Review Article

Snake Venom L-Amino Acid Oxidases and Their Potential Biomedical Applications

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Abstract

L-amino acid oxidase (LAAO) occurs widely in snake venoms. The enzyme is highly specific for L-amino acids, and generally hydrophobic amino acids are the best substrates. LAAO is a flavoprotein consisting of two identical subunits, each with a molecular mass of approximately 60 kDa. The purified enzymes are glycoproteins with 3-4% carbohydrate. Deglycosylation of the enzyme did not alter the enzymatic activity but appeared to alter its pharmacological activities. The amino acid sequences of snake venom LAAOs showed a high degree of homology. X-ray structural analysis of LAAO revealed a dynamic active site and the presence of 3 domains: a FAD-binding domain, a substrate-binding domain and a helical domain. LAAOs were reported to exhibit moderate lethal toxicity. Recent studies showed that LAAOs are multifunctional enzymes exhibiting edema-inducing, platelet aggregation inducing or inhibiting, apoptotic inducing as well as anti-bacterial, anti-coagulant and anti-HIV effects. These effects are mostly mediated by the H_2O_2 liberated in the oxidation process but direct interactions between LAAO and the target cells may play an important role. High resolution X-ray structure of the enzyme revealed the presence of a channel that would direct the H_2O_2 product to the exterior surface of the protein, near the glycan moiety at Asn 172. The glycan moiety was thought to be involved with LAAO-target cell interaction. This may explain the ability of LAAO to localize H_2O_2 to the targeted cells. A better understanding of the pharmacological actions of LAAOs will facilitate the application of snake venom LAAOs in the design of anti-cancer and anti-HIV drugs as well as drugs for the treatment of infectious diseases caused by parasites such as leishmaniasis.

Keywords: L-amino acid oxidase, snake venom

Introduction

L-Amino acid oxidase (L-amino acid:O_2 oxidoreductase, E.C. 1.4.3.2.) is a flavoenzyme that catalyzes the oxidative deamination of an L-amino acid to form the corresponding α-ketoacid and ammonia: $RCH(NH_3^+)COO^- + O_2 + H_2O + RCOCOO^- + NH_4^+ + H_2O_2$

L-Amino acid oxidase (LAAO) occurs widely in nature [1] and snake venoms are perhaps the richest sources of the enzyme. Snake venom LAAOs are generally very active and have been used widely in preparation of α-keto acids because of their chemo- and stereospecificity [2, 3]. α-Keto acids of essential amino acids are useful nutraceuticals as well as therapeutic agents for certain diseases. Recently, snake venom LAAO has become an interesting object for biomedical studies because of its antimicrobial, anti-HIV, anticoagulant, platelet aggregation-inducing and inhibiting, apoptotic-inducing as well as anti-cancer activities. Snake venom LAAO is recognized as a multifunctional protein with promising biomedical application. Several reviews on snake venom L-amino acid oxidases have been published [1, 4-9].

LAAO Assay Methods

Many methods of LAAO assay are available [1]. The O_2 electrode technique has been widely used, particularly in kinetic studies. A commonly used spectrophotometric method was described by Bergmeyer, which measured the rate of oxidation by measuring the rate of formation of color complex between the hydrogen peroxide produced and o-dianisidine [10]. Based on the same principle, a spectrophotometric microplate assay has been developed suitable for processing large numbers of samples [11].

Occurrence in Snake Venoms

LAAO can be found in venoms from most genera [12]. The richest sources of LAAO are the crotalid venoms. The enzyme usually constitutes 1-4% of the venom by weight but in *Calloselasma rhodostoma* (Malayan pit viper) it constitutes up to 30% by weight of the dried venom [8]. Venoms from mamba and sea snakes either contain no or trace amount of L-amino acid oxidase activity.

Purification of Snake Venom LAAOs

Since 1990s, many authors have reported the purification and characterization of LAAOs from various snake venoms (Table 1). In some snake venoms, the enzymes present were in many isoforms. Hayes and Wellner, for example, reported that there were at least 18 isoforms of the LAAO in *Crotalus adamanteus* venom, and that glycosylation contributes to the microheterogeneity

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for the enzyme [13]. However, microheterogeneity was not observed for LAAOs isolated from most other venoms. In general, it is relatively easy to obtain homogenous LAAO from snake venom. For example, the LAAO from *C. rhodostoma* venom can be obtained using a simple two-step procedure: Sephadex gel-filtration chromatography followed by Mono-Q high performance ion exchange chromatography (Figure 1) [14].

Table 1: Physiochemical Properties of Some Purified Venom L-Amino Acid Oxidases

Venom source	Some physical properties	Reference
Agkistrodon controtix laticinctus	120 kDa (dimer), 60 kDa, pI 4.4	Souza *et al.* [32]
A.halys blomhoffii	60 kDa, pI 4.9	Takatsuka *et al.* [22]
Bothrops alternatus	123 kDa (dimer), 66 kDa, pI 5.37	Stabeli *et al.* [28]
B. moojeni	140 kDa (dimer), 69 kDa, pI 4.8	Tempone *et al.* [51]
B. piraja	66 kDa	Izidora *et al.* [29]
Calloselasma rhodostoma	132 kDa (dimer), 66 kDa, pI 4.4, carbohydrate 4.4%	Ponnudurai *et al.* [14]
Crotalus admanteus	58.7 kDa carbohydrate 3-4%	Raibekas and Massey, [19]
C. atrox	100 kDa (dimer), 55 kDa, pI 6.0-6.5	Torii *et al.* [48]
C. dirissus cascavella	120 kDa (dimer), 68 kDa, pI 5.43	Toyama *et al.* [45]
Eristocophis macmahoni	58.7 kDa	Ali *et al.* [44]
Naja naja kaouthia	112 kDa (dimer), 57.4 kDa, pI 8.12	Tan and Swaminathan [54]
Ophiophagus hannah	135 kDa (dimer), 65 kDa, pI 4.5, carbohydarate 3.8%	Tan and Saifuddin [35], Li *et al.* [43]
Pseudechis australis	142 kDa (dimer), 56 kDa	Stiles *et al.* [51]
V. berus berus	126 kDa (dimer), 55.7 kDa	Samel *et al.* [33]
V. lebetina	140 kDa (dimer), 60.9 kDa	Tonismagi *et al.* [34]
Trimeresurus flavoridis	55 kDa	Abe *et al.* [55]
T. mucrosquamatus	140 kDa (dimer), 70 kDa, pI 5.6	Ueda *et al.* [56]
T. stejnegeri	120 kDa (dimer), 58 kDa,	Zhang *et al.* [23]

Physical Properties of Snake Venom LAAOs

General physical properties

Snake venom LAAOs generally have molecular mass ranging from 112 kDa to 140 kDa as determined by gel filtration chromatography and 57-68 kDa by SDS-polyacrylamide gel electrophoresis, indicating that the enzymes are dimers and usually with identical subunits (Table 1). Snake venom LAAOs have a wide range of isoelectric points, ranging from 4.4 to 8.12 [8]. LAAO is a flavoprotein with two molecules of flavin coenzymes. The flavins, which exhibit absorption maximum at 275, 390 and 462 nm, are responsible for the yellowish color of the enzyme as well as for the venoms. Most authors reported that the flavin coenzymes are both FAD though some earlier reports suggested FMN as the coenzymes [8].

Snake venom LAAOs are stable at room temperature and at 4°C [8]. *Ophiophagus hannah* LAAO, for example, at pH 7.4, retained 100% and 80%, respectively, of activity after incubating at 37°C for 5, and 14 days. Many LAAOs, however, are unstable at alkaline condition. Some snake venom LAAOs have highly sensitive active sites. For example, *C. adamanteus* LAAO undergoes reversible pH or temperature-dependent inactivation, accompanied by structural changes in flavin binding site though retaining its overall secondary structure [15]. Earlier, Curti *et al.* reported that *C. adamanteus* LAAO was inactivated by storage at -5°C and -60°C, and by freeze-drying [16]. Many other snake venom LAAOs are also inactivated by freezing. Generally, the inactivated enzyme can be reactivated completely by heating at pH 5. The inactivation was accompanied by shifts in absorption spectrum and optical rotary dispersion

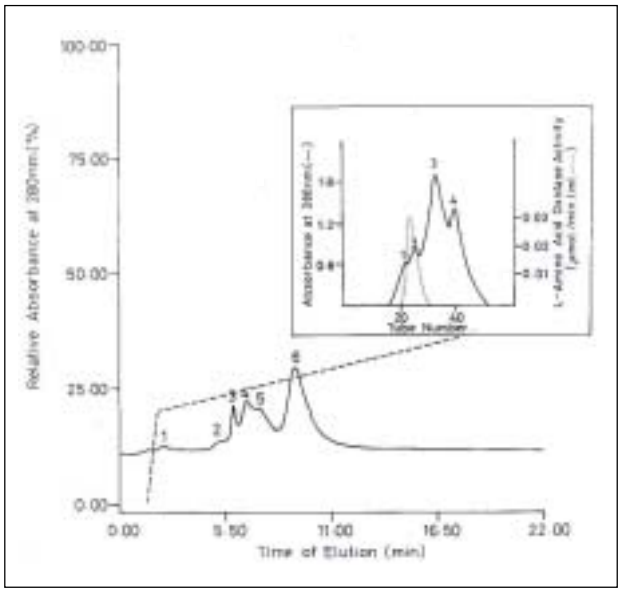

Figure 1: Purification of *C. rhodostoma* LAAO. The LAAO fraction obtained from Sephadex G-200 gel filtration chromatography by the crude venom (100mg; insert, top right) was injected into the column equilibrated with 0.02 M Tris-HCl, pH 8.5, and a linear, 0.2 to 0.4 M sodium chloride gradient was started 2 min after injection of the sample. Flow rate was 1 ml/min. Peak 6 was the purified LAAO. (----): Sodium chloride gradient.

spectrum, and reactivation reverses the spectra changes completely. The inactivation was believed to be due to a limited conformational change of the enzyme structure, presumably also in the vicinity of the flavin biding site [17]. This has been substantiated by X-ray structural studies. Some snake venom LAAOs (for example, LAAOs from *O. hannah* and *C. rhodostoma*), however, are not inactivated by freezing [8].

Reconstitution of LAAO

As a result of the high sensitivity of many snake venom LAAOs to their microenvironment, it was not possible to prepare reconstitutable apoprotein, as reconstitution with the FAD coenzyme often resulted in an inactive protein, with a perturbed conformation of the flavin binding site. Raibekas and Massey reported near complete activation of the reconstituted apoprotein and the restoration of its native flavin binding site in the presence of 50% glycerol [18]. Glycerol as a co-solvent plays a special role in this restorative process by induction of rearrangement in the protein structure. The authors suggested that hydrophobic effect appears to be the dominating force in this *in vitro*-assisted restorative process.

Cloning and Expression of Snake Venom LAAOs

Several snake venom LAAOs have been cloned and sequenced [19-25]. The expression of LAAO's from several animal species has been attempted in various expression hosts but the levels of expression were generally rather low, presumably due to the toxic effects of LAAO to the host cells. Torii *et al.*, however, reported that LAAO from *Crotalus atrox* could be expressed in an active form in mammalian cells [20]. Kommoju *et al.* reported the successful expression of active recombinant LAAO in the methylotropic yeast *Pichia pastoris* [24]. The catalytic properties and substrate specificity of the recombinant LAAO are similar to those of the native enzyme.

Chemical Structure of LAAOs

N-terminal sequences

The N-terminal amino acid sequences of many LAAOs have been elucidated and they are highly similar, except that from *O. hannah.* (Figure 2). There is the presence of a highly conserved Glu rich motif. The structure of LAAO from *C. rhodostoma* revealed that residues 5-25 constituted one part of the substrate-binding domain. The conserved amino acids may therefore play an important role in the substrate binding.

C.rhodostoma	**A**	**D**	**D**				**R**	**N**	**P**	**L**	A	**E**	**C**	**F**	Q	E	N	**D**	**Y**	**E**	**E**	**F**	**L**
C.atrox	**A**	H	**D**				**R**	**N**	**P**	**L**	**E**	**E**	**C**	**F**	**R**	**E**	T	**D**	**Y**	**E**	**E**	**F**	**L**
C.adamanteus	**A**	H	**D**				**R**	**N**	**P**	**L**	**E**	**E**	**C**	**F**	**R**	**E**	T	**D**	**Y**	**E**	**E**	**F**	**L**
B.pirajai	**A**	**D**	**D**				K	**N**	**P**	**L**	**E**	**E**		**F**	**R**	**E**	T	N	**Y**	**E**	V	**F**	**L**
A.h.blomhoffi	**A**	**D**	**D**				**R**	**N**	**P**	**L**	**E**	**E**	**C**	**F**	**R**	**E**	T	**D**	**Y**	**E**	**E**	**F**	**L**
T.stejnegeri	**A**	**D**	**D**				**R**	**N**	**P**	**L**	**E**	**E**	**C**	**F**	**R**	**E**	T	**D**	**Y**	**E**	**E**	**F**	**L**
V.lebetina	**A**	**D**	**D**				K	**N**	**P**	**L**	**E**	**E**	**C**	**F**	**R**	**E**	D	**D**	**Y**	**E**	**E**	**F**	**L**
N.scutatus	**A**	**D**	**D**	R	**R**	R	**P**	**L**	**E**	**E**	**C**	**F**	Q	**E**	A	**D**	**Y**	**E**	**E**	**F**	**L**		
N.n.kaouthia		**D**	**D**	R	**R**	S	**P**	**L**	**E**	**E**	**C**	**F**	Q	Q	N	**D**	**Y**	**E**	**E**	**F**	**L**		
O.hannah	H	V	I				**N**		**L**	**E**	**E**	S	**F**	Q	**E**	P	E	**Y**	X	N	H	**L**	

Figure 2: N-terminal amino acid sequence of some snake venom L-amino acid oxidases. Residues in bold are the same in most enzymes. X: uncertain. Sequences are taken from the following references: Ponnudurai *et al.* [14], Sakurai *et al.* [46], Izidoro *et al.* [29] and Tonismagi *et al.* [34].

Amino acid sequences

Raibekas and Massey reported the cDNA and deduced amino acid sequence of *C. adamanteus* venom LAAO [18]. Analysis and search for protein similarity showed that the enzyme possesses 37% homology with a protein encoded by the mouse B cell interleukin 4-induced *Fig 1* protein, the role of which is yet to be determined. The N-terminus of the protein contains a common fingerprint or βαβ-fold for binding of the adenylate moiety of FAD [9]. Takatsuka *et al.* reported that the full-length cDNA sequence of *Agkistrodon halys blomhoffii* LAAO encodes a putative a signal peptide with 18 amino acid residues and a 486-residue subunit (Figure 3) [22]. Franca *et al.* reported the cDNA sequences of LAAO from *B. moojeni* and *B. jararacussu* venom [25], and phylogenetic analysis showed sequence identities within the range 83-87% being closely related to LAAO from *A. h. blomhoffii* [22] and *Trimeresurus stejnegeri* venoms [23].

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MNVFFMFSLLFLAALGSCADDRNPLEECFRETDYEEFLEIARNGLKATSNPKHVVIV
GAGMSGLSAAYVLSGAGHQVTVLEASERAGGRVRTYRNDKEGWYANLGPMRLPEKHR
IVREYIRKFGLQLNEFSQENDNAWYFIKNIRKRVGEVKKDPGVLKYPVKPSEEGKSA
GQLYEESLGKVVEELKRTNCSYILNKYDTYSTKEYLLKEGNLSPGAVDMIGDLMNED
SGYYVSFPESLRHDDIFAYEKRFDEIVGGMDKLPTSMYRAIEEKVHLNAQVIKIQKN
AEKVTVVYQTPAKEMASVTADYVIVCTTSRATRRIKFEPPLPPKKAHALRSVHYRSG
TKIFLTCTKKFWEDEGIHGGKSTTDLPSRFIYYPNHNFTSGVGVIIAYGIGDDANFF
QALDFKDCADIVINDLSLIHQLPREEIQTFCYPSMIQKWSLDKYAMGGITTFTPYQF
QHFSEPLTASVDRIYFAGEHTAEAHGWIDSTIKSGLRAARDVNRASEQ
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Figure 3: Deduced amino acid sequence of *Agkistrodon halys blomhoffii* L-amino acid oxidase. Adapted from Takatsuka *et al.* [25].

Structure of glycans

LAAO is a glycoprotein with 3-4% carbohydrate, and it has been shown that glycosylation contributes to the microheterogeneity reported for some LAAOs [13]. Glycosylation appears to be important for the secretion and the solubility of the protein. Removal of the glycan moieties from *C. rhodostoma* LAAO leads to insoluble proteins [26]. The glycan moieties also appear to play an important role in the pharmacological actions of the enzyme. It has been suggested that LAAO docks to the cell surface leading to the generation of high local concentrations of hydrogen peroxide [27], which mediates many pharmacological actions of LAAOs. Deglycosylation of the enzyme, however, did not alter the enzyme activity [28, 29].

Geyer *et al.* reported on the glycosylation of LAAO from *C. rhodostom*a [26]. Its glycosylation is remarkably homogeneous with the major oligosaccharide accounting for approximately 90% of the total sugar content. The glycan is identified as a bis-sialylated, biantennary, core-fucosylated dodecasaccharide. An interesting possibility with regard to the homogeneity of the glycan moiety is that it is a functional requirement connected with the biological activities ascribed to LAAO.

Three-dimensional Structure of LAAOs

X-ray structure of C. rhodostoma LAAO

The X-ray structure of *C. rhodostoma* LAAO has been elucidated and the topography of the enzyme most closely resembles that of polyamine oxidase [30]. The data indicate that it is functionally a dimer consisting of two 55 kDa protomers. Each protomer consists of 15 α-helices and 22 β-strands that fold into three well-defined domains: FAD-binding domain, substrate-binding domain and a helical domain. The main structural feature of the FAD-binding domain (consists of residues 35-64, 242-318 and 446-486) is a six-stranded β-pleated sheet sandwiched between three α-helices and a four-stranded β-pleated sheet. The motif makes up the classical nucleotide-binding fold seen in many FAD-binding enzymes. The substrate-binding domain is made up of residues 5-25, 73-129, 233-236 and 323-420. The helical domain is made up of residues 130-230, and comprises one side of a funnel-shaped entrance to the active site of the enzyme. The interface between the substrate-binding and helical domains forms a 25A long funnel, which provides access to the active site. Comparison of the LAAO with the structure of mammalian D-amino acid oxidase reveals significant differences in their modes of substrate entry.

Moustafa *et al.* reported a high resolution (1.8A) X-ray structure of *C. rhodostoma* LAAO with its substrate L-phenylalanine [31]. The data revealed a dynamic active site, as conformational changes were apparent for the isoalloxazine ring. There was a Y-shaped channel system, extending from the external surface of the protein to the active site. The authors suggested that one portion of this channel may serve as the entry path for O_2 during the oxidative half-reaction. On the other hand, the second region, which was separated from the proposed O_2 channel by the N terminus (residues 8-16) of the protein, may play a role in H_2O_2 release. Presumably, the channel would direct the H_2O_2 product to the exterior surface of the protein, near the glycan moiety at Asn 172, which was thought to anchor the enzyme to the host cell. This channel location may explain the ability of the enzyme to localize H_2O_2 to the targeted cell and thus induce the apoptotic effect as well as other pharmacological activities.

The X-ray structure confirmed that the carbohydrate moieties are linked to Asn 172 and Asn 361. The authors speculated that the disialylated oligosaccarides at Asn 172, which is located in the vicinity to the channel leading to the active site of the enzyme, may bind to siglecs (sialic acid-binding Ig superfamily lectins) of the target cells via its sialylated glycan moiety, and may then result in production of locally high concentration of H_2O_2 in or near the binding interface. This, in turn, could lead to oxidative damage of the siglec or another adjacent cell structural element.

Molecular modeling of B. jararacussu and B. moojeni LAAOs

Molecular modeling experiments with overlapping of *B. jararacussu* and *B. moojeni* LAAO models demonstrated that these proteins are almost identical [25]. In addition, the overall fold of the two models is very similar to that of LAAO from *C. rhodostoma* venom. All the essential residues in *C. rhodostoma* LAAO are conserved in the *B. moojeni* and *B. jararacussu* LAAO models, demonstrating the putative functional similarity between the models and the *C. rhodostoma* LAAO structure. It is possible that most snake venom LAAOs have similar three-dimensional structure.

Enzymatic Properties of LAAOs

General enzymatic properties

LAAO required Mg^{2+} and was inhibited by Ca^{2+}, phosphate as well as p-chloromercuribenzoate. Certain amino acids stabilize the enzyme, while at high concentration they become inhibitors. The enzyme is also competitively inhibited by various aliphatic and aromatic acids and had a pH optimum of between 7 and 8.5 [8]. LAAO from different sources differ substantially in their specific activity. When L-leucine was used as the substrate, at pH 8.5, the specific activities of the enzymes isolated from *C. rhodostoma, N. kaouthia* and *O. hannah* were 0.54, 4.59 and 20.9 μmole/min/mg, respectively. Substrate inhibition occurs at high substrate concentrations.

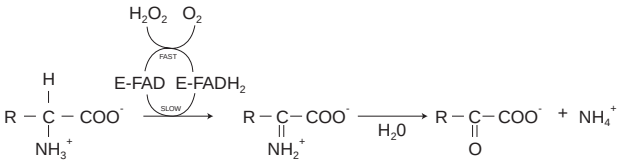
Substrate specificity

LAAO did not oxidize any D-amino acid and was highly specific for the L-enantiomer of amino acids. Effective oxidation of L-amino acid by the enzyme requires the presence of a free primary α-amino group. Many authors have investigated substrate specificity of snake venom LAAO [8]. Generally, the best substrates are L-Leu, L-Met, L-Phe, L-Tyr and L-Tryp, whereas L-Lys, L-Ser, L-Thr, L-Asp and L-Glu were generally hydrolyzed slowly or not at all [14, 28, 29, 32-34]. One exception is the LAAO from *O. hannah*, with which " L-Lys is the best substrate [35]. It is interesting to note that the N-terminal sequence of the *O. hannah* (king cobra) enzyme is very different from the other snake venom LAAOs.

Examination of the substrate specificity data of snake venom LAAOs suggested the presence of an alkyl side chain binding site which comprises at least four sub-sites, each accommodating a methyl/methylene carbon. *O. hannah* LAAO, on the other hand, appears to have an additional amino binding sub-site. Dixon and Kleppe suggested also the presence of an alkyl-binding site which can accommodate a chain of four carbons in D-amino acids [36].

Mechanism of catalysis

The oxidation of L-amino acid by the enzyme proceeds in two steps, forming α-imino acid as the intermediate product as detected by the borohydride trapping experiments [5].



During the reductive half-reaction, the α-hydrogen atom of the amino group is abstracted by FAD, producing the α-imino acid intermediate, which then reacts with water to form the α-keto acid. Two alternative mechanisms have been proposed for the reductive half-reaction: (1) the carbanion mechanism, in which the proton is transferred, leaving a negative charge on the alpha carbon atom, and (2) a hydride-transfer mechanism, in which the hydrogen atom and the two electrons are transferred simultaneously. Recent data are consistent with a direct hydride transfer mechanism [37].

According to data obtained from high resolution X-ray structure of *C. rhodostoma* LAAO [31], conformational changes of the key active site components

His 223, Arg 322 and the FAD cofactor can be related to the direct hydride transfer mechanism: Induced by the presence of the zwitterionic form of the substrate, His 223 alters its side chain conformations (Figure 4; from His 223(A) to His 223(B)). The α-amino group of the substrate is then de-protonated by His 223, and the substrate is then activated. The lone pair of electrons from amino nitrogen atom then moves to the α-carbon atom, followed by hydride transfer to FAD to form the imine. The conserved water molecule at Lys 326 may play a role in the reductive half reaction or may assist in H_2O_2 formation from the flavin-hydroperoxy intermediate.

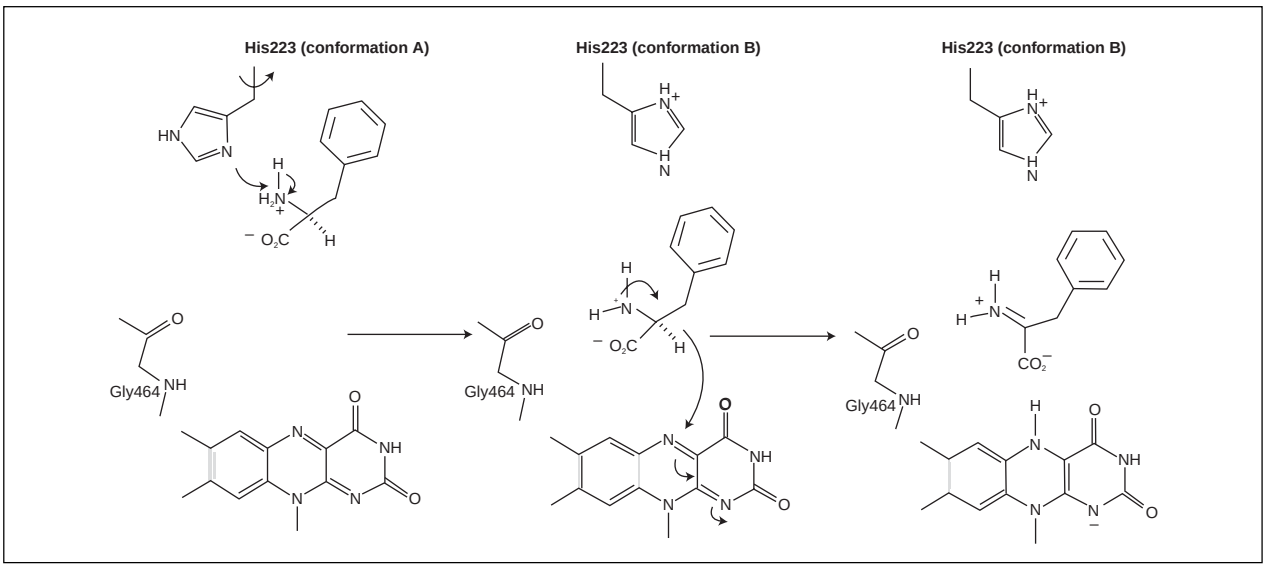


Figure 4: Reaction mechanism for the oxidation of L-phenylalanine by L-amino acid oxidase. The substrate is shown in thick bonds. Two conformations for His 223 are included as labeled (from Moustafa *et al.* [31]; with permission).

Immunological Properties of LAAOs

Snake venom LAAOs are antigenically similar but it has been reported that antibodies to *O. hannah* LAAO yielded indirect ELISA cross-reactions with neurotoxin, hemorrhagin and phospholipase A_2 isolated from the same venom, implying that the LAAO shares common epitopes even with unrelated proteins [38]. This was supported by recent studies by Stabeli *et al.* [39]. They reported that antibodies to homoserine lactone derivatives of the undecapeptide IQRWSLDKYAM (Ile^1-Hse^{11}) excised from *B. moojeni* LAAO cross-reacted with some serine proteases, phospholipase A_2 homologues and LAAOs from venoms of snakes of genera *Bothrops, Lachesis, Crotalus* and *Micrurus.* It was hypothesized that the cross-reactivity of the anti-Ile^1-Hse^{11} antibodies to unrelated venom proteins derives from their mechanism of antigen recognition, whereby complementary is achieved through reciprocal conformational adaptation of the reacting molecules.

Pharmacological Activities of LAAOs

Several LAAOs from snake venoms were reported to exhibit moderate lethal toxicity with i.v. LD_{50} of approximately 5-9 μg/g in mouse. The LD_{50} of LAAO is usually higher than that of the corresponding venom, and hence the enzyme is not a major lethal component of the venom, as the enzyme usually constitutes less than 5% of the venom dry weight [40].

Over the last 15 years, LAAOs have become an interesting object for biomedical studies because of its apoptotic, cytotoxic, platelet aggregation, anticoagulant and other physiological effects. These effects are thought to be mediated by the chemically very reactive hydrogen peroxide generated in the oxidation process, because H_2O_2 scavenger such as catalase neutralizes the effects. Sometimes the toxic effects cannot be attributed to H_2O_2 liberated alone and direct interactions between LAAO and the target cells may play an important role [23].

Edema-inducing and hemorrhagic activities

Several authors reported that venom LAAO was able to induce extensive edema in the mouse paw, and some with slight hemorrhages [9, 28, 29, 40]. Tan and Choy reported that *O. hannah* LAAO exhibited strong edema-inducing activity [41], and the enzyme elicited a 'delayed-type' time course of edema formation, indicating that the edema formation caused by LAAO was not mediated through release of amines subsequent to mast cell degradation, which usually elicited a 'rapid' type of edema formation. The edema-inducing activity of the enzyme was not inhibited by diphenhydramine or dexamethasone. Izidoro *et al.* suggested that edema formation is due to activation of the inflammatory response by the H_2O_2 generated, as administration of glutathione to the mouse paw inhibited the edema-inducing activity of the enzyme [29]. The hemorrhagic effect of LAAO results from complex effects, and may involve apoptosis of endothelial and other vascular cells.

Anticoagulant effects

Sakurai *et al.* reported that LAAO purified from *A. h. blomhoffii* venom possesses anticoagulant activity [42]. The enzyme significantly delayed the onset and progress of blood coagulation, prolonged the activated partial thromboplastin time but had little effect on the prothrombin time. The results indicated that LAAO interferes primarily with the intrinsic blood coagulation pathway, and further studies by the authors indicated that the anticoagulant effect of LAAO is due to its inhibitory action on clotting factor IX.

Effects on platelet aggregation

Reports on the effects of snake venom LAAOs on platelet aggregation seems to be contradictory. Some authors reported that LAAOs induce platelet aggregation, whereas others reported that LAAOs have an inhibitory action on platelet aggregation [9]. Catalase, a H_2O_2 scavenger, inhibited both platelet aggregation inducing and inhibiting effects, indicating that both effects are due primarily to the action of H_2O_2 produced by the enzyme during the oxidation.

LAAOs from venoms of *C. durissus cascavella, E. macmahoni, B. alternatus, B. pirajai, O. hannah* induce platelet aggregation [28-29, 43-45]. Toyama *et al.* reported that the platelet aggregation inducing activity was inhibited by cyclooxygenase pathway inhibitors such as aspirin and indomethacin, suggesting that the H_2O_2 liberated leads to activation of inflammatory enzymes [45]. Du and Clemetson suggested that the H_2O_2 production promoted a rapid increase of thromboxane A2 synthesis and consequently the platelet aggregation [9].

On the other hand, LAAOs from *A. h. blomhoffi, V. lebetina* and *N. naja kaouthia* dose-dependently inhibited both agonist-induced platelet aggregation and shear-induced platelet aggregation [22, 34, 46]. One mechanism for platelet aggregation inhibition may be connected with a reduced binding for ADP in platelet exposed to H_2O_2, or the interference of the peroxide in the interaction between activated platelet integrin GPIIb/IIIa and fibrinogen [18, 33].

It is still not clear why some LAAOs induce and others inhibit platelet aggregation. Sakurai *et al.* suggested that the controversies may be connected with differences in the experimental procedure or preparation of blood samples [46]. Other possibilities include the difference in specific activity of the enzyme, or the involvement of mechanisms other than H_2O_2 liberation that are present only in certain LAAOs. Takatsuka *et al.*, for example, suggested that only LAAOs with very high specific activities would induce platelet aggregation as initiation of aggregation requires relatively high peroxide concentration (mM range) [22]. However, since LAAOs generally liberate H_2O_2 in only micro-molar concentrations, it has been further suggested that presumably these enzymes bind to the platelet and is thus able to generate high concentrations of H_2O_2 locally to induce platelet aggregation.

Apoptosis-inducing effect

Apoptosis is the programmed cell death characterized by a distinct pattern of cellular events, including cleavage of nuclear DNA into fragments that produce a typical nucleosomal DNA ladder in agarose gel. Snake venom is known to exhibit apoptosis-inducing effect. Suhr and Kim [47] and Torii *et al.* [48] reported that the snake venom component that induced apoptosis was an LAAO, and that the LAAO induced apoptosis in human umbilical vein endothelial, human promyelocytic leukemia HL-60, human ovarian carcinoma A2789 and mouse endothelial KN-3 cells. Since then, many snake venom LAAOs were reported to also exhibit apoptosis-inducing activity [22, 29, 32-33, 49-50], and the apoptosis was usually demonstrated by the DNA fragmentation gel pattern. The apoptosis-inducing activity was abolished by catalase and other H_2O_2 scavengers, indicating that the H_2O_2 generated by LAAO action plays an important role in the apoptosis. Tempone *et al.* suggested that cells submitted to oxidative stress induced by LAAO generated H_2O_2 that could activate heat shock proteins and initiate cell membrane disorganization, DNA fragmentation, apoptosis and therefore cell death [51]. Sun *et al.* suggested that the generated peroxide activates the transcription of such factors as the nuclear factor B, the activator protein 1, Fas/Apo-1 and p53 [49].

Suhr and Kim, however, demonstrated that LAAO-induced apoptotic mechanism was clearly distinguishable from the one stimulated directly by exogenous H_2O_2, suggesting that the LAAO-induced apoptosis was not solely triggered by the peroxide produced by the oxidation [27]. Takatsuka *et al.* demonstrated that venom LAAOs directly bind to cell surface thereby increasing the local peroxide concentration [22]. On the other hand, Torii *et al.* reported that the venom LAAO did not associate with human embryonic kidney cells [20]. The cause of these discrepancies is not clear.

Ande *et al.* [50] and Samel *et al.* [33], using Jurkat and K562 (human chronic myeloid leukemia) cells, respectively, reported that at low concentration LAAO induced apoptosis, but caused necrosis of the cells at higher concentrations. According to Ande *et al.* the factors contributing to apoptosis are: (i) generation of toxic intermediates from fetal calf serum and (ii) binding and internalization of LAAO, which appears to be mediated by the glycan moiety of the enzyme, as desialylation of the enzyme reduces cytotoxicity [50]. D-Amino acid oxidase, which lacks glycosylation, also triggers necrosis by the H_2O_2 liberated, but it does not cause apoptosis. Thus, just like its effect on platelet aggregation, induction of cell death by LAAO also appears to involve both the generation of H_2O_2 and the molecular interaction of the

glycan moiety of the enzyme with structures at the cell surface.

Antibacterial activity

Stiles *et al.* reported that two LAAOs from the venom of *Pseudechis australis* (Mulga snake) have a powerful antibacterial effect against Gram-positive and Gram-negative bacteria [52]. Compared to tetracycline, the *in vitro* antibacterial effects of the enzymes were 18-70 times more effective, on a molar basis. Recently, many authors reported LAAO from other snake venoms also exhibited similar antibacterial activity [28-29, 34, 45]. It is believed that the antibacterial effect of LAAO is also due to the H_2O_2 liberated, as addition of catalase completely suppressed the antibacterial activity. Electron microscopic studies suggested that the H_2O_2 generated in the oxidation process induced bacterial membrane rupture and then cell death [45]. Zhang *et al.* reported that the *A. halys* LAAO was able to bind to the surfaces of bacteria and generate high concentrations of H_2O_2 locally, which enables the enzyme to inhibit bacterial growth at low concentrations [53]. It is not clear whether this happens to other snake venom LAAOs.

Leishmanicidal activity

Leishmaniasis includes a spectrum of human infectious disease ranging from self-healing cutaneous ulceration to a progressive and lethal visceral infection. It is a disease that probably affects 12 million people and is prevalent in 88 nations throughout the world. Tempone *et al.* [51] and Toyama *et al.* [45] reported that snake venom LAAO possesses strong leishmanicidal activity, as the H_2O_2 generated by the enzyme was a strong inducer of apoptosis in promastigotes of *Leishmania ssp.* cells. At present, few drugs are available for treatment of leishmaniasis. The understanding of the mode of action of LAAO upon parasites may trigger the design of new drugs or therapeutical approaches for leishmaniasis. For example, if one was able to target a H_2O_2 generator, (such as snake venom LAAO) towards the parasitophorous vacuole, this would represent a highly specific treatment not only for leishmaniasis but also for other intracellular parasites.

Anti-HIV activity

Zhang *et al*. reported that LAAO isolated from *T. stejnegeri* venom possesses antiviral activity [23]. The enzyme exhibited dose-dependent inhibition on HIV-I infection and replication at concentrations that showed little effect on cell viability. Under the same experimental conditions, no anti-HIV-1 activity was observed by exogeneous addition of H_2O_2. Furthermore, the presence of catalase causes a decrease in its antivirus activity but resulted in an increase of its antiviral selectivity. The authors suggested that while liberated H_2O_2 is involved in the anti-HIV-1 activity of the LAAO, the dosages of H_2O_2 and relative molecular pathways mediating suppression in virus infection and replication are independent and/or different from those of causing cell death. Presumably, the mechanism of the anti-HIV-1 effect of LAAO involves specific binding of the enzyme to cell membrane, which helps to generate high local concentrations of H_2O_2 to trigger certain signal reactions and activation of host cells, resulting in the inhibition of HIV infection and/or replication.

Conclusion

Prior to the 1990s, studies of snake venom LAAO dealt mainly with their enzymatic properties and industrial applications. In the past 15 years, there has been considerable progress in the studies of the structure and mechanism of the enzyme but the focus has shifted to the investigations of the pharmacological actions of the enzyme and its potential biotechnological and medical applications. Snake venom LAAOs are interesting multifunctional enzymes exhibiting edema-inducing, platelet aggregation inhibiting or inducing, apoptotic inducing and anti-HIV-1 activities as well as anticoagulation effect. Their toxicological actions are due mainly, but not entirely, to the H_2O_2 liberated during the oxidation. The exact mechanism of the toxicological actions of snake venom LAAO awaits further studies. Sun *et al.* suggested that LAAO may be applied clinically in glioma therapy by cloning the cDNA of the enzyme and transfect to the tumor cells of patients to induce the apoptosis in the target tumor cells [49]. Many authors have demonstrated the apoptotic effect of snake venom LAAO on various malignant cells (eg, S180 tumor, human breast, acute T cell leukemia, Erlich ascetic tumor cell lines). There is therefore great potential in the application of LAAO in cancer therapy. The understanding of the LAAO mode of action upon parasites may also trigger the design of new drugs or therapeutical approaches for leishmaniasis as well as other intracellular parasites. In addition, investigation on the anti-HIV activity of LAAO would also provide valuable information on the therapeutic development of new generations of anti-HIV drugs.

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