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SHORT COMMUNICATION

SOME AMINES AS INHIBITORS OF PEA DIAMINE OXIDASE

PAVEL PEČ and IVO FRÉBORT

Department of Analytical and Organic Chemistry, Faculty of Science, Palacký University, Tř. Svobody 8, 771 46 Olomouc, Czechoslovakia

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The inhibition of pea diamine oxidase (DAO; EC 1.4.3.6) by some amines and chelating agents has been studied. Ethylenediamine (competitive, K_i = 5.2 mmol.l^{-1}), cis-1,2-diaminocyclohexane (noncompetitive, K_i = 2.9 mmol.l^{-1}) and trans-1,2-diaminocyclohexane (competitive, K_i = 3.6 mmol.l^{-1}) are weak inhibitors whereas diethylenetriamine (noncompetitive, K_i = 7.2 μmol.l^{-1}), triethylenetetraamine (partial noncompetitive, K_i = 0.57 μmol.l^{-1}) are potent inhibitors. The chelating agents 1,10-phenanthroline (K_i = 0.031 mmol.l^{-1}), and 2,2′-bipyridyl (K_i = 0.058 mmol.l^{-1}) are noncompetitive inhibitors. The pH-inhibition profile for the inhibitors indicates that the protonated forms are the active species.

KEY WORDS: Pea diamine oxidase, amines, chelating agents, inhibitors.

INTRODUCTION

Diamine oxidase [DAO; EC 1.4.3.6; diamine: O_2 – oxidoreductase (deaminating; copper containing)] from pea cotyledons oxidizes diamines to aminoaldehydes with the stoichiometry:

$$H_2N(CH_2)_nNH_2 + O_2 + H_2O \rightarrow H_2N(CH_2)_{n-1}CHO + H_2O_2 + NH_3 \quad (1)$$

The reaction mechanism involves only binary enzyme-substrate complexes, the catalytic sequence being amine on, aminoaldehyde and ammonia off, O_2 on, followed by release of H_2O_2.[1,2] Pyrroloquinoline quinone serves as a cofactor[3] and with M_r 180 000 the enzyme has 2 g.atom.mol^{-1} Cu-2$^+$.[4,5] The amines most readily oxidized are the diamines putrescine, cadaverine, agmatine, 1,6-diaminohexane, histamine[6] and spermidine,[7] and the phenylalkylamines β-phenylethylamine, di-β-phenylethylamine, tyramine and adrenaline and tryptamine.[6] Diamine oxidase is inhibited by copper-chelating reagents such are 2,2′-bipyridyl and 1,10-phenanthroline[7-9] although the character of the inhibition has not been described. We have already described the inhibition of pea DAO[10] by phenylcyclopropylamine (noncompetitive) and trans-2-phenyl-cyclopropylamine (competitive). The substrate *E*-1,4-diamino-2-butene inhibits the enzyme[11] at high concentration and the diamines 1,5-diamino-3-pentanone[12] and 1,4-diamino-2-butanone[13] are potent inhibitors of the enzyme. 1-Amino-3-phenyl-3-propanone and 1-amino-3-phenyl-2-propanone[14] are weak competitive inhibitors. Other known inhibitors are histamine and histidine and the strong noncompetitive dipeptide inhibitors anserine and carnosine.[15] β-Aminopropionitrile is a potent inhibitor[16] (I_{50} = 8.0 μmol.l^{-1}) and is probably a mechanism-based DAO inhibitor.

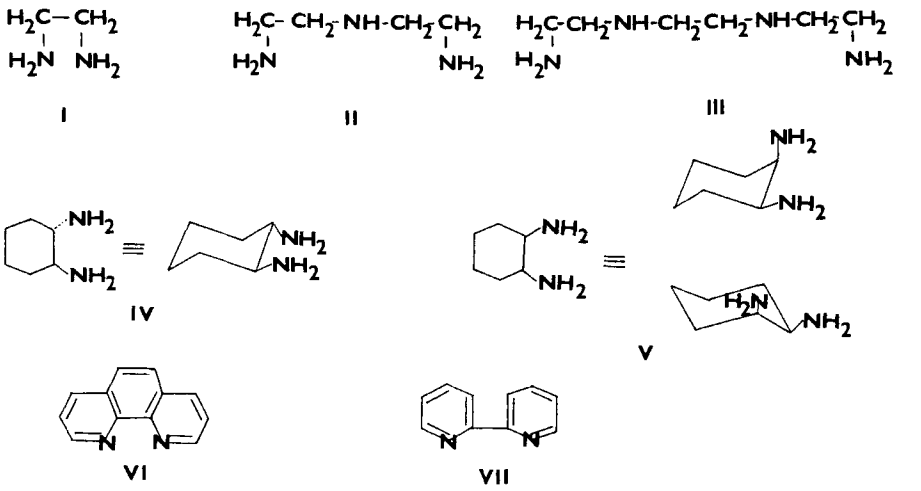

FIGURE 1 Structural formulas of inhibitors ● **I**, ethylenediamine; **II**, diethylenetriamine; **III**, triethylenetetraamine; **IV**, trans-1,2-diaminocyclohexane; **V**, cis-1,2-diaminocyclohexane; **VI**, 1,10-phenanthroline; **VII**, 2,2′-bipyridyl.

The problem of studying interaction of amines and diamines with pea DAO is to decide between their behavior as substrates or inhibitors e.g. N- and C-alkylputrescines, which are substrates and inhibitors.[17]

The aim of the present study was to investigate the kinetics of the inhibition of DAO by some amines and copper-chelating reagents to determine their mechanism of inhibition. The structures of the studied inhibitors are shown in Figure 1.

MATERIAL AND METHODS

Enzymes and chemicals

Diamine oxidase (EC 1.4.3.6) from the cotyledones of pea (*Pisum sativum*) was isolated by a five step procedure and purification method.[18] The protein content was determined according to the method of Bradford[19] as 2.9 mg.ml^{-1}, and the specific activity by the guaiacol method[20] which was 254.2 nkat.mg prot.$^{-1}$. Peroxidase (EC 1.11.1.7) from horse radish (*Armoracia rusticana*) was a salt free lyophilisate from Boehringer Manheim, Germany with specific activity 1850 nkat. mg^{-1}. Ethylenediamine dihydrochloride (EDA), diethylenetriamine (DITA), triethylenetetraamine tetrahydrochloride (TETA), 1,10-phenanthroline 2,2′-bipyridyl and 2,2′-biquinolyl were obtained from Lachema Brno as analytical grade. Cis-(c-DAC) and trans-1,2-diaminocyclohexane (t-DAC) were obtained from Aldrich, Germany. The hydrochoride of putrescine (1,4-diaminobutane) was obtained from Koch Light, England.

Enzyme activity determination

Spectrophotometric determination of the enzyme activity[20] was performed in a 2 cm cell in a light-tight chamber of an EK 5 adapter of a Specol 10 spectrophotometer (Carl Zeiss Jena, Germany), thermostated to 30°C. The reaction mixture (3.45 ml)

contained 0.1 mol.l^{-1} potassium phosphate buffer (pH = 7.0 or 8.0 for type of inhibition and pH = 5.8, 6.5, 7.0, 7.4 and 8.0 for pH profiles), guaiacol in a final concentration 0.5 mmol.l^{-1}, peroxidase (18.5 nkat), diamine oxidase (16.7 nkat) and the inhibitor. The enzyme was usually preincubated for 10 min with the inhibitor (with 1,10-phenanthroline and 2,2′-bipyridyl for 20 min and with ethylenediamine for 5 min only) in the reaction mixture and the reaction was started by injecting 0.05 ml of putrescine (final concentration 0.1–0.5 mmol.l^{-1}) directly into the cell, in which the reaction mixture was for a short time bubbled with air. The bubbling was then stopped and the time-dependent increase in absorption at 436 nm (for 3 min) was recorded on a PMD 85-1 computer with our own hardware-software system.[21]

RESULTS

Throughout the present study, the initial rates of reaction, as measured by change in absorbance at 436 nm, were linear after substrate addition. The results are summarized in Table 1. Using putrescine as substrate, ethylenediamine is a weak competitive inhibitor (K_i = 5.2 mmol.l^{-1}) of the enzyme. In agreement with the results of Kenten *et al.*[6] EDA is a substrate but in comparison with putrescine the velocity is only 4.5%. The other two amines DITA and TETA (Figure 2.) are potent inhibitors of pea DAO and inhibit it in a noncompetitive manner. TETA is the most potent noncompetitive inhibitor of pea DAO yet known ($K_i = 5.7 \cdot 10^{-7}$ mol.l^{-1}). The two isomers of 1,2-diaminocyclohexane are weak inhibitors but surprisingly t-DAC is a competitive inhibitor (Figure 3.) with K_i = 3.6 mmol.l^{-1} whereas c-DAC is a weak noncompetitive inhibitor.

The secondary graph in Figure 2 shows partial inhibition of DAO by TETA. The dependence of $v_0/(v_0 - v_i)$ on 1/[I] (not shown), where v_0 is initial reaction rate without inhibitor and v_i is initial reaction rate with inhibitor and [I] is the concentration of the amine, is linear and the intersect on the verticle axis gives a value for $v_0/(v_0 - v_i)$ of 1.49. This value is indicative of partial noncompetitive inhibition.[22] Dependence of the activity of DAO and inhibition effects on pH is shown in Figure 4. In the range pH < 7 the dependence of the inhibition on pH follows the pH-activity curve for DAO. In the range pH > 7 the activity of DAO decreases, but the inhibition effects with c-DAC and t-DAC are unchanged, while with EDA, DITA and TETA they are decreased.

TABLE I
Type of inhibition and inhibition constants for some amines with pea diamine oxidase

Inhibitor	Type of inhibition	K_i (mmol.l^{-1})	$-G°$ (kJ.mol^{-1})
cis-1,2-diaminocyclohexane	noncompetitive	2.9	14.7
trans-1,2-diaminocyclohexane	competitive	3.6	14.2
ethlenediamine	competitive	5.2	13.3
diethylenetriamine	noncompetitive	0.0072	29.8
triethylenetetraamine	partial, noncomp.	0.00057	36.3
1,10-phenanthroline	noncompetitive	0.031	26.2
2,2′-bipyridyl	noncompetitive	0.058	24.6

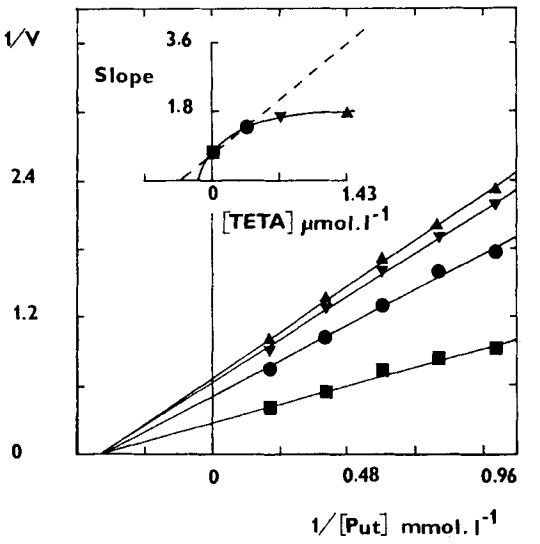

FIGURE 2 Double reciprocal plot of partial noncompetitive inhibition of pea DAO by triethylenetetraamine (TETA) (with putrescine (Put) as a substrate) in concentration of: ■ – without inhibitor, ● – 0.357, ▼ – 0.714 and ▲ – 1.43.μmol.l^{-1}. The inhibition constant is 0.57 μmol.l^{-1}.

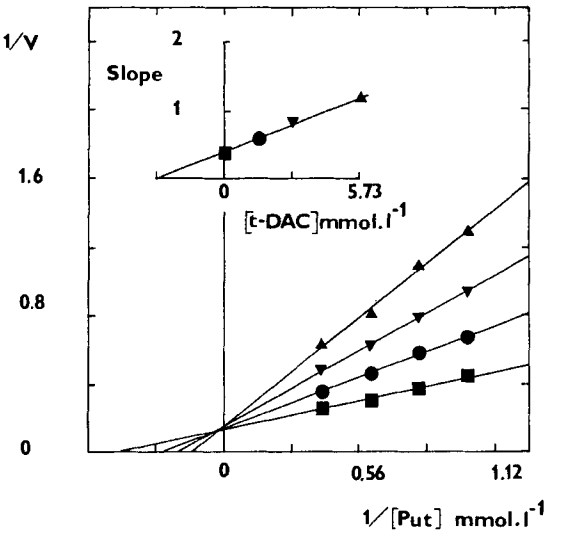

FIGURE 3 Double reciprocal plot of competitive inhibition of pea DAO by trans-1,2-diaminocyclohexane (t-DAC) with putrescine (Put) as a substrate) in concentration of: ■ – without inhibitor, ● – 1.43, ▼ – 2.86 and ▲ – 5.73 mmol.l^{-1}. The inhibition constant is 3.6 mmol.l^{-1}.

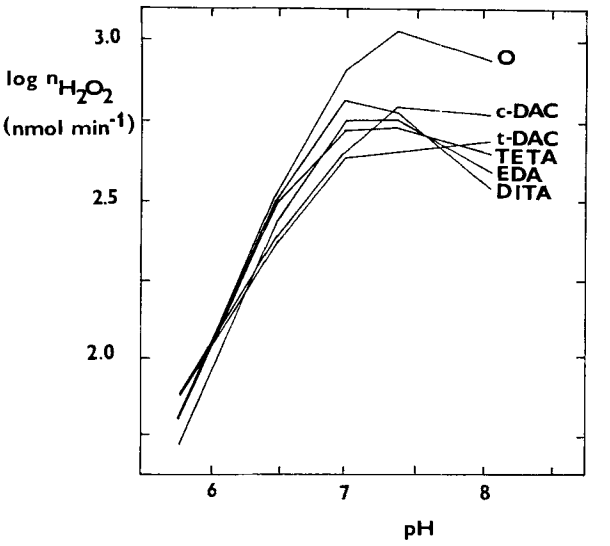

FIGURE 4 pH-activity curves for pea DAO (0 = alone) and with inhibitors: 5.71 mmol.l^{-1} ethylenediamine (EDA), 5.71 μmol.l^{-1} diethylenetriamine (DITA), 1.43 μmol.l^{-1} triethylenetetraamine (TETA), 5.71 mmol.l^{-1} trans-1,2-diaminocyclohexane (t-DAC) and 5.71 mmol.l^{-1} cis-1,2-diaminocyclohexane (c-DAC).

2,2′-Bipyridyl and 1,10-phenanthroline are noncompetitive inhibitors of DAO with K_i values about 10^{-5} mol.l^{-1}. Here, equilibration between the chelating reagents and DAO is very slow and is only obtained by preincubating the reaction mixture for 20 minutes.

DISCUSSION

In the present study we have paid attention to the interactions of some diamines with pea DAO. Consideration of aliphatic diamines as substrates of DAO shows that the best substrate is 1,5-diaminopentane, and that 1,4-diaminobutane > agmatine > 1,6-diaminohexane.[6] The oxidation of 1,3-diaminopropane is insignificant by comparison and at higher concentrations is an inhibitor. A protonated diamine or monoamine with protonated amine group and a five carbon chain or aromatic ring is required in substrates for substantial binding in the active site of pea DAO. We have found that EDA is a weak competitive inhibitor of DAO using 1,4-diaminobutane as substrate and is very slowly oxidized. The rate of oxidation is only 4.8% that of the oxidation rate of 1,4 diaminobutane.

DITA with a secondary amine group in its chain (Figure 1) is a strong noncompetitive inhibitor. DITA has a similar structure to cadaverine, except that it has a secondary and two primary amine groups. The secondary and one primary amine groups are protonated[23] at pH = 7.

TETA has a longer chain than DITA, and contains two intrachain secondary amine groups. TETA is a strong partial noncompetitive inhibitor of the enzyme with the lowest K_i value for noncompetitive inhibition of DAO yet known (Figure 2).

Partial noncompetitive inhibitors have no influence on the binding of the substrate in the active site of DAO, but the ternary complex, enzyme-inhibitor-substrate, breaks down into products more slowly than the enzyme-substrate complex.[22] Possibly the cause of the noncompetitive character of the inhibition of DAO by DITA and TETA is due to formation of a complex between DITA or TETA with copper in the active site of the enzyme.

It is known, that pea DAO contains pyrroloquinolino quinone (PQQ) as a cofactor.[24] In this connection it is interesting that the vicinal diamines c-DAc and t-DAC are weak inhibitors of the pea DAO, being noncompetitive and competitive inhibitors respectively, since Gacheru *et al.*[25] have shown that c-DAC is an irreversible inhibitor of lysyl oxidase which has PQQ as a cofactor. These authors showed that binding of c-DAC to the ortho-quinone group of the PQQ occurs so that the bifunctional Schiff base formed does not break down into products and t-DAC is without effect on the lysyl oxidase. In c-DAC (Figure 1), the position of the equatorial and axial amine groups is sterically less suitable for reaction with the ortho-quinone group of the DAO than in the trans-isomer where both amine groups are diequatorial. This view gains support from our results since c-DAC is a noncompetitive inhibitor and does not bind to the active site of DAO, while t-DAC is a weak competitive inhibitor. Production of hydrogen peroxide was not observed when either isomers was used as a substrate.

The chelating reagents 2,2′-bipyridyl and 1,10-phenathroline have been described as inhibitors of pea DAO but without determination of the type of inhibition and the value of K_i. A long preincubation time is necessary for formation of the DAO-inhibitor complex indicating interaction with Cu^{2+}.

All the amines examined are strong bases and their inhibition-pH profiles were studied in the range of pH = 5.8–8.0 (Figure 4). In general the inhibition effect increased with pH and the inhibition is related to the degree of protonization of the amine groups of the inhibitors.[23] Surprisingly c-DAC and t-DAC, which have a different type of inhibition, are activators (118% of control) of DAO at pH = 5.8.

Acknowledgement

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