

L-Amino Acid Oxidases-Microbial and Snake Venom

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Abstract

L-amino acid oxidases (EC 1.4.3.2, L-aa) are flavoenzymes that catalyse the stereospecific deamination of an L-amino acid to their corresponding α -keto acid with the production of hydrogen peroxide and ammonia. These enzymes are widely distributed across diverse phyla from bacteria, fungi to mammals and many venomous snakes. Although they are mainly involved in cellular amino acid catabolism, many other physiological functions are attributed to L-aa including their antibacterial property and ability to protect from infection. L-aa has also been correlated with penicillin production, violacein synthesis and biofilm development and cell dispersal. Snake venom L-aaos are studied extensively for their ability to induce apoptosis, aggregate platelets, induce haemorrhage, edema and many other toxic effects. L-aaos have been characterized biochemically and found to differ in terms of biochemical parameters not only among different species but also among members of the same species. L-aa act on L-amino acids, preferentially basic, aromatic and aliphatic L-amino acids. The snake venom enzyme shows broad oxidizing activity towards aromatic and hydrophobic L-amino acids such as leucine, phenylalanine and isoleucine.

L-aaos have practical value in biochemical and chemical investigations as they have been used to destroy L-isomer of a racemic DL-amino acid and thus yield an optically pure preparation of the D-isomer. As such, L-aa finds numerous applications as catalysts in biotransformation and for production of keto acids. L-aa has also been used for the determination of L-amino acids as part of biosensors. L-amino acids are reported to be found in physiological fluids of patients with certain diseases and disorders. In addition, the content of certain amino acids essentially controls the nutritional quality of the food. L-aa is useful in this aspect by development of biosensors to detect the L-amino acids.

Snake venom L-aaos are known to induce apoptosis and antibacterial effects mediated by the hydrogen peroxide produced during the L-aa reaction. The hydrogen peroxide induces oxidative stress which in turn activates the heat shock proteins and initiates an array of functions ultimately leading to apoptosis and cell death. In this aspect, L-aa can be greatly useful for the development of efficient therapeutics and drugs to control tumor cells, bacterial, leishmanicidal, viral and protozoal infections. L-aa is also reported to display dose dependent inhibition on HIV-1 infection and replication and as such can be studied for development of anti HIV medicine.

Keywords: L-amino acid oxidases; Function; Characterization; Substrate specificity; Structure; Applications

Introduction

Enzymes that catalyse the oxidation of amino acids have been known for many years. L-amino acid oxidases (EC 1.4.3.2) (L-aaos) are flavoenzymes that catalyse the stereospecific deamination of an L-amino acid substrate to their corresponding α -keto acid with the production of hydrogen peroxide and ammonia via an imino acid intermediate. These enzymes are widely distributed across diverse phyla from bacteria to mammals and many venomous snakes. L-aaos in microorganisms are involved in the utilization of nitrogen sources and those in animals have been characterized as having distinct biological and physiological functions.

Much attention has been given on the snake venom L-aaos which have become an interesting subject for pharmacological as well as structural and molecular biology studies. Snake venom L-aa has been characterized extensively in terms of their molecular mass, substrate preference, apoptosis, cytotoxicity, bactericidal activities etc.

In the present review emphasis will be given on the relatively recent advances in my knowledge of the flavoprotein L-aaos of organisms other than snakes and also to some extent on the snake venom L-aaos.

Physiological role of L-aa

L-aa are reported to be extracellular enzymes in *Aplysia californica* [1], and *Myoxocephalus polyacanthocephalus* [2] while the enzyme was found to be localized in the cell envelope of *Proteus mirabilis*

[3] and cytoplasmic membrane of *Proteus* sps [4]. In *Myoxocephalus polyacanthocephalus*, the skin mucus isozyme MPLAO3 contains a signal peptide, comprising residues Met1-Ala26. *Synechococcus elongatus* L-aa was found in the membrane where it helps in photosynthesis as part of photosystem II particles [5]. Different localization of L-aa was reported in *Meleagris gallopavo* [6] in mitochondria, while *Chlamydomonas reinhardtii* [7] and *Synechococcus elongatus* L-aa were found in the periplasm [8]. Soluble L-aa was also reported from *Corynebacterium* sp [9] and *Neurospora crassa* [10].

Fungal L-aa is involved in the utilization of amino acids as nitrogen sources. *Neurospora crassa* expresses an L-aa whose synthesis is induced in nitrogen starved cultures by amino acid addition [11]. Depending on the amino acid used as nitrogen source, the catabolic pathway using a broad range L-aa can coexist with alternative pathways in *Aspergillus nidulans* [12]. An L-aa having antitumour

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activity has been identified in the fungus *Trichoderma viride*. This enzyme is highly specific and is an L-lysine oxidase which also has promising antibacterial and cytotoxic properties [13].

Lysyl oxidase is a different type of L-aa found in mammalian tissues which catalyse the ϵ -oxidative deamination of lysyl residues in mammalian sclerotic proteins, especially collagen and elastin to yield allysyl residues that rapidly crosslink those proteins during the formation of the extracellular matrix and therefore play an important role in the development, elasticity and extensibility of the connective tissue [14].

Mouse milk is enriched in various nutrients like proteins, carbohydrates, lipids, minerals and vitamins together with many bioactive substances and hence bears a great risk for bacterial infection and proliferation. But milk also contains antibacterial factors that protect the mother and the offspring. This antibacterial capacity of milk can be attributed to some extent on the presence of L-aa [15]. It displays antibacterial effect through the production of hydrogen peroxide from the oxidative deamination of free amino acids [16].

An interesting observation was made by Knight [17], on the correlation between the penicillin producing ability and the L-aa content of the penicillin producing molds. The highest penicillin producers (*P. chrysogenum*, *P. notatum*) are more active and have more L-aa content as compared to the low penicillin producers like *P. expansum*, *P. sanguineum*, *Aspergillus niger* etc.

Hebeloma sp and *Laccaria* bicolour contain L-aa that is involved in cellular amino acid catabolism. They are potential candidates for causing nitrogen mineralization from amino acids at the ecosystem level [18].

Since the first finding of antibacterial activity in an L-aa from snake (*Crotalus adamanteus*) venom [19], antibacterial L-aos have been reported from various snake venoms of *Pseudochis australis* [20], *Trimeresurus jerdonii* [21] and *Bothrops alternatus* [22], the body surface of the giant African snail, *Achatina fulica* Ferussac (termed as achacin) [23], the albumen gland of the sea hare, *Aplysia kurodai* (termed as aplysianin A) [24] and the ink of the sea hare *Aplysia californica* (termed as escapin) [1]. Antibacterial LAO isozymes may be involved in the innate immunity which was demonstrated in the rockfish *Sebastes schlegelii* and the sculpin *Myoxocephalus polyacanthocephalus* skin. The skin secretion of the rockfish *Sebastes schlegelii* produces an L-aa which is a potent antibacterial protein with strict selectivity against Gram negative bacteria like *Aeromonas hydrophila*, *A. salmonicida*, *Photobacterium damsela* ssp piscida and *Vibrio parahaemolyticus*, but not against enteric bacteria such as *Escherichia coli* and *Salmonella typhimurium* suggesting the importance of the antibacterial protein as a primary innate immunity strategy in the rockfish skin. The antibacterial action is elicited by hydrogen peroxide generated from the enzyme reaction [25]. The skin mucus of *Myoxocephalus polyacanthocephalus* contain an L-aa which has antibacterial activity against Gram positive and Gram negative bacteria and is most active against *Aeromonas salmonicida*. This enzyme containing the antibacterial activity help in the innate immunity of the sculpin skin [2].

Yang et al. [1] described a monomeric antibacterial protein from the purple ink of sea hare *Aplysia californica* which was named as escapin because of its potential role in sea hare defence. The escapin is released when the sea hare is attacked by predators and it has cytotoxic effects against a predatory sea anemone. This protein which is an L-aa has a wide spectrum of antimicrobial activities including that against

bacteria, yeasts and fungi but with variable efficacies. The hydrogen peroxide generated from the enzyme reaction plays a prominent role in the bacteriostatic effect and a weak role in bactericidal effect.

The physiological role of L-aa in bacteria is greatly unknown. A tryptophan oxidase from *Chromobacterium violaceum* is involved in violacein synthesis [26]. In *Marinomonas mediterranea* Lod A shows lysine oxidase activity and it is described as a novel antimicrobial protein [27].

Pseudoalteromonas tunicata also expresses a protein similar to Lod A that has important role in biofilm development and cell dispersal [28,29].

The L-aa produced from *Pseudoalteromonas luteoviolacea* is also a broad range L-aa which has antimicrobial activity [30].

Snake venom is useful sources of bioactive substances showing a wide range of pharmacological activities. This complex cocktail of both toxic and nontoxic components includes several peptide and enzymes such as L-aos which may represent 1-9 % of the total venom proteins. Although the exact biological function of snake venom L-aos is still unknown, these enzymes are postulated to be toxins that may be involved in the allergic inflammatory response and specifically associated with mammalian endothelial cell damage [31,32], cytotoxic activities [33], induction of apoptosis, platelet aggregation, hemorrhage, edema and other toxic effects [34-36].

Enzymatic Properties of L-aos

L-aos differ in the parameters of enzymatic properties greatly, not only among different species but also among the same species. A comparison of various L-aos and their properties is given in Table 1.

In terms of substrate preference, L-aa show great variations. The *Pseudoalteromonas* L-aa has broad substrate specificity, oxidizing several amino acids but it shows some preference for L-glutamine [30].

Mouse milk L-aa accepts a broad substrate range i.e., phenylalanine, methionine, tyrosine, leucine, lysine and histidine but they do not oxidize isoleucine [15].

L-aa produced from *Marinomonas mediterranea* has high affinity for L-lys. α -N-acetyl-L-lys is a good substrate indicating that the amino group in position α is not oxidized by this enzyme. On the contrary, ϵ -N-acetyl-L-lys is not a substrate which points out that the modification of the ϵ -group abolished the enzyme activity. Other substrates with lower affinity are L-orn, D-lys and 5-hydroxy-L-lys. This shows the importance of the appropriate distance between the amino and the carboxyl groups in L-lys, the stereospecificity and the negative effects of the introduction of a hydroxyl group adjacent to the ϵ -amino group on the side chain of lysine. Other compounds with structural similarity to L-lysine, such as the tetrapeptide LSKL, amino acids such as arginine and p-amino-L-phenylalanine are not substrates of this enzyme [37].

The mold (*Penicillium chrysogenum*) enzyme deaminates simple short chain amino acids like L-alanine, L-methionine, L-cysteine more rapidly than longer, branched or substituted amino acids [17] while the substrate specificity of *N. crassa* enzyme is broad with the best utilized including L-histidine, L- α amino butyrate, L-canavaine and L-tyrosine [38].

Basic, aromatic and aliphatic L-amino acids are generally good substrates for *Rhodococcus opacus* L-aa. Threonine, proline and

Species	Metal and ions	Inhibitors	pl value	pH optima	Temp. optima	Mol.wt. and subunits	Reference
<i>Anacystis nidulans</i>	NI	2-napthol, Ba ²⁺ , Ca ²⁺ , Cd ²⁺ , Co ²⁺ , chloropromazine, Cu ²⁺ , La ³⁺ , Mg ²⁺ , Mn ²⁺ , Na ⁺ , Ni ²⁺ , o-phenanthroline, NaN ₃ , Sr ²⁺ , Zn ²⁺	NI	NI	NI	98 kDa (dimer of 49 kDa)	[5]
<i>Rhodococcus opacus</i>	NI	Glycine (competitive inhibition)	4.8	8	30°C	99-104 kDa(dimer)	[39]
<i>Proteus sp.</i>	NI	Atebrine, HgCl, KCN, quinine sulphate, NaN ₃	NI	7.0-7.6	NI	NI	[4]
<i>Proteus vulgaris</i>	NI	Caprylic alcohol, Ag, Cu, Hg, 0.01 M HCN (88% inhibition) under aerobic condition	NI	6.8	50°C	NI	[43]
<i>Proteus rettgeri</i>	NI	KCN, α,α'-dipyridyl, salicylaldehyde, 1, 10-phenanthroline, 8- hydroxyquinoline	NI	7.4-7.8	NI	NI	[42]
<i>Meleagris gallopavo</i>	Mn ²⁺ (activator)	Mg, Fe and molybdene	NI	NI	NI	NI	[6]
<i>Chlamydomonas reinhardtii</i>	CaCl ₂ (2 mM, 50% activation)	EDTA (10 mM, 90% inhibition), hydroxylamine (5mM,75% inhibition) KCN (10mM, complete inhibition) NaF (10mM,85%inhibition)	NI	9	NI	900-1300 kDa (oligomer)	[7]
<i>Aplysia californica</i>	NI	NI	NI	7	37°C	60 kDa (monomer)	[1]
<i>Neurospora Crassa</i>	NI	NI	NI	9.5	49°C	300 kDa	[10]
<i>P. luteoviolacea</i>	NI	NI	NI	NI	NI	110 kDa, oligomeric	[30]
<i>Lechevalieria aerocolonigenes</i>	NI	NI	NI	NI	NI	101 kDa (dimer of 55 kDa)	[68]
<i>Marinomonas mediterranea</i>	NI	B-APN, cadaverine, 6-aminocaproic acid (strong inhibitors) 5-aminovaleric acid, amiloride aminoguanidine, (weak inhibitors)	NI	NI	NI	140 kDa	[27]
<i>Penicillium chrysogenum</i>	NI	Capryl alcohol, CuSO ₄ , (NH ₄) ₂ SO ₄ , 2-4-dinitrophenol, benzoic acid, iodoacetic acid	NI	8-8.5	50-55°C	NI	[17]
<i>Streptomyces endus</i>	NI	Ag ⁺ and Hg ²⁺ ions	6.2-6.3	6.5-8.0	30-45°C	90 kDa, dimeric	[41]
<i>Bombyx mori</i>	NI	CuSO ₄ , HgCl ₂ (10 ⁻³ M), KCN, EDTA (10 ⁻² M), riboflavin, isoriboflavin.	NI	7.2	55°C	NI	[81]
<i>Calloselasma rhodostoma</i>	NI	Anthranilate (competitive)	NI	9	NI	132 kDa (dimer of 66 kDa)	[58,32]
<i>Crotalus durissus cascavella</i>	NI	Aspirin, indomethacin	5.4	6.5	NI	68 kDa	[82]
<i>Trimeresurus mucrosquamatus</i>	NI	Benzoic acid, CdCl ₂ , HgCl, Iodoacetamide, KCN, MnCl ₂ , p-aminobenzoic acid, ZnCl ₂ p-chloromercuribenzoate	5.6	9 (L-trp), 8(L-his), 7(L-leu, phe, tyr)	NI	140 kDa (dimer of 70 kDa)	[59]
<i>Crotalus adamanteus</i>	Cl ⁻ (activator)	Aromatic carboxylates, benzoate, iodoacetic acid, orthanilic acid, vinylglycine, mandelate	NI	7.5	NI	Dimeric with 58.7 kDa subunit	[83,84]
<i>Agkistrodon piscivorus piscivorus</i>	NI	Benzoic acid, Iodoacetic acid, NH ₄ ⁺	NI	7.2-7.5	NI	150 kDa	[56]
<i>Rattus norvegicus</i>	NI	Benzeneearsonic acid, Benzoic acid, CuSO ₄ , iodoacetic acid, p-chloromercuribenzoate, NH ₄ ⁺	NI	8.8-9.2	NI	138 kDa	[56]
<i>Bothrops pirajai</i>	NI	NI	4.9	6.0-7.4	37°C	130 kDa (dimer of 66 kDa)	[50]

NI = No information

Table 1: Comparison between different L-aa in terms of some enzymatic properties.

glycine are the only proteogenic amino acids that are not accepted by this broad range enzyme. Among the aliphatic amino acids, alanine showed the highest activity followed by leucine, valine and isoleucine. Phenylalanine, tyrosine and tryptophan showed similar activity among the aromatic amino acids. Among the basic amino acids, asparagine and arginine are good substrates. The S-containing amino acids like serine, methionine and cysteine are also good substrates for this enzyme [39].

The *Saccharomyces cerevisiae* has a specific lysine oxidase activity; however it also accepts L-arginine, L-asparagine and other L-amino acids like alanine, leucine, glutamic acid and tryptophan as substrates [40].

Streptomyces endus is a specific L-glutamate oxidase which oxidizes only L-glutamate. L-aspartate, even in high concentrations is not

converted to any extent. However the L-glutamic acid 4-benzyl ester is oxidized by the enzyme but the diester is not. The 4-substituted L-glutamic acid derivatives, L-glutamine and glutathione as well as peptide derivatives are also not substrates for this enzyme [41].

Proteus rettgeri contains two separable L-aaos that differ in their substrate specificities. One of them catalyses the oxidative deamination of aromatic, monoaminomonocarboxylic, sulphur containing, imino and β-hydroxy L-amino acids with no affinity for the basic amino acid, L-citrulline. The other oxidase catalyses the oxidative deamination of L-arginine, histidine, ornithine, citrulline and lysine only [42] while the *Proteus vulgaris* enzyme oxidizes the unsubstituted monocarboxylicmonoamino, primary amino acids like nor-leucine, phenylalanine, leucine, tryptophan, methionine with the exceptions of alanine and valine [43].

A. fumigatus L-aao also shows certain degree of substrate preference. The enzyme has a greater specificity towards hydrophobic aromatic amino acids namely tyrosine and phenylalanine. D-amino acids are not attacked. The enzyme does not act on basic amino acids [44] and the best substrates for this enzyme are the L-isomers of phenylalanine, tyrosine, leucine and isoleucine.

L-aaos from various organisms exhibit different substrate specificity. For e.g. leukocyte L-aao (IL411) prefers aromatic L-amino acids such as phenylalanine [45] while the enzyme from ink of sea hare is most active to positively charged L-amino acids like arginine and lysine [1,46].

The snake venom enzyme shows broad oxidizing activity towards aromatic and hydrophobic L-amino acids such as leucine, phenylalanine and isoleucine [47,48]. In general, the best substrates for this enzyme are the L-isomers of phenylalanine, tyrosine, leucine, isoleucine, methionine and tryptophan [49-51].

Bungarus fasciatus L-aao (BF L-aao) substrate specificity is similar to that of L-aao from *Calloselasma rhodostoma*, *Naja naja kaouthia*, *Agkistrodon blomhoffii ussurensis*, *Bothrops jararaca*, *Daboia russellii siamensis*, *Vipera lebetina* in that these enzymes have affinity towards hydrophobic amino acids including phenylalanine, tryptophan, tyrosine and leucine. However, BF L-aao is also active towards acetic L-amino acids aspartic acid and glutamic acid [33,52,53]. The *Agkistrodon contortrix laticinctus* enzyme shows greater activity against hydrophobic L-amino acids but a significant activity was also present for the basic L-amino acids arginine and histidine but not detected for lysine [49].

FAD acts as a cofactor for these enzymes. L-aao family members possess in common, flavin as a coenzyme and two motifs, a dinucleotide binding motif comprising of β -strand/ α -helix/ β -strand of the secondary structure and a GG motif (R-X-G-G-x-x-T/S) shortly after the dinucleotide binding motif [54]. In *Anacystis nidulans* [5], 1 mol of FAD is bound per mol of enzyme while in *Bacillus carotarum* [55], *Rhodococcus opacus* [39] and most other organisms, the enzyme is a homodimer complex containing 2 FAD molecules.

Snake venom L-aao of *Agkistrodon piscivorous piscivorous*, *Crotalus adamanteus* [56], *Ophiophagus hannah* [57] binds to 2 FAD molecules per enzyme, while *Calloselasma rhodostoma* [58], *Trimeresurus jerdoni* [21], and *T. mucrosquamatus* [59] contain 2 mol of FMN per mol of enzyme.

An exception to this observation is that, the enzyme from *Marinomonas mediterranea* is an unusual amine oxidase. This melanogenic marine bacterium synthesizes marinocine, which is a type of lysine oxidase that has antibacterial activity. It does not depend on flavin as a cofactor, and is copper enzyme, requiring tyrosine derived quinone as a cofactor [37].

Although L-aao are flavoprotein enzymes, glycoprotein L-aao are reported in many organisms like *Aplysia californica* where the L-aao sequence contains one potential glycosylation site but glycosylation is not essential for its antimicrobial activity [1], *Chlamydomonas reinhardtii* [7] and *Sebastes schlegelii* where the protein contains N-linked glycochains [25]. Most snake venom L-aao are reported to be glycoproteins [22,60].

Structural properties of L-aao

Primary structures of snake venom L-aao were determined for

Crotalus adamanteus [61], *A. contortrix laticinctus* [49], *Crotalus atrox* [62], *Agkistrodon halys blomhoffii* [63], *Trimeresurus stejnegeri* [64] and *Bothrops* sp [65]. Phylogeny analysis shows that *Bothrops* and *Crotalus adamanteus* L-aao form a cluster and are more closely related to each other.

The complete nucleotide sequence of *Rhodococcus opacus* L-aao gene was determined and its primary structure was deduced [39]. The nucleotide sequence revealed that the L-aao is synthesized as a precursor carrying a signal peptide of 45 amino acids, which is processed after translation. The proteolytic cleavage site of the precursor protein does not agree with the predicted cleavage site. The nucleotide sequence of *Neurospora crassa* L-aao gene was obtained from a partial c-DNA and a complete genomic DNA clone [66]. The gene encodes a protein consisting of 695 amino acids and unlike most of the cloned genes of *N. crassa*, the L-aao gene is devoid of introns. The enzyme is synthesised as a precursor exceeding the mature form (566 amino acids) by 129 amino acids.

Various workers report the cloning of the L-aao gene into vectors for their expression in large quantities. Geueke and Hummel [39] report the expression of *Rhodococcus opacus* L-aao in *E. coli* but, this yields only inclusion bodies, while the expression in a *Streptomyces lividans* strain yields both soluble and active enzyme, but at low yields. The *Calloselasma rhodostoma* L-aao (CRL-aao) was cloned in a yeast expression system (*Pichia pastoris*) after the α -MF-signal sequence that promotes secretion. The expression was repressed when glycerol was used as the sole carbon source while switching the carbon source to methanol leads to the secretion of recombinant CRL-aao with good yields [67]. The L-aao of *Aplysia californica* (escapin) was cloned, sequenced and functionally expressed in *E. coli* [1]. The bioactive recombinant escapin level was relatively low because much of the escapin is present in the form of insoluble inclusion bodies. Also the escapin inhibits growth of *E. coli* which likely inhibits the level of bacterial expression. *Lechevalieria aerocolonigenes* produces an L-aao (rebecamycin) (RebO) which was overexpressed in *E. coli* using an expression vector (pDHS5514). A KLAALAEHHHHHH amino acid sequence was engineered onto the C-terminus of RebO to facilitate purification by a Ni-nitrilotriacetic acid strategy. The co-expressed plasmid was used to facilitate protein folding and to prevent aggregation and degradation of RebO in *E. coli*. Thus, the recombinant RebO protein was found to be >95% pure [68]. Nagashima et al. [2] report the c-DNA cloning of *Myoxocephalus polycanthocephalus* L-aao (MPL-aao) which show that the full length of c-DNA was 2659 bp and it encodes the signal peptide (Met1-Ala26) and the mature protein (Val28-Phe520). MPL-aao shares 74% sequence identity with the antibacterial L-aao from skin mucus of the rockfish *Sebastes schlegelii*.

Applications of L-aao

L-amino acid oxidases have practical value in biochemical and chemical investigations. The usefulness of these enzymes arises from the fact that they exhibit absolute antipodal specificity. It is therefore possible to detect as little as one part of a susceptible amino acid isomer in the presence of ten thousand times the concentration of its enantiomorph. The kinetic resolution of race mates is a highly successful strategy for the synthesis of enantiomerically pure chiral compounds and has found widespread usage in industry. The amino acid oxidases have been used to destroy one isomer of a racemic amino acid and thus yield an optically pure preparation of the other isomer [69]. We demonstrated the racemic resolution of DL-

amino acids to yield optically pure D-amino acids. DL-tyrosine, DL-phenylalanine and DL-alanine were successfully resolved with the help of *Aspergillus fumigatus* L-aao to yield D-tyrosine, D-phenylalanine and D-alanine respectively [70]. Further applications of this enzyme were demonstrated as catalysts in biotransformation [71] and could also be used for production of keto acids, which can function as siderophores [72].

The determination of amino acids is important for several purposes. Determination of certain amino acids in tissue or physiological fluids may be a useful indicator of certain diseases and disorders. Also the content of certain amino acids in food essentially controls the nutritional quality of the food. L-aao can be useful in this aspect by development of amperometric biosensors to detect the amino acids. Amperometric biosensors based on screen printed electrodes have been developed by Sarkar et al. [73]. An amperometric microbial biosensor based on *Saccharomyces cerevisiae* cells was developed for selective and rapid determination of L-lysine [40]. In addition, L-aao was physically immobilized on diamond paste to construct an amperometric biosensor that detects L-leucine by measuring the hydrogen peroxide formed when L-aao catalyses the conversion of L-leucine to their keto acids and H₂O₂ [74]. L-aao was immobilized on a preactivated nylon membrane by using glutaraldehyde to develop an enzyme sensor for L-amino acids that can detect ammonia [75].

Snake venom L-aaos are known to induce apoptosis and antibacterial effects mediated by the hydrogen peroxide produced during the L-aao reaction. The hydrogen peroxide is a strong inductor of apoptosis in promastigote forms of *Leishmania* spp. The hydrogen peroxide induces oxidative stress which in turn activates the heat shock proteins and initiates cell membrane/ cytoplasmic disorganization, DNA fragmentation, apoptosis and therefore cell death [76]. In this aspect, L-aao can be greatly useful for the development of efficient therapeutics and drugs to control tumor cells, bacterial and leishmanicidal infections. Also the *Trimeresurus stejnegeri* L-aao displays dose dependent inhibition on HIV-1 infection and replication [64].

Antiviral (against Dengue virus) and antiprotozoal (trypanocidal and leishmanicidal) activities have been reported from *Bothrops jararaca* L-aao (LAAO-I) [77]. The *B. jararaca* L-aao was found to significantly inhibit Ehrlich ascites tumour growth and induce an influx of polymorphonuclear cells, as well as spontaneous liberation of H₂O₂ from peritoneal macrophages. Later, LAAO-I induce mononuclear influx and peritoneal macrophage spreading. Animals treated with LAAO-I show higher survival time [78] and thus, the application of these enzymes in tumour inhibition was implicated.

In *Deinagkistrodon acutus*, L-aao (ACTX-6) demonstrates cytotoxicity in vitro and inhibits tumour growth in vivo and can markedly increase accumulation of sub-G1 phase, which suggests that this enzyme can induce apoptosis. ACTX-6 is a potential substance to develop into an antitumor drug [79] since it induces apoptosis in HeLa cervical cancer cells in a concentration- and time-dependent manner. Caspase activation and PARP cleavage are involved in ACTX-8-induced apoptosis. ACTX-8 activates a mitochondrial pathway of apoptosis, which is regulated by Bcl-2 family members. Reactive oxygen species generated by ACTX-8 are involved in apoptosis [80].

In *Viridovipera stejnegeri* the L-aao enzyme displays dose dependent inhibition on HIV-1 infection and replication [64] and as such can be studied for development of anti HIV medicine. Thus

L-aaos are useful enzymes due to their biotechnological potential as model of therapeutic drugs and medicine.

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