J. Enzyme Inhibition, 1998, Vol. 13, pp. 457–463
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CYTOKININS AS INHIBITORS OF PLANT AMINE OXIDASE

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(Received 12 February 1998)

The interaction of pea seedling amine oxidase with cytokinins was examined to probe a possible connection between cytokinin oxidase and amine oxidase by determining whether cytokinins are substrates or inhibitors of the latter. Kinetic measurements suggest that cytokinins are weak competitive inhibitors of amine oxidase while their behaviour as substrates was not observed. The absence of enzymatic activity with cytokinins as substrates denies the identity or even any similarity of these two enzymes which was previously considered [Hare, P.D. and van Staden, J. (1994) *J. Physiol. Plant.*, **91**, 128]. From the values of the inhibition constants obtained it seems unlikely that cytokinins take part in the regulation of amine oxidase activity *in vivo*. Their inhibitory effect on amine oxidase may be similar to that of some alkaloids studied earlier.

Keywords: Pea seedling amine oxidase; Cytokinins; Cytokinin oxidase; Active site; Competitive inhibitors

INTRODUCTION

Cytokinins represent a class of natural and artificial plant growth regulators. Their major physiological effect is to induce cell division. About thirty natural cytokinins from various sources have been identified up to date. They occur both in free form and in form of tRNA nucleotides. Naturally occurring cytokinins are derivatives of the purine base adenine which carries a specific substituent bound to the amino group in the 6-position of the purine ring.[1] Naturally occurring cytokinins, such as zeatin, are often

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transformed by a substitution on the purine ring or modification, cleavage, and substitution of the side chain. The metabolic pathways used considerably depend on the physiological state of the studied plant. Among the processes identified as possibly playing a role in cytokinin metabolism are the formation of cytokinin nucleosides and nucleotides from the free bases, N-glucosylation, N-alanyl conjugation, O-glucosylation, side-chain reduction and side-chain oxidation.[1] Enzymes of cytokinin catabolism that have been purified and characterized are cytokinin oxidase, cytokinin-7β-glucosyl transferase (E.C. 2.4.1.118) and β-(9-cytokinin)alanine synthase (E.C. 4.2.99.13).[1,2] Cytokinins with unsaturated side-chain are degraded by cytokinin oxidase in plant organism.[3,4] The substrate specificity is determined by the nature of N^6 side chain and does not depend on other substituents on the purine ring. Investigated cytokinin oxidases so far present no affinity for natural cytokinins with a saturated or aromatic side chain.[3] Although cytokinin oxidase has not yet been extensively studied, Hare and van Staden[5] supposed that the mechanism of degradation of isopentenyladenine to adenine and 3-methyl-2-butenal is the same as the mechanism for degradation of biogenic amines by amine oxidases since it requires molecular oxygen and is stimulated by copper ions. Hence, they have classified cytokinin oxidase as a copper containing amine oxidase, although it is generally known that amine oxidases do not oxidize secondary amines.[6]

The pea seedling amine oxidase used in this study belongs to the group of copper containing amine oxidases (E.C. 1.4.3.6; amine: O_2 oxidoreductase (deaminating)) catalyzing 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 role in these organisms is not completely understood with the exception of their role in several physiological processes connected to the metabolism of amines and polyamines.[6] Amine oxidase contains the carbonyl cofactor, recently identified as topa quinone,[7,8] that is generated from a specific tyrosyl residue by a self-oxidating mechanism catalyzed by cupric copper.[9]

The main aim of this work was to study possible interactions between cytokinins and copper amine oxidase.

MATERIALS AND METHODS

Enzymes and Other Reagents

Amine oxidase was isolated from seven-day-old etiolated seedlings of the pea (*Pisum sativum*) by the standard purification method.[10] The specific

activity of the enzyme preparation was 749 nkat mg^{-1} with putrescine as a substrate as measured spectrophotometrically by the guaiacol method.[11] Homogeneity of the enzyme preparation was checked by SDS-PAGE. Horseradish peroxidase (E.C. 1.11.1.7) was a salt free lyophilisate from Fluka (Buchs, Switzerland) with specific activity 100 U mg^{-1}. Bovine liver catalase (E.C. 1.11.1.6) was purchased from Reanal (Budapest, Hungary) with specific activity 2000 Bergmeyer units mg^{-1}.

The cytokinins isopentenyladenine, isopentenyladenosine, benzyladenine, benzyladenosine, kinetin (N^6-(2-furfuryl)adenine), zeatin (N^6-(*trans*-4-hydroxy-3-methyl-2-buten-1-yl)adenine) and their ribosides were purchased from Sigma (St. Louis, USA). Cytokinins with a hydroxylated aromatic ring substituted at N^6, naturally occurring in poplar (*Populus x Robusta*), named topolins (6-(3-hydroxybenzyl)adenine and 6-(2-hydroxybenzyl)-adenine) and their ribosides, and olomoucine (6-benzyl-2-[(2-hydroxy-ethyl)amino]-9-methyladenine) were isolated or synthesized in the Laboratory of Growth Regulators, Faculty of Science, Palacký University in Olomouc.[12]

Kinetics of Interaction of Pea Seedling Amine Oxidase with Cytokinins

The enzyme activity with cytokinins as substrates was determined by the measurement of oxygen consumption in the reaction mixture by a Clark oxygen electrode on a digital oxygen system Model 10 (Rank Brothers, Cambridge, England). The enzyme reaction product of amine oxidase is hydrogen peroxide which could be degraded by catalase to water and oxygen that could be re-cycled. Since catalase contamination of a not entirely pure enzyme preparations could cause a measuring error, an excess of catalase and ethanol was added to the reaction mixture. Hydrogen peroxide originating from the enzyme reaction is then consumed in the oxidation of ethanol to ethanal by catalase and the reaction course is stoichiometric. The typical spectrophotometric assay[11] could not be applied because the complex enzyme-cytokinin caused colouring of the reaction mixture that made measurements inaccurate.

The inhibition properties of the cytokinins were determined in the following manner: Determination of enzyme activity was performed in the incubation chamber which was thermostated to 30°C. The reaction mixture (3.4 ml) contained 0.2 M potassium phosphate buffer (pH 7.0), 0.1 ml catalase, 0.1 ml ethanol, amine oxidase (5 nkat), and the respective cytokinin (final concentration 0.51–1.17 mM). The enzyme was usually preincubated for 10 min with the inhibitor in the reaction mixture which was aerated for a short period before the reaction was started by addition of 0.1 ml of

putrescine (final concentration 0.25–2.0 mM). The time-dependent decrease of oxygen was then recorded over a 3 min period. The obtained values were evaluated on a PC using the program GraFit 3.0 from Erithacus Software.

RESULTS AND DISCUSSION

Thirteen naturally occurring and synthetic cytokinins (structures shown in Figure 1) at a concentration of 0.8–2.4 mM were tested as possible substrates or inhibitors of pea seedling amine oxidase (5 nkat). None of the cytokinins tested showed any activity as substrate, when measured by the guaiacol method[11] or by measuring oxygen consumption with the Clark oxygen electrode. On the other hand, all thirteen cytokinins tested inhibited putrescine oxidation by the amine oxidase. All the compounds inhibited in a competitive manner as determined from double reciprocal plots (Figure 2). The values for the inhibition constant, K_i, were calculated from the dependence of the primary graph slopes on the inhibitor concentration and are summarized in Table I.

Using putrescine as substrate, we have found that cytokinins are weak inhibitors of pea seedling amine oxidase and do not exhibit any substrate activity. The inhibition was competitive, which indicates that the cytokinin molecule binds to the same site as the substrate. Hence, it is likely that there is some degree of similarity between the active sites of the amine oxidase and cytokinin oxidase. However, there is definitely a large structural difference between the substrates of the amine oxidase which are all primary amines and cytokinins which are secondary amines. Other differences between amine oxidases and cytokinin oxidases are apparent in their molecular properties and pH optima as published earlier.[5,6] Since the values of the inhibition constants are in the millimolar range, it is not likely that the cytokinins could have any regulatory role towards amine oxidase activity *in vivo*, since naturally occurring cytokinins are usually found in micromolar or lower concentrations and are metabolized by enzymes with K_m in the micromolar range.[1]

Certain conclusions can be made concerning the structure of cytokinins and their ability to bind into the active site on the basis of their K_i values. The inhibitory effect of ribosylated cytokinins is about 7-fold lower than that of cytokinins as the free form. The hydrophilic structure of ribose seems to restrain the cytokinin molecule from binding into the active site. Inhibitory potency is affected by the nature of the N^6 side chain. The isoprenoid structure of the side-chain in N^6-(Δ^2-isopentenyl)adenine increases

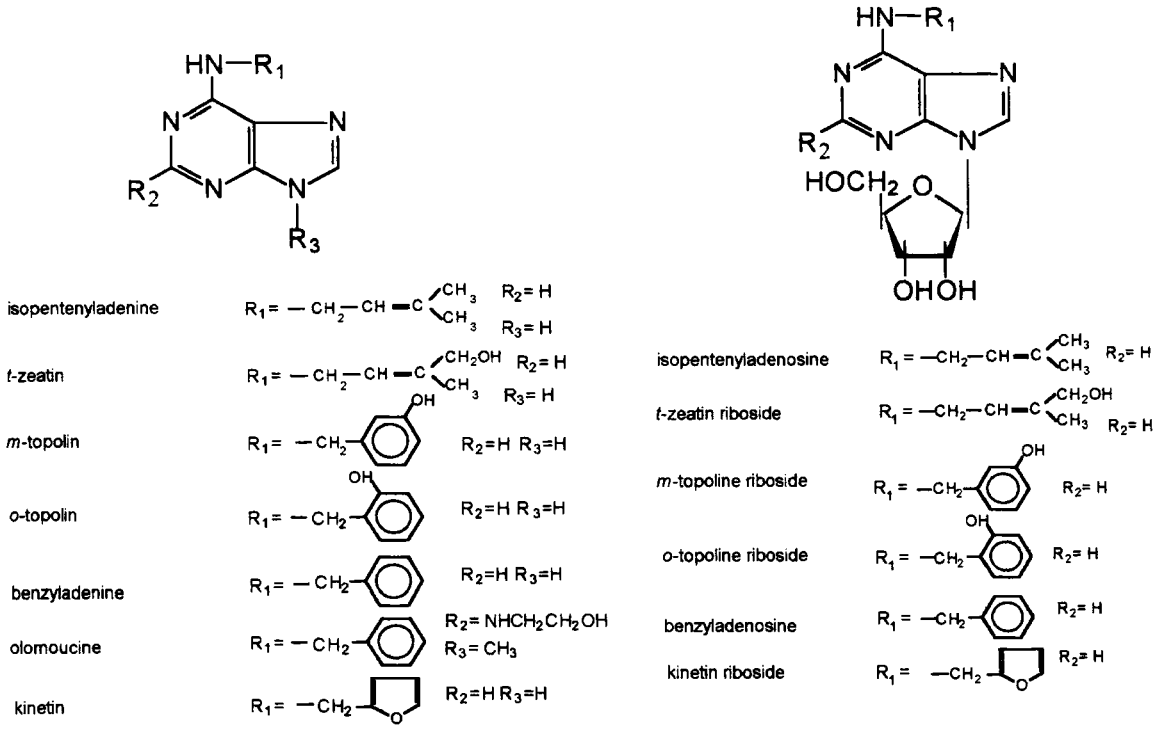

FIGURE 1 Structures of cytokinins tested as substrates and inhibitors of pea seedling amine oxidase.

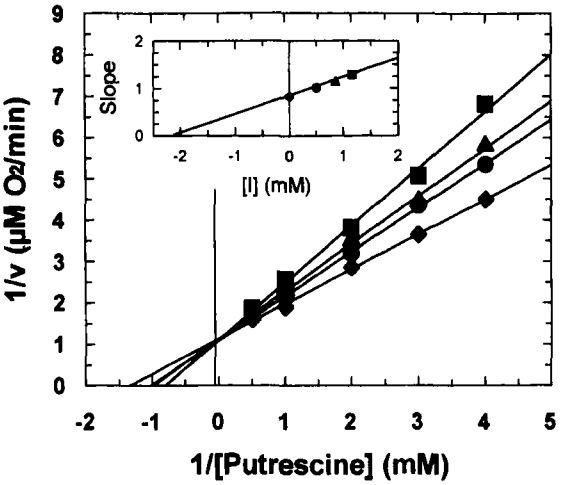

FIGURE 2 Double reciprocal plot of competitive inhibition of pea seedling amine oxidase by isopentenyladenine. Inhibitor concentrations [I]: 0(◆), 0.51(●), 0.86(▲) and 1.17 mM(■).

TABLE I Cytokinins acting as competitive inhibitors of pea seedlings amine oxidase and their inhibition constants

Inhibitor	K_i (mM)
N^6-(Δ^2-Isopentenyl)adenine	2.1
N^6-(Δ^2-Isopentenyl)adenosine	4.8
N^6-(*t*-4-Hydroxy-3-methyl-2-buten-1-yl)adenine (zeatin)	0.9
N^6-(*t*-4-Hydroxy-3-methyl-2-buten-1-yl)adenosine (zeatin riboside)	4.5
N^6-(2-Furfuryl)adenine (kinetin)	0.7
N^6-(2-Furfuryl)adenosine (kinetin riboside)	1.8
N^6-(3-Hydroxybenzyl)adenine (*m*-topolin)	0.6
N^6-(3-Hydroxybenzyl)adenosine (*m*-topolin riboside)	3.8
N^6-(2-Hydroxybenzyl)adenine (*o*-topolin)	0.5
N^6-(2-Hydroxybenzyl)adenosine (*o*-topolin riboside)	3.5
N^6-Benzyl-2-[(2-hydroxyethyl)amino]-9-methyladenine (olomoucine)	6.6
N^6-Benzyladenine	0.6
N^6-Benzyladenosine	3.9

the value of the inhibition constant *c* approx., 3-fold in comparison with zeatin bearing a hydroxyl group and 4-fold in comparison with a side chain bearing an aromatic ring. This suggests that the cytokinin may be bound into the active site by the side chain hydroxyl group or aromatic ring. Neither hydroxylation on the aromatic ring nor its location affect the inhibitor potency as seen from the similar K_i values for the topolines. The furfuryl group of artificial cytokinin, kinetin, allows better access into the active site of the enzyme. Substitution in the 2-position of the purine ring (olomoucine)

considerably restrains binding into the active site. Mechanistically, the interaction of the cytokinins studied with amine oxidase resembles the inhibition by some alkaloids that bind to a hydrophobic site close to the topa quinone cofactor and thus prevent substrate binding.[13]

The results from this work suggest that the classification of cytokinin oxidase as a copper containing amine oxidases[5] is incorrect. Isolation of cytokinin oxidase followed by study of its molecular properties and the mechanism by which it degrades the cytokinins is in progress in our laboratory.

Acknowledgements

This work was supported by the grants 204/96/K235 and 204/96/0391 from the Grant Agency and VS96154 from the Ministry of Education, Czech Republic.

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