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Review Article

INHIBITORS OF PLANT COPPER AMINE OXIDASES *

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In this review, inhibitors of plant copper amine oxidases from *Lens esculenta* seedlings, *Pisum sativum* seedlings, and *Euphorbia characias* latex are described. Reversible competitive inhibitors and non-competitive inhibitors, irreversible active-site directed inhibitors and mechanism-based inactivators are reviewed in regard to their mechanisms of action.

Keywords: Amine oxidase; 6-Hydroxydopa; Copper; Inhibitors

Abbreviations: TPQ, 6-hydroxydopa quinone; LSAO, lentil seedling amine oxidase; PSAO, pea seedling amine oxidase; ELAO, *Euphorbia characias* latex amine oxidase; DDC, diethyldithiocarbamate; MGBG, methylglyoxal-bis-guanylhydrazone; MBTH, 3-methyl-2-benzothiazolinone hydrazone; DABI, 1,4-diamino-2-butyne

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INTRODUCTION

Amine oxidases (amine oxygen oxidoreductase deaminating, copper containing; EC 1.4.3.6) are soluble dimeric enzymes containing two copper atoms and two organic prosthetic groups identified as TPQ, the quinone of 2,4,5-trihydroxyphenylalanine,[1,2] formed from tyrosine in a post-translational event.[3–5] Amine oxidases catalyze the oxidative deamination of biogenic primary amines, including mono-, di- and polyamines, to aldehydes, ammonia and hydrogen peroxide. The catalytic cycle of these enzymes has now been outlined.[6,7] Briefly, an aminotransferase mechanism is operative (Figure 1) in which the oxidized enzyme (**a**) reacts with an amine substrate giving a Schiff base formation at C(5) of the TPQ (the Cu(II)-quinone ketimine, **b**) that owing to proton abstraction (**c**), gives rise to a Cu(II)-quinolaldimine (**d**). After hydrolysis and release of the aldehyde, an aminoresorcinol species is formed (**e**), and the reduced cofactor is reoxidized by molecular oxygen via the Cu(I)-semiquinone intermediate (**f**).

Under standard conditions the velocity (v) of the amino oxidase catalyzed reaction can be defined as: $v = -d[R-CH_2-NH_2]/dt = -d[O_2]/dt = d[R-CHO]/dt = d[NH_3]/dt = d[H_2O_2]/dt$, where v can be altered by the presence of modifier substances.

A useful tool for enzymologists has been to find and use specific inhibitors so as to follow the effects of these inhibitors on the enzyme action and probe structure–function relationships. Inhibitors can be divided into two groups: reversible and irreversible. Reversible inhibition is characterized by an equilibrium formed between the enzyme and inhibitor that depends on the concentration of the inhibitor, value of the inhibitor constant (K_i), and is time independent. The reversible inhibitor binds the enzyme non-covalently to modulate the enzymatic activity and enzyme activity can be restored when the inhibitor is removed by a suitable procedure such as gel filtration or dialysis. On the contrary, irreversible inhibition is characterized by a progressive decrease of enzymatic activity with time and becomes complete when all the enzyme is combined with the inhibitor. After irreversible inhibition the enzyme activity cannot be restored by gel filtration or dialysis.

Two main types of reversible inhibitors acting on plant amine oxidase may be recognized, competitive and non-competitive, whereas only sodium azide is known as an uncompetitive inhibitor of PSAO[8,9] that blocks the hydrolysis of the enzyme–substrate complex (Dooley, D., personal communication). Mixed-type inhibitors have not been encountered.

Competitive inhibition Substances that bind to the enzyme at the same site as the substrate produce the competitive type of inhibition. These

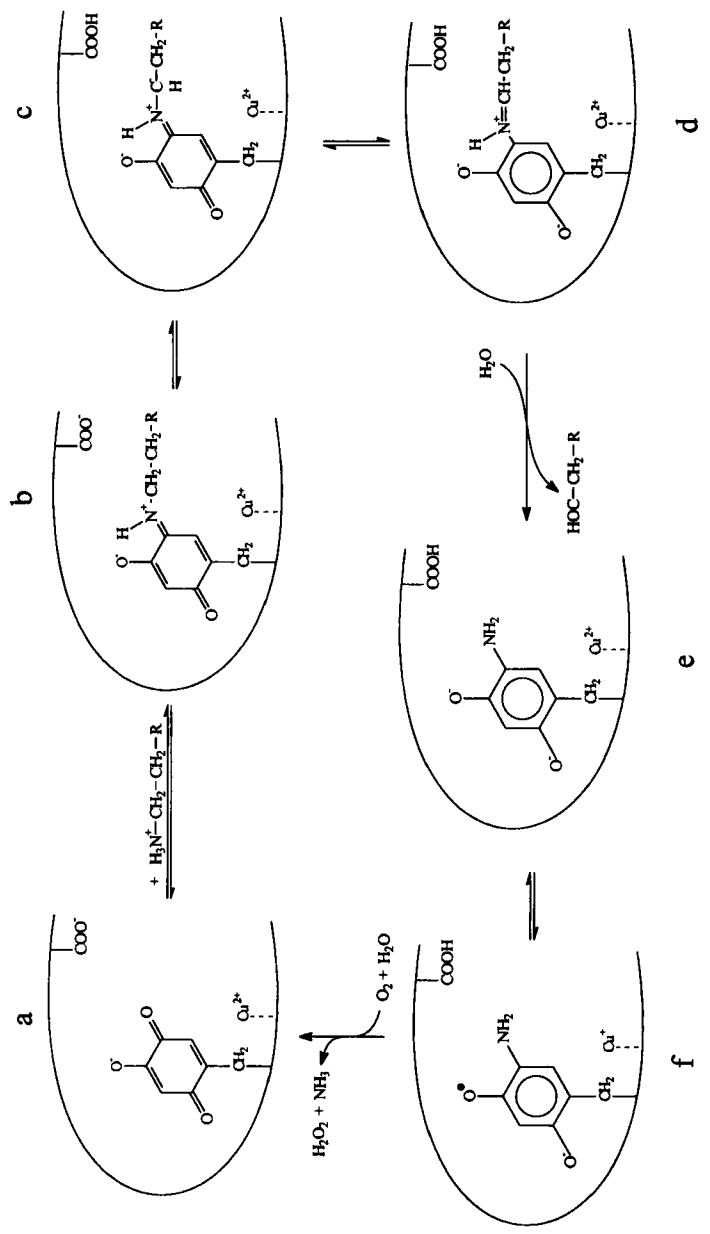

FIGURE 1 Mechanism of the reaction catalyzed by plant copper amine oxidases.

inhibitors are often structural analogs of the substrate that block the active site of the enzyme. The system may be represented by the following scheme:

$$\begin{array}{l} \mathrm{E} + \mathrm{S} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \mathrm{ES} \overset{k_{cat}}{\rightleftharpoons} \mathrm{E} + \mathrm{P} \\ + \\ \mathrm{I} \\ \Big\| K_i \\ \mathrm{EI} \end{array} \tag{1}$$

$$K_i = \frac{[\mathrm{E}] \cdot [\mathrm{I}]}{[\mathrm{EI}]}, \tag{2}$$

forming either an enzyme–substrate complex ES or an enzyme–inhibitor complex EI.

Non-competitive inhibition Substances that bind to the enzyme at a site different from that for the substrate, and have no influence on the binding of the substrate, produce the non-competitive type of inhibition. The system may be represented by the following scheme:

$$\begin{array}{ccccc} & & \mathrm{E} & & \\ & K_i \swarrow & & \searrow K_s & \\ \mathrm{EI} & & & & \mathrm{ES} \xrightarrow{k_p} \mathrm{E} + \mathrm{P} \\ & K_s \searrow & & \swarrow K_i & \\ & & \mathrm{EIS} & & \end{array} \tag{3}$$

$$K_i = \frac{[\mathrm{E}] \cdot [\mathrm{I}]}{[\mathrm{EI}]} = \frac{[\mathrm{ES}] \cdot [\mathrm{I}]}{[\mathrm{ESI}]}. \tag{4}$$

Hence, the non-competitive inhibitor can bind either to the enzyme giving an inactive complex enzyme–inhibitor EI, or to the ES complex giving an inactive ternary complex ESI.

Active-site directed inhibitors The irreversible inhibition of enzymes is characterized by the formation of stable covalent bond(s) between the inhibitor and essential functional groups of the enzyme. The irreversible inhibition is progressive with time reaching complete inhibition of the enzyme. When the irreversible inhibitors are substrate analogs, they are called affinity labels or active-site directed inhibitors.

Mechanism-based inactivators A mechanism-based enzyme inactivator is a substrate analog that binds to the active site of the enzyme being accepted

and processed by the normal catalytic mechanism of the enzyme. During the reaction, however, a reactive functional group of the intermediate formed is positioned proximal to an active-site residue. This results in formation of an equilibrium between normal product formation and covalent modification of the proximal active-site residue, leading to irreversible inactivation of the enzyme. The system may be represented by the following scheme:

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \overset{k_2}{\rightleftharpoons} EI \overset{k_4}{\dashrightarrow} E\text{-}I \tag{5}$$

$$\begin{array}{ccccccc} & & & & E + I & & \\ & & & & \Big\uparrow k_5 & & \\ E + S & \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} & ES & \overset{k_2}{\rightleftharpoons} & EI & \overset{k_4}{\dashrightarrow} & E\text{-}I \\ & & & & \Big\downarrow k_3 & & \\ & & & & EP & \overset{k_6}{\rightleftharpoons} & E + P \end{array} \tag{6}$$

In the first approximation, the kinetics of inactivation can be accounted for by the minimum scheme represented in Equation (5). In most cases, however, the inhibitor can be turned over many times before the inactivation occurs. The kinetic scheme of inactivation is then well described by Equation (6).

Mechanism-based enzyme inactivation must follow these criteria:[10] time-dependent loss of enzyme activity with pseudo-first order and saturation kinetics, kinetic protection by normal substrate, a rate of inactivation proportional to the concentration of inactivator and irreversibility of the inactivation of the target enzyme.

This review deals with the inhibitors of the best-known and studied amine oxidases from *Lens* and *Pisum* seedlings and from *Euphorbia characias* latex.

COMPETITIVE INHIBITORS

Diamino- and monoamino-ketonic compounds were synthesized and evaluated as inhibitors of PSAO.[11] 1,5-Diamino-3-pentanone is the most potent competitive inhibitor of the enzyme (Figure 2(a)).[12] An approximately one magnitude increase in the inhibition constant is observed when the carbon chain of the inhibitor is shortened by one methylene group as in 1,4-diamino-2-butanone. Significant decrease in inhibition occurs when one of the

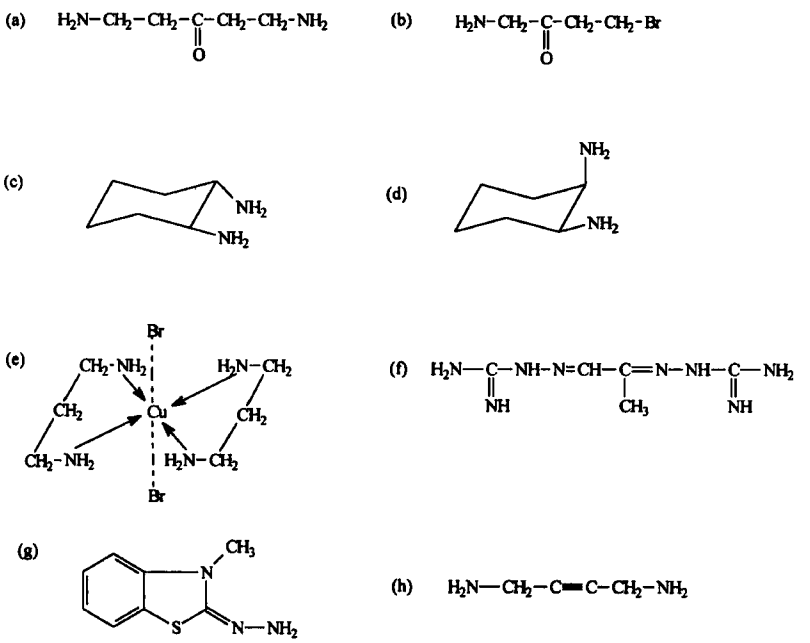

FIGURE 2 Structural formulas of inhibitors of pea and lentil seedling amine oxidase (a) 1,5-diamino-3-pentanone (b) 1-bromo-4-amino-3-butanone, (c) *trans*-1,2-diaminocyclohexane, (d) *cis*-1,2-diaminocyclohexane, (e) Cu(1,3-diaminopropane)$_2$Br$_2$ complex, (f) methylglyoxal-bis-guanylhydrazone, (g) 3-methyl-2-benzothiazolinone hydrazone, and (h) 1,4-diamino-2-butyne.

amino groups is eliminated as in 1-amino-2-butanone or replaced with the bromine atom as in 1-bromo-4-amino-3-butanone (Figure 2(b)), whereas the short-chained 1-amino-2-propanone is almost ineffective. All these compounds show strong affinity to bind to the TPQ cofactor as normal substrates, but their complexes are hydrolyzed only very slowly at a rate about 1% of that of putrescine.[12]

PSAO is competitively inhibited by some alkaloids with a piperidine skeleton, piperidine derivatives[13] and by cinchona alkaloids.[14]

The shortest diamine 1,2-diaminoethane[15] is a weak substrate of PSAO (its oxidation velocity is only 4.5% of that of putrescine) that shows a weak competitive inhibition of putrescine oxidation ($K_i = 5.2$ mM). The vicinal diamine *trans*-1,2-diaminocyclohexane ($K_i = 3.6$ mM) is also a weak competitive inhibitor of PSAO[15] (Figure 2(c)).

Bivalent transition-metal complexes with diamines have been prepared and characterized by elemental analyses, conductance measurements, IR spectrometry, and diffuse reflectance electronic spectra.[16] The complexes have the general formula $M(L)_nX_2$, where $M = Cu^{2+}$, Ni^{2+}, Co^{2+}, Zn^{2+},

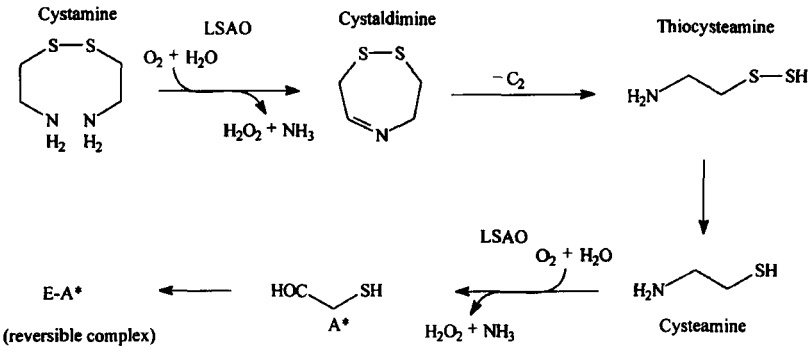

FIGURE 3 Mechanism of the reaction of lentil amine oxidase with cystamine and cysteamine.

Cd^{2+}, Hg^{2+}, Pd^{2+}, or Pl^{2+}, L = en (1,2-diaminoethane) or tn (1,3-diaminopropane), $n = 1$ or 2, and X = Cl^-, Br^-, I^-, or NO_3^-. $Cu_3(put)_2Cl_6$ and $Cu_3(cad)_2Cl_6$ (put = putrescine, cad = cadaverine) have also been prepared and characterized. All complexes show a competitive type of inhibition toward LSAO, the strongest inhibitory effect being shown by $Cu(tn)_2Br_2$ ($K_i = 9\,\mu M$), $Zn(en)_2Cl_2$ ($K_i = 20\,\mu M$) and $Cu_3(cad)_2Cl_6$ ($K_i = 10\,\mu M$). No inhibitory effect was found when free ligands or metal salts were used at the same concentration as in the complexes. The structure of the complex $Cu(tn)_2Br_2$ is shown in Figure 2(e).

Cysteamine was found to be both substrate and competitive inhibitor of LSAO.[17] It shows saturation kinetics with $K_m = 0.2\,mM$ like other substrates, but its interaction leads to loss of enzymatic activity which can be restored by dialysis. The Lineweaver–Burk plot shows that the inhibition is competitive. The apparent inhibition constant determined from the Dixon's plot is 0.05 mM. The mechanism of interaction involves oxidation of cysteamine to form thioacetaldehyde as a primary product, which then reacts with the TPQ cofactor causing inactivation of the enzyme. An interesting reaction mechanism (Figure 3) for the oxidation of cysteamine catalyzed by plant amine oxidases[17,18] that differs from the mechanism for mammalian amine oxidases[19] has been postulated.

NON-COMPETITIVE INHIBITORS

Non-competitive inhibition of plant amine oxidases has been observed with chelating agents such as cyanide and azide that strongly perturb the EPR spectrum of the copper and are likely to bind with different affinity

to the enzyme.[20] They act as chelating agents for copper and seem to displace water from its equatorial coordination sites. Diethyldithiocarbamate (DDC) gives precipitation of the copper chelate complex causing a loss of enzymatic activity, and therefore has been often used to obtain copper-free enzymes.[21] When DDC reacts with a Cu-amine oxidase, it forms a DDC–enzyme complex that dissociates to give copper-free protein and a copper–DDC complex. This type of inhibition can be reversed only by adding Cu^{2+} ions.[22] DDC ($K_i = 45\,\mu M$) was used to obtain copper-free LSAO[23] in a number of studies on the active site.[6,24,25] LSAO is inhibited non-competitively by sodium cyanide ($K_i = 3\,mM$) and sodium azide ($K_i = 50\,mM$).[26] Surprisingly PSAO is inhibited uncompetitively by sodium azide[8,9] which is the only uncompetitive inhibitor known for plant Cu-amine oxidases.

Imidazole derivatives, such as carnosine, anserine, and impromidine behave as non-competitive inhibitors of PSAO.[27] On the other hand, some physiologically important derivatives of 4,5-dihydroimidazole such as 2-benzyl-, 2-N(*p*-tolyl)-N-(*m*-hydroxyphenyl)aminomethyl-, 2-(1-naphthyl)-methyl-, (2,2,6-dimethyl-4-*tert*-butylphenyl)methyl-, and 2-(N-phenyl-N-benzylamino)methyl-4,5-dihydroimidazole are competitive inhibitors of PSAO with inhibitor constants in the range of 1.6–8.2 mM.[28]

The chelating agents 1,10-phenanthroline ($K_i = 31\,\mu M$) and 2,2-bipyridyl ($K_i = 58\,\mu M$) are non-competitive inhibitors of PSAO. Relatively slow formation of complexes between enzyme-bound copper and the chelating agents requires a longer preincubation time (~ 20 min) to achieve full inhibition.[16] Diethylenetriamine ($K_i = 72\,\mu M$) and triethylenetetramine ($K_i = 0.57\,\mu M$) are strong non-competitive inhibitors of PSAO.[16] In contrast to *trans*-1,2-diaminocyclohexane, a previously described competitive inhibitor, the vicinal diamine *cis*-1,2-diaminocyclohexane ($K_i = 2.9\,mM$) is a weak non-competitive inhibitor of PSAO[15] (Figure 2(d)).

COMPETITIVE–NON-COMPETITIVE INHIBITORS (PREINCUBATION TIME-DEPENDENT)

Methylglyoxal-bis-guanylhydrazone (MGBG; Figure 2(f)) is an interesting inhibitor of PSAO since it shows inhibition which depends on the preincubation time. The inhibition without any preincubation is competitive with $K_i = 0.26\,\mu M$. When the enzyme is preincubated for 1 hour with the inhibitor, the kinetics seem to reflect non-competitive inhibition with $K_i = 0.017\,\mu M$.[29] An affinity chromatography technique which uses

MGBG–Sepharose to purify PSAO to homogeneity with a 35% recovery has been developed.[29]

IRREVERSIBLE INHIBITORS

Irreversible inhibitors of amine oxidases are mostly carbonyl group reagents that interact with the TPQ cofactor. Carbonyl group reagents form irreversible adducts with all plant Cu-amine oxidases with concomitant loss of the catalytic activity. Typical reaction with phenylhydrazine is characterized by high affinity formation of an adduct that shows strong absorption with a maximum at 415–445 nm (Figure 4(a)). The most effective irreversible inhibitors of LSAO[30] are the substituted hydrazines, phenylhydrazine (adduct

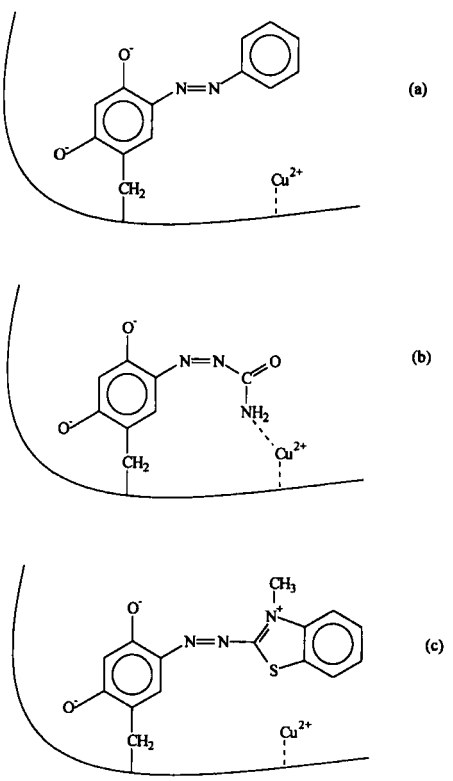

FIGURE 4 Structure of the TPQ-adduct after the reaction of lentil amine oxidase with irreversible inhibitors: (a) phenylhydrazine, (b) semicarbazide, and (c) 3-methyl-2-benzothiazolinone hydrazone.

with $\varepsilon_{445} = 64\,000\ M^{-1}\,cm^{-1}$) and benzylhydrazine ($\varepsilon_{380} = 58\,000\ M^{-1}\,cm^{-1}$), and the hydrazides, semicarbazide ($\varepsilon_{492} = 8600\ M^{-1}\,cm^{-1}$ and $\varepsilon_{360} = 35\,000\ M^{-1}\,cm^{-1}$), thiosemicarbazide ($\varepsilon_{460} = 42\,000\ M^{-1}\,cm^{-1}$ and $\varepsilon_{390} = 31\,000\ M^{-1}\,cm^{-1}$), and phenylsemicarbazide ($\varepsilon_{400} = 38\,000\ M^{-1}\,cm^{-1}$). Carbonyl reagents were used to solve the problem of conflicting data for the number of functional active sites in the dimeric enzyme from lentil seedlings.[30] The binding ratio of all carbonyl reagents extrapolates to 1 mol of the inhibitor per mol of enzyme subunit indicating the presence of one TPQ cofactor at each enzyme subunit (Figure 5). Using ESR spectra of native and copper-free LSAO, the interaction between the terminal amino group of the TPQ-semicarbazone and the copper may be evidenced.[6] This interaction provides direct evidence for the location of the copper atom at a distance of approximately 3.0 Å from TPQ as predicted earlier[31](Figure 4(b)). In the crystal of PSAO, however, the shortest distance from TPQ to copper has been found to be ~ 6 Å.[32]

The pea seedling enzyme is inhibited in a time-dependent manner by the hydrazides of acetic, benzoic, nicotinic, picolinic, and other acids.[33]

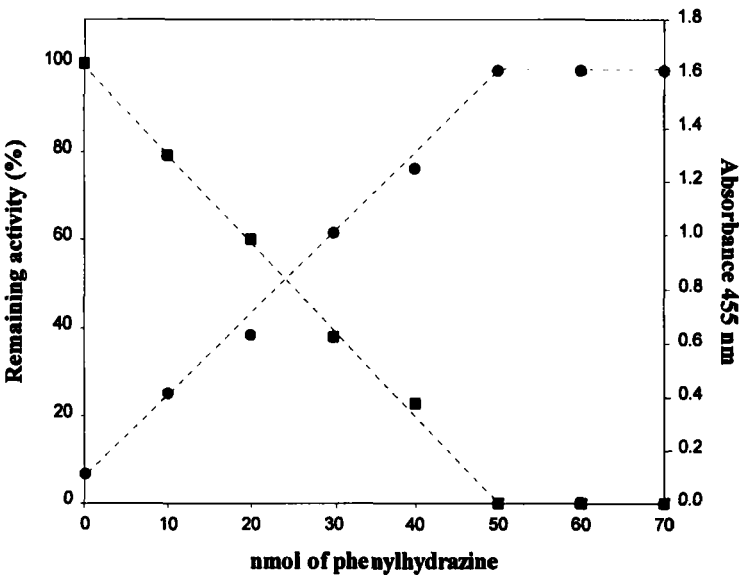

FIGURE 5 Correlation between spectral changes and inhibition of lentil amine oxidase by phenylhydrazine. Aliquots of 10 nmol phenylhydrazine were added to 47 nmol of LSAO active sites in 100 mM potassium phosphate buffer, pH 7. Absorbance readings were taken at 455 nm (●) and simultaneously 10 µl portions of the reaction mixture were taken out for activity assay (■).

COMPETITIVE-IRREVERSIBLE INHIBITORS (INCUBATION TIME-DEPENDENT)

3-Methyl-2-benzothiazolinone hydrazone (MBTH; Figure 2(g)) is a colorimetric standard hydrazone reagent for the detection of minute quantities of aldehydes in solution.[34] MBTH was found to be a competitive inhibitor of LSAO[6] with $K_i = 0.2\,\mu M$ obtained from a Dixon's plot. Surprisingly upon prolonged incubation with MBTH the enzyme gradually loses its activity in an irreversible manner, different from the competitive inhibition seen for short time incubation. When MBTH is added to LSAO at pH 7 the intensity of the broad band *ca* 500 nm increases while the maximum shifts to 510 nm indicating the formation of an azino adduct MBTH–TPQ (Figure 4(c)).

MECHANISM-BASED INACTIVATORS

Several types of mechanism-based plant amine oxidase inactivators can be distinguished on the basis of their interaction:

(A) Compounds forming a reactive enzyme–inhibitor complex that is sensitive to nucleophilic attack by an active-site side-chain residue.
(B) Compounds showing suicide product mechanism in which the enzyme forms a product that irreversibly inhibits the enzyme.
(C) Compounds showing substrate-mediated inhibition in which a compound may become an inhibitor of the enzyme only in the presence of substrate.

(A) 1,4-Diamino-2-butyne (DABI; Figure 2(h)) is a mechanism-based inhibitor of pea cotyledon amine oxidase.[35] It shows saturation kinetics with $K_m = 1\,mM$, but its interaction leads to a time-dependent and irreversible loss of the enzyme activity. The substrate-saturation kinetic data for DABI and the pseudo-first-order time-dependent irreversible inactivation of pea amine oxidase indicate that DABI is a mechanism-based inhibitor of this enzyme with a partition ratio = 17 and characteristic constants $K' = 0.32\,mM$ and $k_{in} = 4.9\,min^{-1}$ ($K' = (k_{-1} + k_2) \cdot (k_3 + k_4)/k_1 \cdot (k_2 + k_3 + k_4)$ and $k_{in} = k_2 \cdot k_4/(k_2 + k_3 + k_4)$, see Equation (6)). The mechanism of the interaction involves an intermediate aminoallenic compound, which forms covalently bound pyrrole in the reaction with an active site nucleophile (Figure 6).

(B) The alkylamines 2-bromoethylamine and 2-chloroethylamine, and the shortest diamine 1,2-diaminoethane are irreversible inhibitors of several

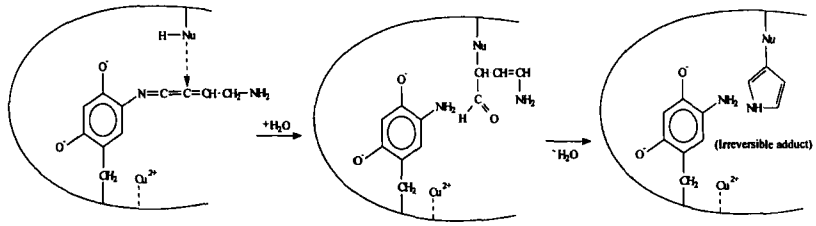

FIGURE 6 Mechanism of the reaction of pea amine oxidase with 1,4-diamino-2-butyne.

Cu-amine oxidases. These compounds were found to be both poor substrates and irreversible inhibitors of lentil amine oxidase where their inhibition mechanism has been demonstrated.[24] Lentil enzyme catalyzes the oxidation of these compounds via reversible formation of an enzyme–inhibitor complex [E · I] followed by an irreversible inactivation due to the generation of a stable enzyme–inhibitor complex [E-I] as shown in the following scheme:

$$E + I \rightleftharpoons E \cdot I \rightarrow E\text{-}I \tag{7}$$

The irreversible inhibition is caused by the aldehyde produced reacting with a highly reactive species of the TPQ-derived free radical catalytic intermediate that, after β-elimination, forms a stable 6-membered ring (Figure 7(a)). Inactivation takes place in both presence and absence of oxygen. On the contrary, the copper-free protein is neither inactivated by haloamines nor by 1,2-diaminoethane which indicates that the aldehydes react only with the free radical, formed only in the holoenzyme and only in the absence of oxygen, to form a covalently modified enzyme. The corresponding propylamine compounds 1,3-diaminopropane, 3-bromopropylamine, and 3-chloropropylamine are reversible inhibitors of lentil amine oxidase.[24] These compounds, by analogy with the mechanism presented above, may form the E-I* complex, an unstable reversible 7-membered ring (Figure 7(b)), that causes reversible inhibition as follows:

$$E + I \rightleftharpoons E \cdot I \rightarrow E\text{-}I^* \rightarrow E + P \tag{8}$$

Different from the case of LSAO, 1,2-diaminoethane is a competitive inhibitor of PSAO,[15] whereas 1,3-diaminopropane is a mechanism-based inhibitor of PSAO.[36]

LSAO is able to catalyze the oxidative deamination of some indoleamines, such as tryptamine, 5-hydroxytryptamine, and 5-methoxytryptamine,[25] with K_m values nearly the same as for the normal substrate putrescine. However, their oxidation leads to irreversible loss of the

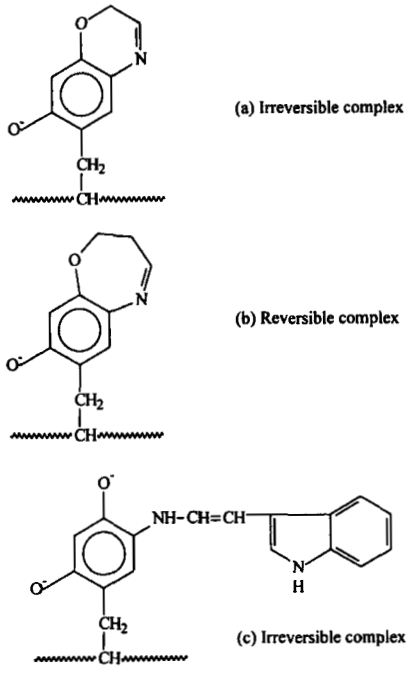

FIGURE 7 Structure of the TPQ-adduct after reaction of LSAO with (a) 2-bromoethylamine, (b) 3-bromopropylamine, and (c) tryptamine.

enzymatic activity only in the absence of oxygen due to formation of a stable adduct between indoleacetaldehydes and TPQ semiquinone as shown in Figure 7(c).

(C) Amine oxidase from *Euphorbia characias* latex was shown to contain two sulfhydryl groups per mole of dimeric enzyme.[37] The sulfhydryl groups are not reactive in the native enzyme, but can be titrated with 4,4′-dithiodipyridine after protein denaturation in 8 M urea or under anaerobic conditions in the presence of amine substrates. These sulfhydryl groups show different reactivity toward sulfhydryl reagents. Half-site reactivity is found toward N-ethylmaleimide and iodoacetate since both reagents produce 50% inactivation and modification of only one sulfhydryl group per dimer. On the other hand, 4,4′-dithiodipyridine produces 100% inactivation and modification of both reactive sulfhydryl groups. The values of the pseudo-first-order rate constants for the inactivation of ELAO are $k = 0.02\,\text{min}^{-1}$ by 1 mM 4,4′-dithiodipyridine and $k = 0.06\,\text{min}^{-1}$ for 1 mM N-ethylmaleimide, corresponding to bimolecular rate constants of 20 and $60\,\text{min}^{-1}\,\text{M}^{-1}$ respectively.

ELAO is not sensitive to hydrogen peroxide in the absence of substrate.[37] In the presence of substrate and in absence of catalase, however, either hydrogen peroxide formed by the reaction or more rapidly the hydrogen peroxide added before starting the enzymatic reaction causes total inactivation. The rate constant of the autoinhibitory reaction is $k = 0.07\,\text{min}^{-1}$, whereas the addition of hydrogen peroxide before starting the reaction produces an increase in the rate constant of inactivation ($k = 0.20\,\text{min}^{-1}$). Since no free sulfhydryl groups are titrated in the enzyme after inactivation by hydrogen peroxide and denaturation in 8 M urea, the inactivation by hydrogen peroxide is considered to be related to the sulfhydryl group modification. The insensitivity of the native enzyme to specific sulfhydryl group reagents and to hydrogen peroxide suggests that the sulfhydryl groups are located in the interior of the enzyme and their reactivity in the presence of the amine substrate is probably caused by a conformational change occurring upon substrate binding or after substrate oxidation.

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