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Probing the Active Site of Pea Seedlings Amine Oxidase with Optical Antipodes of Sedamine Alkaloids

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Interactions of pea seedlings amine oxidase (PSAO, EC 1.4.3.6) with sedamine derivatives were studied. All compounds exhibited a competitive inhibition with the inhibition constants in the range 0.03–1.0 mM. The inhibition effect increased in the order allosedamine < sedamine ≪ norallosedamine < norsedamine. The *nor*-derivatives are about five-fold stronger inhibitors and the *allo*-isomers are slightly weaker inhibitors than the others. Interestingly, the (−)-diastereomers of the studied sedamines were considerably stronger inhibitors than the (+)-antipodes. Absorption spectroscopy was used to differentiate between two known groups of competitive inhibitors of PSAO. A representative of substrate analogues, 1,5-diamino-3-pentanone, bleached the spectrum of the TPQ cofactor producing a very stable intermediate of the enzyme catalytic cycle that was only slowly converted to the product. On the other hand, the alkaloids did not perturb the spectrum of TPQ so they may interact with some other residue near the active site.

Keywords: Copper amine oxidase; Pea seedlings; Sedamine alkaloids; Competitive inhibition; Stereoisomers

Abbreviations: PSAO, pea seedlings amine oxidase

INTRODUCTION

Pea seedling amine oxidase (PSAO) belongs to the group of copper containing amine oxidases (EC 1.4.3.6), [amine: O_2 oxidoreductase (deaminating)], catalysing the oxidative deamination of biogenic amines to the corresponding aldehydes and ammonia, accompanied by two electron reduction of molecular oxygen to hydrogen peroxide. These enzymes have been found in bacteria, fungi and various plants and animals, but their actual function in these organisms is not completely understood with the exception of several physiological processes connected to the metabolism of amines and polyamines.[1,2] Moreover, plant amine oxidases participate in the biosynthesis of some alkaloids[2] and also provided hydrogen peroxide for the lignification and stiffening of the cell wall.[3] Amine oxidase contains the organic cofactor topa quinone,[4] that

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is generated from a specific tyrosyl residue by a self-oxidising mechanism catalysed by cupric copper.[5] PSAO is the best studied enzyme of this class regarding its substrate and inhibitory specificity. The enzyme readily converts the diamines putrescine, cadaverine and polyamine spermidine, less effectively oxidises a wide range of both biogenic and synthetic amines, and is inhibited by substrate analogues, carbonyl reagents, copper complexing agents and some alkaloids.[6]

In this study, we have used synthesised optical antipodes of sedamine derivatives,[7,8] some of them naturally occurring in plants such as (−)-sedamine from *Sedum acre* L., (−)-allosedamine and (+)-norallosedamine from *Lobelia inflata* L, to further pursue our earlier studies on the inhibition of pea seedling amine oxidase by alkaloids.[9–11]

MATERIALS AND METHODS

Enzymes and Other Reagents

PSAO was isolated from seven days old etiolated seedlings of pea (*Pisum sativum*) by a standard purification method.[12] The specific activity of the enzyme preparation was 150 and 750 nkat. mg^{-1} with *E*-2-butene-1,4-diamine[13] and putrescine[12] (butane-1,4-diamine) as substrates, respectively.

Bovine liver catalase (EC 1.11.1.6) was purchased from Reanal (Budapest, Hungary) with a specific activity of 2000 Bergmeyer units mg^{-1}.

Sedamine alkaloids were kindly provided by Prof. G. G. Habermehl from the Institute of Chemistry, Hanover School of Veterinary Medicine, Germany, as synthetic products from Prof. C. Schöpf's laboratory formerly at the Institute of Organic Chemistry, Technical University, Darmstadt. The following compounds (Fig. 1) were synthesised as racemates[7,8] and their optical antipodes were separated using fractional crystallisation with dibenzoyl-D-tartrate: (+)-sedamine,[7] m.p. 72–74°C, $[\alpha]_D^{20} = +80.4°$ ($c = 5.0$, methanol); (−)-sedamine,[7] m.p. 68–70°C, $[\alpha]_D^{20} = -82.4°$ ($c = 5.0$, methanol); (+)-allosedamine,[7] m.p. 81–82°C, $[\alpha]_D^{20} = +31.0°$ ($c = 5.0$, methanol); (−)-allosedamine,[7] m.p. 81–82°C, $[\alpha]_D^{20} = -31.2°$ ($c = 5.0$, methanol); (+)-norsedamine,[8] m.p. 108–109°C, $[\alpha]_D^{20} = +33.2°$ ($c = 2.0$, methanol); and (−)-norsedamine,[8] m.p. 108–109°C, $[\alpha]_D^{20} = -32.5°$ ($c = 2.0$, methanol). Norallosedamine

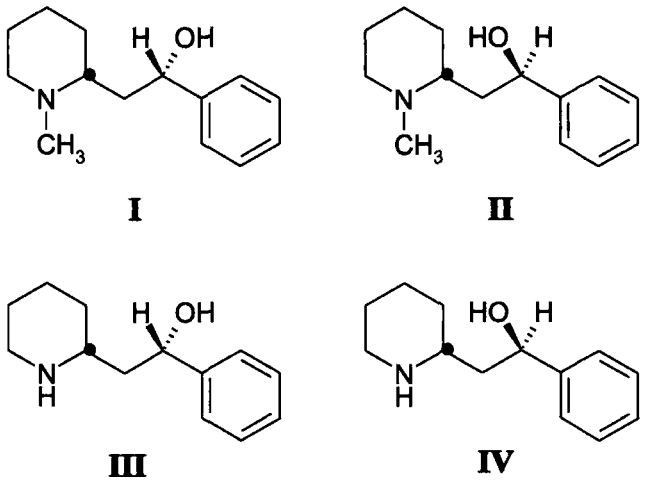

FIGURE 1 Structure of sedamine alkaloids tested as inhibitors of pea seedling amine oxidase: **I**—sedamine [(2S:8S)-8-phenyl-lobelol], **II**—allosedamine [(2S:8R)-8-phenyl-lobelol], **III**—norsedamine [(2S:8S)-8-phenyl-norlobelol] and **IV**—norallosedamine [(2S:8R)-8-phenyl-norlobelol].

was synthesised as a racemate[8] and its optical antipodes were separated by crystallisation with (+)-6,6′-dinitrodiphenic acid: (+)-norallosedamine, m.p. 104°C, $[\alpha]_D^{20} = +37.2°$ ($c = 3.0$, ethanol); (−)-norallosedamine, m.p. 104–105°C, $[\alpha]_D^{20} = -37.0°$ ($c = 3.0$, ethanol). The (+)-antipodes were obtained as crystalline salts with dibenzoyl-D-tartrate or (+)-6,6′-dinitrodiphenic acid, respectively, while the (−)-antipodes were isolated from the mother liquors. The salts were neutralised by $NaHCO_3$ and respective free bases extracted with petrolieum ether.

L-Lobeline hydrochloride was obtained from Spofa (Prague, Czech Republic), cinchonine from Fluka (Buchs, Switzerland) and 1,5-diamine-3-pentanone dihydrochloride was synthesised.[14] Stock solutions (20 mM) of the inhibitors were made in 20 mM potassium phosphate buffer, pH 7.0. For dissolving those inhibitors obtained as free bases, a stoichiometric amount of HCl was added and the solution was briefly heated up to 80°C.

Kinetics of the Interaction of PSAO with the Inhibitors

The assay method with *E*-2-butene-1,4-diamine as a substrate based on the reaction of the formed product pyrrole with *p*-dimethylaminobenzaldehyde[13] was used for the kinetic measurements. The reaction mixture with a total volume of 3 ml contained 0.1 M potassium phosphate buffer, pH 7.0, catalase (80 units), PSAO (2 nkat) and the inhibitor in concentrations 0.2–2.0 mM. The mixture was preincubated at 30°C for 10 min prior to adding the substrate (final concentration 0.025–0.6 mM). The reaction was stopped after 5 min by adding 2 ml of Ehrlich's reagent (15% *p*-dimethylaminobenzaldehyde in 80% *n*-propanol and 7% HCl). The mixture was then incubated at 30°C for 30 min and chilled on ice before measuring the absorbance at 563 nm. The obtained values were evaluated on a PC using the program GraFit 4.0 from Erithacus Software (Horley/Surrey, UK).

Spectral Measurements

Rapid scanning experiments were carried out using a DU 7500 photodiode array spectrophotometer (Beckman, Fullerton, CA, USA) in the multiwavelength kinetics mode which allowed it to acquire spectra of photodiode readings in 0.1 s interval and store up to 99 recorded spectra at chosen time intervals. Longer-time-interval spectral measurements were performed on a Lambda 11 UV-Vis spectrophotometer (Perkin-Elmer, Überlingen, Germany) connected to a PC equipped with UV WinLab Version 2.70.01 software (Perkin-Elmer). Absorption spectra of the respective enzyme-substrate reaction mixture were recorded with a time interval of 3–60 min. Changes in absorption spectrum of PSAO (22.5 μM) were monitored in 20 mM potassium phosphate buffer, pH 7.0, after addition of the following competitive inhibitors (final concentration 1 mM): 1,5-diamino-3-pentanone, L-lobeline, cinchonine and (−)-norallosedamine.

RESULTS AND DISCUSSION

Catalytic reaction of PSAO can be altered by a wide range of compounds acting as reversible inhibitors.[6] In particular, copper chelating agents, e.g. diethyldithiocarbamate, 1,10-phenathroline and diethylenetriamine, act as non-competitive inhibitors, whereas diaminoketones, cinchona alkaloids and alkaloids derived from piperidine are competitive inhibitors.

It has been speculated that the inhibition of an amine oxidase can serve as a feedback control in some plants in the biosynthesis of alkaloids derived from ornithine and lysine, where the enzyme forms in one of the earlier steps 1-pyrroline (or *N*-methyl-1-pyrrolinium salt) and 1-piperideine, respectively.[15–20] Some of

these alkaloids are optically active compounds containing one or more chiral carbon atoms and in plants they are produced only as certain enantiomers.

From this point of view, it is indeed interesting to study the inhibition effect of different stereoisomers on the amine oxidase. Copper amine oxidases are enzymes that show stereochemical preferences in the catalysed reaction, e.g. dopamine and tyramine are oxidised with the abstraction of the pro-R hydrogen by the porcine plasma enzyme, the pro-S hydrogen by PSAO, and non-stereospecifically by bovine plasma, rabbit and sheep serum amine oxidases.[21]

In this study we used the PSAO as a model enzyme with four types of synthesised compounds as shown in Fig. 1, sedamine, norsedamine, allosedamine and norallosedamine, all of them were separated into their dextrorotatory (+) and levorotatory (−) enantiomers. Among the eight compounds used, three have been found naturally occurring in plants: (2S:8S)-(−)-sedamine in *Sedum acre*, (2S:8R)-(−)-allosedamine and (2S:8R)-(+)-norallosedamine from *Lobelia inflata*. All compounds exhibited a competitive inhibition towards oxidation of *E*-2-butene-1,4-diamine by the enzyme with a K_i value in the range 0.03–1.0 mM as shown in Table I. Examples of typical Lineweaver–Burk double reciprocal plots are shown in Fig. 2. The inhibition effect increases in the order allosedamine < sedamine ≪ norallosedamine < norsedamine. There is a clear decrease of the inhibition effect with *N*-methylation of the piperidine ring, the *nor*-derivatives are about five-fold stronger inhibitors. Also the diastereomers with 2S:8R configuration (*allo*-isomers) are slightly weaker inhibitors than the 2S:8S ones (referring to the hydroxyl group on C-8). Similar, but much more notable dependence of the inhibition effect on the absolute configuration of the hydroxyl group bound at the chiral carbon (C-9) has been previously found for cinchonine (8S:9S, $K_i = 0.2$ mM), cinchonidine (8S:9R, $K_i = 1.1$ mM), quinidine (8S:9S, $K_i = 1.1$ mM) and quinine (8S:9R, unspecified higher value of K_i).[9]

TABLE I Inhibition constants of sedamine alkaloids on pea seedling amine oxidase. Roman numerals (I)–(IV) in the Configuration column refer to the structures shown in Fig. 1

Alkaloid	Configuration	K_i (mM)	Reference
(+)-Sedamine	2S:8S, (I)	0.90	10
(−)-Sedamine	2R:8R,	0.30	this work
(+)-Allosedamine	2S:8R, (II)	1.00	this work
(−)-Allosedamine	2R:8S,	0.40	this work
(+)-Norsedamine	2S:8S, (III)	0.16	this work
(−)-Norsedamine	2R:8R,	0.03	10
(+)-Norallosedamine	2S:8R, (IV)	0.22	this work
(−)-Norallosedamine	2R:8S,	0.06	10

Most strikingly, the (−)-enantiomers of the studied sedamines are considerably stronger inhibitors than the (+)-antipodes, showing 2.5–5.3 fold lower K_i. Structure of the pea amine oxidase has already been solved, electron densities of the residues constituting the substrate channel being well defined.[22] The side chain, however, was modelled in a single conformation, which obscured the active site when the structure was viewed from the direction of the channel.[24] Thus the data do not allow precise interpretation and modelling of the chirality of this part of the enzyme active site, and the possible stereochemically-specific binding of the above inhibitors cannot be evaluated.

Spectral measurements presented in this study were conducted to assess the difference between two groups of competitive inhibitors of PSAO. As has been found earlier by kinetic analysis, the substrate analogues such as 1,5-diamino-3-pentanone and 1,4-diamino-2-butanone bind the TPQ cofactor as normal substrates, but their complexes are slowly hydrolysed at about hundred-fold lower rate than that of normal substrates.[13] Therefore they show kinetics similar to that of a competitive inhibitor. On the other hand, the alkaloids studied do not possess a primary amino group that can be oxidised, so they presumably act as "true" competitive inhibitors. Spectral measurements indeed con-

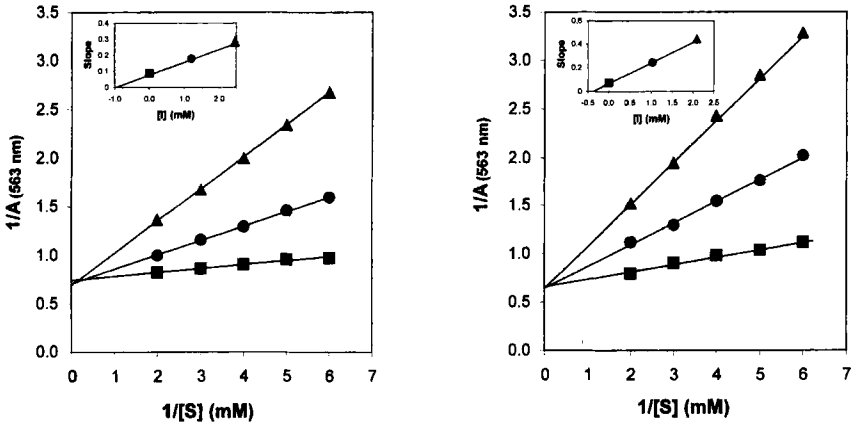

FIGURE 2 Double reciprocal plot of competitive inhibition of pea seedling amine oxidase by optical isomers of allosedamine with *E*-2-butene-1,4-diamine as substrate. Left: (+)-allosedamine, final concentrations ■-0, ●-1.22 and ▲-2.45 mM, $K_i = 1.0$ mM; right: (−)-allosedamine, ■-0, ●-1.05 and ▲-2.10 mM, $K_i = 0.4$ mM. The corresponding insets show secondary plots of slopes against the inhibitor concentration.

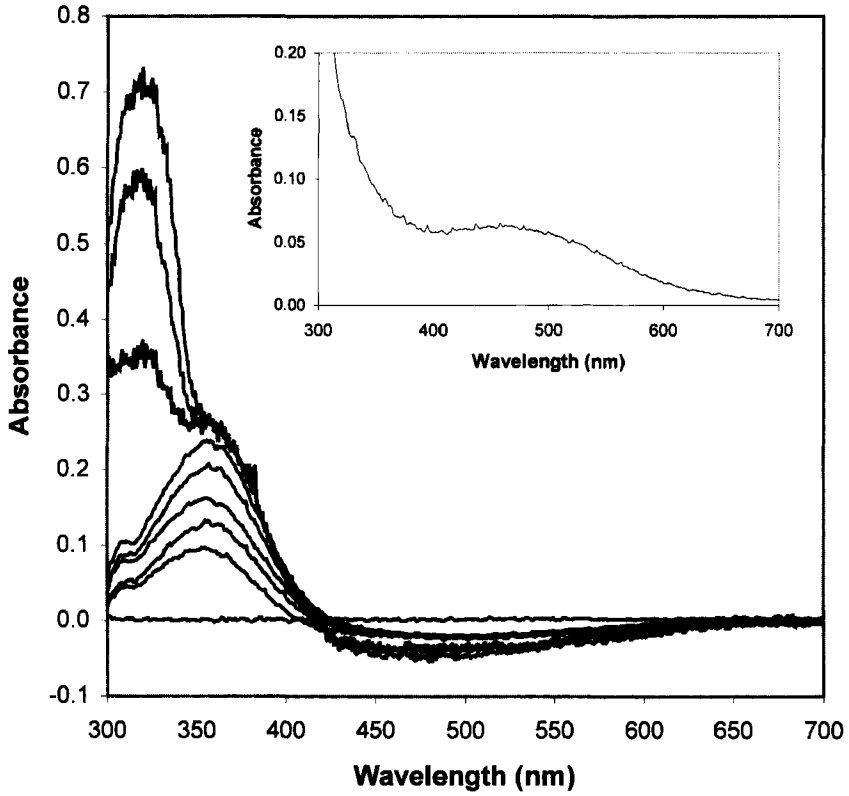

FIGURE 3 Spectral changes (difference absorption spectra) of pea seedling amine oxidase (22.5 μM) after addition of 1 mM 1,5-diamino-3-pentanone. Detailed description of the experiment is given in Material and Methods. The traces show selected spectra recorded at the following time intervals (from the bottom): 0, 0.4, 0.5, 0.6, 0.7, 0.8, 60, 240, and 420 s. The inset shows absorption spectrum of native amine oxidase from pea seedlings.

firmed that the addition of 1,5-diamino-3-pentanone rapidly bleaches the TPQ absorption at 500 nm as usual substrate and shows a typical spectrum for a Schiff base intermediate with absorption maximum at 350 nm that is slowly converted to the corresponding aldimine absorbing at 315 nm[23] (see Fig. 3). The aldimine then remains stable and does not undergo rapid hydrolysis, thus it is not possible to detect the typical three-banded semiquinolamine radical spectrum (maxima at 360, 435 and 465 nm).[23]

On the contrary, the alkaloids L-lobeline, cinchonine and (−)-norallosedamine did not affect the spectrum of TPQ and at the same time they showed a marked inhibition effect. It seems very likely that they interact with some residue other than TPQ in the active site of the enzyme, possibly via hydrophobic interaction as already suggested.[10] A substrate-leading channel to the active site in the PSAO has been identified recently by structural similarity with the amine oxidase from *Arthrobacter globiformis*.[24] From the above structures, one possible candidate for the hydrophobic interaction with the alkaloids might be the residue Phe298 that lies in the substrate channel of PSAO and acts as a plug.

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