2**)[22]. SV-LAAOs have been extensively studied regarding their function, but

structural studies were so far little explored, except for C. rhodostoma venom. The amino acid composition of LAAO from various species of snakes does not differ substantially[4].

LAAO Activity

Determination of LAAO activity can be done spectrophotometrically using method modified from Bergmeyer (1983) (23) and assay method can be done according to Ponnudurai et al.[24] According to spectrophotometric method one unit of enzyme activity was defined as the oxidation of 1 µ mol of L-leucine per min. According to Ponnudurai et al.[24] one unit (1 U) of enzyme activity was defined as the amount of enzyme able to produce 1 m mol of hydrogen peroxide/min, under the described conditions. In order to find out the affinity for different substrates, other amino acids (10 nmol) were assayed at different temperatures under the same experimental conditions. L-Leu is the favorite substrate for evaluating the enzymatic activity of purified SV-LAAOs (**Table 3**)[1, 23].

L-Lysine, the best substrate for the king cobra LAAO, is oxidated very slowly by other snake venom LAAOs. For all other LAAOs, including N. naja oxiana enzyme, the best substrates are hydrophobic amino acids L-Phe, L-Met, L-Leu, and L-Trp[10,12,16,22].This catalytic preference can be explained by differences of side-chain binding sites in the enzyme responsible for substrate specificity .The substrate specificity of snake venom LAAOs has been studied by Tan's group[16,25,26.] According to their tentative model the enzyme comprises three hydrophobic binding sites (a, b, c) for substrate accumulating one or two methyl/methylene carbons (subsite "a" accommodates one methylene carbon, subsite "b" — two), and an amino binding subsite (d)[25]. Moustafa et al. (2006)[27] have crystallized C. rhodostoma LAAO with L-Phe as substrate. This study provides a deeper understanding of the catalytic mechanism arriving at the conclusion that hydrophobic amino acids suit perfectly the hydrophobic pocket in the catalytic cavity. Ph determination can be done by incubating SV-LAAOs at 25ºC 2hrs at different pH conditions and enzyme activity could be measured using L-Leu as substrate.

Platelet aggregation activity of SV-LAAOS

Platelet aggregation is the clustering together of thrombocytes, which are the small, cell-like structures made in the bone marrow that help prevent bleeding. The reported effects of SV-LAAOs on platelet function are quite controversial: LAAOs from Trimeresurus jerdonii, B. moojeni, T. mucrosquamatus, B. atrox and B. pauloensis venom induce platelet aggregation, while LAAOs from V. lebetina, V. b. berus, A. b. ussurensis and Naja naja oxiana venom dose-dependently inhibit ADP- or collagen-induced platelet aggregation. The contradiction appears even with LAAO from the same sourced[28] claimed that O. hannah LAAO induces platelet aggregation in the concentration range of 10–60 mg/ml, and Jin et al. (2007)[20] have shown dose dependent inhibition of ADP-induced platelet aggregation in the concentration range of 4.8–48 mg/ml (IC50 0.15 mM).

It is not clear yet why some SV-LAAOs inhibit and others induce platelet aggregation. Sakurai et al. (2001)[9] consider that the controversies may be connected with differences in the experimental procedure or preparation of blood samples. Up to now, it was generally recognized that SV LAAOs exert their function via H_2O_2 production. Activities of induction or inhibition platelet aggregation of SV-LAAOs were dose-dependently reduced by catalase, a H_2O_2, indicating that the effects of SV-LAAOs on platelet function are related to H_2O_2 production[1, 29]. In opinion of Takatsuka *et al.* (2001)[11] the aggregation inducing enzymes must have extraordinarily high specific activities. Thus generating high concentrations of H_2O_2 (millimolar range), SV-LAAOs are perhaps able to induce platelet aggregation. Hydrogen peroxide itself induces aggregation in high concentrations (millimole). The induction of platelet aggregation has been explained by promotion of thromboxane A2 synthesis by H_2O_2, and consequent platelet aggregation[1, 29]. The concentrations of H_2O_2 liberated by SV-LAAOs are insufficient to induce platelet aggregation and both H_2O_2 production and binding to platelet membrane proteins might be involved in its action. In micromolar concentrations, H_2O_2 elicited an inhibition of platelet aggregation[11]. One reason for inhibition may be connected with a reduced binding for ADP in platelets exposed to H_2O_2[1]. The inhibitory activity might also be explained by the interference of peroxide in the interaction between activated platelet integrin GPIIb/IIIa and fibrinogen.

It is still unclear how H_2O_2 functions in SV-LAAO induced/inhibition platelet aggregation. Several recent studies showed that H_2O_2 production might not be fully responsible for the biological activities of SV-LAAOs. At least, the liberated H_2O_2 has not to be the only factor affecting platelet function. Some yet unknown mechanisms may be connected with the effects.

Antimicrobial susceptibility of SV-LAAOS

Since Skarnes (1970)[30] first reported bactericidal effects elicited by SV-LAAO from Crotalus adamanteus venom, more and more SV-LAAOs have been found to be effective against many bacteria. Like LAAOs from T. jerdonii , T. mucrosquamatus , A. halys, C. durissus cascavella, V. lebetina, B. moojeni, N. n. oxiana venom, B. pauloensis and B. jararaca snake venom.

Inhibition of the antibacterial activity of SV-LAAOs by catalase confirms the role of hydrogen peroxide in the process[1]. But the amount of H_2O_2 generated in the oxidative reaction of L-amino acid by LAAO is significantly less than that of H_2O_2 itself needed to inhibit the bacterial growth. Zhang *et al.* (2004)[13] determined that AHP LAAO from A. halys venom inhibited the growth of E. coli with an IC50 of 2.0 mg/ml which produced 0.21 mM H_2O_2 for 2.5 h, while H_2O_2 itself did with the IC50 of 8.5 mM. They demonstrated that the effective antibacterial action of AHP-LAAO could result from the binding of AHPLAAO to E. coli cells by fluorescence detection. Ehara *et al.* (2002)[17] also revealed that achacin, an LAAO isolated from the giant snail, showed significant bacterial-binding activity against E. coli and S. aureus by Western blotting. As a result, the production of highly localized concentration of H_2O_2 in or near the binding interfaces may elicit a potent antibacterial effect. Toyama et al. (2006)[7], on the contrary, showed that H_2O_2 produced by Casca LAO induced bacterial membrane rupture, and consequently loss of cytoplasmatic content. Furthermore, the results suggest that the ability of Casca LAO to bind to bacterial membranes does not seem to play an important role in its antibacterial activity. It remains to be clarified if H_2O_2 is fully responsible for antibacterial-activities of SV-LAAOs.

MRSA is a bacterium responsible for difficult-to-treat infections in humans. It is a resistant variation of the common bacterium S. aureus. MRSA has evolved an ability to survive treatment with beta-lactamase resistant betalactam antibiotics, including methicillin, dicloxacillin, nafcillin, and oxacillin. In the past decade or so the number of MRSA infections in the United States has increased significantly. A 2007 report in Emerging Infectious Diseases, a publication of the Centers for Disease Control and Prevention (CDC), estimated that the number of MRSA infections treated in hospitals doubled nationwide, from approximately 127,000 in 1999 to 278,000 in 2005, while at the same time deaths increased from 11,000 to more than 17,000 . In recent years, MRSA has become a source of public fear and outcry. Up to now, MRSA infection is said to be the third most common cause of death all over the world. SV-LAAOs also show high antibacterial activity against MRSA clinical isolates. The results suggested that the enzyme has potential anti-MRSA effect. The exploration for why LAAOs has high antibacterial effect against S. aureus strain may provide clues for new antimicrobial drug designing.

As far as known, LAAOs from bacterial, fungal and plant species are involved in the utilization of nitrogen sources. But the physiological role of SV-LAAOs in snake envenomation is not well understood. Although more and more SV-LAAOs have been isolated and well-studied, the mechanisms of effects on platelet and bacteria are inconsistent.

Anti tumor activity of SV-LAAOS

The use of snake venoms as tumor growth inhibitors has raised several studies. There are controversial works in the literature, but LAAOs isolated from the venoms of various snake species are described as enzymes with antitumor and apoptotic effects in various types of cells[15]. This type of inhibitory effect on tumor growth caused by LAAO has been demonstrated in earlier works with different cell lines, including human promyelocytic leukemia HL-60, HeLa, glioma , human ovary carcinoma A2780, endothelial cells from the human umbilical cord, mouse NR-3 endothelial cells and murine EL-4 lymphoma cells. However, reports evaluating the effect of LAAO on tumor growth in vivo are rare.

It has been reported that SV-LAAOs, present in the venom of various snakes such as crotalids, elapids and viperids, can induce cell death[1, 12]. Most of the biological effects of LAAO are believed to be due the secondary effect of H_2O_2 produced by enzymatic reactions[31]. Batrox LAAO induced cell death in Jurkat cells (acute T cell leukemia), and LAAO from Malayan pit viper induced both necrosis and apoptosis in Jurkat cells. Cell death by necrosis is attributed to H_2O_2 produced by oxidation of α-amino acids. The generation of toxic intermediates from fetal calf serum, binding and internalization of LAAO could contribute to apoptosis[32]. LAAO from B. pirajai induced cytotoxic effects for S180 tumor cells, human breast (SKBR-3) cells, acute T cell leukemia (Jurkat) cancer cells, and Erlich ascitic tumor (EAT) cells. No significant cell death was observed for macrophages[12]. LAAO from Vipera berus berus induced apoptosis in K562 cells after 24 h of incubation[10], LAAO from Agkistrodon contortrix laticinctus in 25 µg/mL caused DNA fragmentation in HL-60 cells, after 24 h of treatment with the enzyme. ACTX-8, an L-amino acid oxidase from Agkistrodon acutus, induced cell death in Hela cells. Apoptotic changes such as phosphatidyl serine externalization and DNA fragmentation were detected in ACTX-8-treated cells. This enzyme was able to stimulate ROS generation which was involved in cell death. ACTX-8 was involved in the activation of caspase-3 and -9. The apoptosis was accompanied by release of cytochrome c which indicated that mitochondrial pathway played a role in ACTX-8-induced cell apoptosis.

Apoptosis induction is probably the most potent defense against cancer progression, as most of the currently-used

chemotherapeutic drugs inhibit cancer cell proliferation by inducing apoptosis[33, 34]. The activation of caspases-9 and -3 and phosphatidylserine exposure are classic markers of the apoptotic process. Many LAAOs demonstrate apoptosis-inducing activity[32, 35] and it is partially due to the generation of hydrogen peroxide. LAAOs from snake venom are interesting multifunctional enzymes owing, at least partly, their effects to hydrogen peroxide liberating in the oxidation process. The underlying mechanism of the physiological effects produced by these enzymes needs further investigation.

Leishmanicidal activity of SV-LAAOS

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 species involved and host's immune response[36].

Studies carried out by Tempone et al. (2001)[37] showed that hydrogen peroxide generated by LAAO was a strong inducer of apoptosis in promastigotes of Leishmania ssp. cells, where oxidative stress induced by hydrogen peroxide could activate heat shock proteins and initiate cell membrane and cytoplasmatic disorganization and consequently cell death. The ability of peroxide hydrogen to induce apoptosis in promastigotes is well known and basically involves a balance between the production of ROS and the ability of the cell's antioxidant system to neutralize it. In some cases, when the antioxidant system fails, the ROS produced triggers the apoptotic event[38]. Murray and Cartelli, 1983[39] demonstrated that amastigotes from the Leishmania donovani contained three times more catalase, 14 times more glutathione oxidase, and were four times more resistant to enzymatically generated hydrogen peroxide than to promastigotes.

Despite the antioxidant ability of Leishmania, LAAO induces a high production of hydrogen peroxide and the minimal concentration of LAAO necessary to induce a death rate of 50%or inhibited the growth rate of cells (IC50) of cells in Leishmania donovani promastigotes was 2.39 mg, similar to the ones found by Tempone *et al.,* (2001)[37]. Other important considerations involve the production of hydrogen peroxide also occurring in the Leishmania and other intracellular blood parasites, such as Trypanosoma. Recently Das et al.(2001)[40] demonstrated that apoptosis is an important control event maintain Leishmania ssp cell numbers.

CONCLUSION

From this review it is clear that the physiological role of SV-LAAOs in snake envenomation is not well understood. Although more and more SV-LAAOs have been isolated and well-studied, the mechanisms of effects on platelet and bacteria are inconsistent. The pharmacological activities of the enzymes are connected with the production of hydrogen peroxide, but the real mechanisms of action need further and deeper investigation. Better knowledge of the mechanisms allows us to consider the use of SV-LAAOs as models for therapeutic agents, as tools in biochemical research to investigate cellular processes, and gives better understanding of the envenomation mechanism. Some functional and structural studies with SV LAAOs have demonstrated their high biotechnological potential for design of novel therapeutic agents. In conclusion, the biological effects of snake venom LAAOs are connected with the production of hydrogen peroxide but the real mechanisms of action need further investigation. Better knowledge of the mechanism of action allows us to consider the use of L-amino acid oxidases as models for therapeutic agents, as tools in biochemical research to investigate cellular processes, and gives better understanding of the envenomation mechanism. SV-LAAOs are therefore interesting multifunctional enzymes, not only for a better understanding of the ophidian envenomation mechanism, but also due to their biotechnological potential as model for therapeutic agents.

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Table 1: Species of snakes with L-Amino Acid Oxidases in their venom

Family	Description
Atractaspididae (atractaspidids)	Burrowing asps, mole vipers, stiletto snakes.
Colubridae (colubrids)	Most are harmless, but others have toxic saliva and at least five species, including the boomslang (*Dispholidus typus*), have caused human fatalities.
Elapidae (elapids)	Cobras, coral snakes, kraits, mambas, sea snakes, sea kraits and Australian elapids
Viperidae (viperids)	True vipers and pit vipers, including rattlesnakes.

Table 2: The first 20 amino acid sequence of SV- LAAOs of certain species of snakes by Edman Degradation method

SNAKES	N-TERMINAL SEQUENCES	IDENTITY (%)
B. alternatus	ADVR**N**P**L**-**EE**-F**R**ETD**Y**E**V**-**L**	100
Echis macmahoni	ADDK**N**P**L**-**EEA**F**R**EAD**Y**E**V**F**L**	80
Cotralus atrox	AHDR**N**P**L**-**EEC**F**R**ETD**Y**EEF**L**	80
C. adamanteus	AHDR**N**P**L**-**EEC**F**R**ETD**Y**EEF**L**	80
T. flavoviridis	AHDR**N**P**L**-**EEY**F**R**ETD**Y**EEF**L**	20
C. rhodostoma	ADDR**N**P**L**A**EE**-F**Q**E**NN**Y**EEF**L**	70
Agkistrodon c. laticinctus	ADSR**N**P**L**E**EE**-F**R**ETN**Y**EEF**L**	69
Naja n. kaouthia	DDRRSP**L**-**EEC**F**Q**QND**Y**EEF**L**	51
O. hannah	-SVI**N**-**L**-**EESFQ**EPE**Y**E**N**H**L**	45

Table 3: Substrate specificity of SV-LAAOs

TYPES OF L-AMINO ACIDS	NAMES OF L-AMINO ACIDS	RELATIVE ACTIVITY (%)
Non-polar amino acids	L-Leu	100.0
	L-Ile	66.7
	L-Phe	86.7
	L-Pro	0
Polar, uncharged amino acids	L-Asn	0
	L-Gly	0
	L-Ser	0
Acidic amino acids	L-Asp	16.7
	L-Glu	16.7
Basic Amino Acids	L- Lys	13.3
Others	L-Cys	0

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