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Cytokinin Oxidase from Auxin- and Cytokinin-Dependent Callus Cultures of Tobacco (*Nicotiana tabacum* L.)

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Abstract. Cytokinin oxidase was extracted and partially purified from auxin- and cytokinin-dependent callus tissue of tobacco (*Nicotiana tabacum* L. cv. Wisconsin 38). The activity of the enzyme preparation was examined using an assay based on the conversion of tritiated N^6-(Δ^2-isopentenyl)adenine ([2,8-^{3}H]iP) to adenine. Cytokinin oxidase exhibited a temperature optimum at 45–50°C and a relatively high pH optimum (8.5–9.0). The apparent K_m value of the enzyme was 4.3 μM for iP. On the basis of the substrate competition assays, iP was determined to be the preferred substrate of the enzyme. Substrate competition was also observed with zeatin and the cytokinin-active urea derivative Thidiazuron. Cytokinins bearing saturated isoprenoid side chains or cyclic side chain structures, as well as auxins and abscisic acid, had no effect on the conversion of [2,8-^{3}H]iP. The cytokinin oxidase exhibited increased activity in the presence of copper-imidazole complex in the reaction mixture. Under optimal concentrations of copper (15 mM $CuCl_2$) and imidazole (100 mM), the enzyme activity was enhanced ca. 40-fold. Under these conditions the pH optimum was lowered to pH 6.0, whereas the temperature optimum, the apparent K_m value, and the substrate specificity were not altered. Most of the enzyme moiety did not bind to the lectin concanavalin A. The characteristics of cytokinin oxidase presented here suggest that a novel molecular form of the enzyme, previously identified and characterized in *Phaseolus lunatus* callus cultures (Kamínek and Armstrong (1990) Plant Physiol 93:1530), also occurs in cultured tobacco tissue.

Cytokinin oxidase is the key enzyme for cytokinin degradation in plant tissues. It specifically catalyzes the oxidative N^6-side chain cleavage of isoprenoid cytokinins to adenine or adenosine and the corresponding side chain aldehyde (Brownlee et al. 1975, McGaw and Horgan 1983a).

The presence of an enzyme activity catalyzing the conversion of isopentenyladenosine to adenosine was originally demonstrated by Pačes et al. (1971) in crude homogenates from cultured tobacco cells. Subsequently, the cytokinin oxidase activity was detected in tissues from a number of higher plants (Whitty and Hall 1974, McGaw and Horgan 1983b, Chatfield and Armstrong 1986, Laloue and Fox 1989, Kamínek and Armstrong 1990), in cellular slime molds (Armstrong and Firtel 1989) and in mosses (Gerhäuser and Bopp 1990). A careful examination of chromatographic properties and pH optima of cytokinin oxidase presented by several authors (Whitty and Hall 1974, McGaw and Horgan 1983b, Chatfield and Armstrong 1988) indicate the occurrence of the enzyme in several molecular forms. In callus cultures of *Phaseolus vulgaris* and *Phaseolus lunatus*, the presence of two distinct forms of cytokinin oxidase, differing in both their biochemical properties and possibly localization within plant cells, was reported (Kamínek and Armstrong 1990). A protein exhibiting cytokinin oxidase activity has recently been isolated and purified to homogeneity from *Zea mays* kernels (Burch and Horgan 1989), and antisera to the protein have been used to isolate a λgt11 clone carrying a part of the cytokinin oxidase gene (Burch and Horgan 1992).

Abbreviations: Ade, adenine; iP, N^6-(Δ^2-isopentenyl)adenine; [2,8-^{3}H]iP, [2,8-^{3}H]-N^6-(Δ^2-isopentenyl)adenine; [9R]iP, N^6-(Δ^2-isopentenyl)adenosine; (diH)iP, N^6-isopentyladenine; (diH)Z, dihydrozeatin; BAP, N^6-benzyladenine; (*o*OH)[9R]BAP, N^6-(*o*-hydroxybenzyl)adenosine; (*m*OH)[9R]BAP, N^6-(*m*-hydroxybenzyl)adenosine; IAA, indole-3-acetic acid; IBA, indole-3-butyric acid; NAA, naphthalene-1-acetic acid; ABA, abscisic acid; Con A, concanavalin A.

Cytokinin oxidases from tissues of different plant species possess the same substrate specificity. Naturally occurring substrates are iP, zeatin and their ribosides, N-glycosides, and N-alanyl conjugates. Cytokinins bearing saturated N^6-side chains (dihydrozeatin-type cytokinins) or bulky substituents on the side chains (O-glucosides, cytokinins with cyclic side chain structures) are resistant to attack by the enzyme (Whitty and Hall 1974, McGaw and Horgan 1983b, Chatfield and Armstrong 1986, Kamínek and Armstrong 1990).

The in vitro activity of cell-free cytokinin oxidase preparations is strongly inhibited by cytokinin-active urea derivatives (Chatfield and Armstrong 1986, Laloue and Fox 1989, Burch and Horgan 1989, Kamínek and Armstrong 1990, Abdelnour-Esquivel et al. 1992). The supply of exogenous cytokinins to cultured tissues of *Phaseolus* sp. (Chatfield and Armstrong 1986, Kamínek and Armstrong 1990) and tobacco (Motyka and Kamínek 1990) induces a relatively rapid increase in the levels of the enzyme activity in vivo. The increase in the activity is sensitive to antibiotic inhibitors of transcription and translation (Chatfield and Armstrong 1986) and is induced by both substrate and nonsubstrate cytokinin-active compounds.

The mechanism of cytokinin oxidase action is still unclear, but an iminopurine intermediate which requires molecular oxygen for degradation, has been identified (Laloue and Fox 1985). The oxygen requirement is reduced or eliminated if copper–imidazole complexes are included in the reaction mixture (Chatfield and Armstrong 1987).

The metabolism of cytokinins in tobacco tissues has been extensively investigated (for review see Letham and Palni 1983). Although cytokinin oxidase activity was first detected in cultured tobacco cells (Pačes et al. 1971), the enzyme preparation has not been further characterized in this material. The characterization of cytokinin oxidase activity in auxin- and cytokinin-dependent tobacco callus cultures is presented in this report.

Materials and Methods

Chemicals

Polymin P (polyethyleneimine, 50% w/v) was obtained from Miles Laboratories and PVP (polyvinylpyrrolidone) was purchased from Serva. Con A-Sepharose 4B was obtained from Sigma and methylmannose (methyl-α-D-mannopyranoside) from Fluka. Microcrystalline cellulose TLC plates were products of Kavalier Glass Works (Votice, Czech Rep.).

The cytokinin [2,8-^{3}H]iP used to assay the cytokinin oxidase activity was synthesized as described by Chatfield and Armstrong (1987). The synthesis of cytokinins (*o*OH)[9R]BAP and (*m*OH)[9R]BAP has been described elsewhere (Kamínek et al. 1987). Thidiazuron (N-phenyl-N′-1,2,3-thidiazol-5-ylurea, DROPP) and (diH)iP were kind gifts of D. J. Armstrong from Oregon State University. Other cytokinins, auxins, and abscisic acid were obtained from Sigma.

Plant Material

Auxin- and cytokinin-dependent callus cultures of tobacco (*Nicotiana tabacum* L. cv. Wisconsin 38) were derived from stem pith tissues and subcultured at 4- to 5-week intervals. Callus tissues were cultivated on Murashige and Skoog solid medium (Murashige and Skoog 1962) containing sucrose (30 g/L), *myo*-inositol (100 mg/L), thiamine (0.4 mg/L), nicotinic acid (0.25 mg/L), pyridoxine (0.25 mg/L), NAA (1 mg/L), and BAP (0.285 mg/L). The callus cultures were grown in 100-ml Erlenmeyer flasks containing 50 ml medium in the dark at 26°C for 26 days and then used for the enzyme extraction.

Extraction of Cytokinin Oxidase Activity

The enzyme preparations were extracted and partially purified by a modification of the method of Chatfield and Armstrong (1986). All operations were performed at 4°C. The callus tissues were homogenized with an equal volume of cold extraction buffer (0.1 M Tris-HCl, pH 7.5). An aliquot of the homogenate equivalent to 5 g tissue was mixed with 1.5 g (dry weight) of acid-treated polyvinylpyrrolidone hydrated with 0.05 M Tris-HCl (pH 7.5). The resulting suspension was filtered through a glass filter and washed with two 5-ml aliquots of the same buffer. Combined rinses were clarified by centrifugation (10,000 *g;* 10 min). Polymin P (1% v/v, pH 7.5) was added dropwise with stirring to the supernatant (40 μl/ml supernatant), and the precipitated nucleic acids and associated proteins were removed after 10 min by centrifugation (10,000 *g;* 10 min). Solid ammonium sulphate was added to the supernatant to 80% saturation. After 30 min, the resulting precipitate was collected by centrifugation (13,000 *g;* 40 min). The pellets were frozen under liquid N_2 and stored in sealed centrifugation tubes at −15°C for no longer than 3 weeks.

Protein concentration was determined according to the method of Bradford (1976) with bovine serum albumin (BSA) as a standard.

Assay of Cytokinin Oxidase Activity

Cytokinin oxidase activity was determined by in vitro assays based on the conversion of [2,8-^{3}H]iP to adenine. The assay mixture (50 μl, final volume) contained H_3BO_3-KCl-NaOH buffer (100 mM KCl in 100 mM H_3BO_3, adjusted by NaOH to pH 8.5), 10 μM substrate ([2,8-^{3}H]iP, 42.8 μCi/μmol), and an enzyme preparation equivalent to 200 mg tissue fresh weight. After 4 h of incubation at 37°C, the reaction was terminated by the addition of 10 μl Na_4 EDTA (200 mM) and 120 μl of cold 95% (v/v) ethanol containing unlabeled iP and adenine (0.75 mM each).

Separation of the intact substrate and labeled product of the enzyme reaction was performed by thin-layer chromatography on microcrystalline cellulose plates developed with the upper phase of a mixture containing ethyl acetate/*n*-propanol/water (4:1:2, v/v). Zones containing iP (R_f 0.8–0.9) and adenine (R_f 0.2–0.3) were located under ultraviolet (UV) light and cut into

strips. Radioactivity was counted by liquid scintillation technique using a Packard TRI-CARB 300c scintillation counter.

The assay mixture for the copper–imidazole-enhanced reaction contained 100 mM imidazole buffer (pH 6.0), 25 mM sodium acetate, 15 mM $CuCl_2$, 10 μM [2,8-^{3}H]iP (42.8 μCi/μmol), and the enzyme (equivalent to 50 mg tissue fresh weight) in a total volume of 50 μl. The assays were incubated for 30 min at 37°C. The reactions were terminated, and the chromatography was performed in the same manner as that in the standard cytokinin oxidase assay procedure.

Determinations of Substrate Specificity and K_m

The substrate specificity was determined by testing the effects of unlabeled cytokinins on the in vitro degradation of [2,8-^{3}H]iP in the standard cytokinin oxidase assay. Unlabeled cytokinins were added to the 50-μl assay volumes at concentrations equal to that of the labeled substrate (10 μM) and in tenfold excess (100 μM). The effects of auxins and abscisic acid on the in vitro degradation of [^{3}H]iP by cytokinin oxidase were tested in the same way.

The apparent K_m value for iP was determined using the standard cytokinin oxidase assay procedure. The concentration range of [2,8-^{3}H]iP in the assay was 1.25–25 μM.

Chromatography on Con A–Sepharose 4B Column

Con A–Sepharose 4B chromatography was performed by a modification of methods described elsewhere (Chatfield and Armstrong 1988, Kamínek and Armstrong 1990). Con A–Sepharose 4B was packed into 0.75-cm diameter chromatography columns to give 3-ml bed volumes and washed with 30 ml of 25 mM bisTris-HCl (pH 6.5) containing $(NH_4)_2SO_4$ (200 mM), $CaCl_2$ (1 mM) and $MnCl_2$ (1 mM). The enzyme preparation equivalent to 15 g tissue fresh weight was dissolved in 3 ml of 25 mM bisTris-HCl (pH 6.5) and loaded onto the column. The column was washed with 24 ml of 25 mM bisTris-HCl (pH 6.5) containing $(NH_4)_2SO_4$ (200 mM) and eluted with the same solution containing methylmannose (200 mM). Fractions of 3 ml were collected and assayed for cytokinin oxidase activity using the copper–imidazole-enhanced assay described above. The absorbance of the fractions was measured at 280 nm.

Results

Reaction Characteristics

The effects of incubation time and enzyme concentration on iP degradation are shown in Fig. 1. From these results, an incubation of 4 h with 80 μg protein preparation (i.e., equivalent to 200 mg of tissue fresh weight) per assay were chosen to be used in the standard assay procedure. Under these conditions, 20–25% iP was degraded.

A single radioactive product of the [2,8-^{3}H]iP degradation by the tobacco callus enzyme was detected by thin-layer chromatography (TLC). It co-chromatographed with authentic adenine on both cellulose plates developed with the upper phase of a

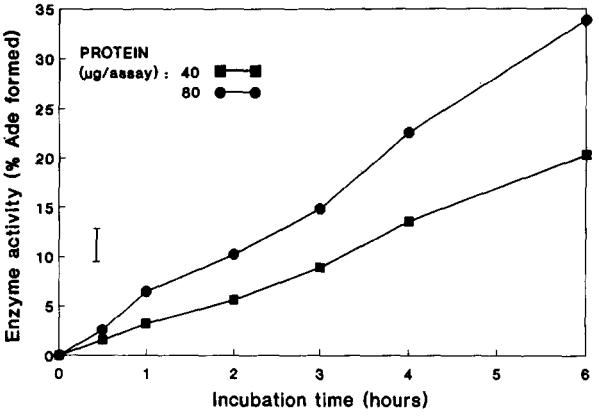

Fig. 1. The effect of incubation time and enzyme concentration on the in vitro activity of cytokinin oxidase from tobacco callus tissue. The cytokinin oxidase assays were performed as described in Materials and Methods except for the indicated modifications in incubation time. The protein concentrations were 40 μg, that is, equivalent to 100 mg tissue fresh weight (■—■) or 80 μg, that is, equivalent to 200 mg tissue fresh weight (●—●) per assay. Vertical bar represents L.S.D. (three replicates).

mixture containing ethyl acetate/*n*-propanol/water (4:1:2, v/v) and silica gel plates using chloroform/methanol (3:1, v/v) as the solvent (data not shown).

The degradation of iP by the tobacco callus enzyme was inhibited considerably when cytokinin oxidase activity was assayed in the presence of the antioxidants dithiothreitol and 2-mercaptoethanol in the reaction mixture. Both reducing agents lowered the enzyme activity by up to 90% (at concentrations 20 mM and 200 mM for dithiothreitol and 2-mercaptoethanol, respectively); complete inhibition was not achieved (Fig. 2).

Characterization of Cytokinin Oxidase Activity

The optimum temperature for cytokinin oxidase activity from tobacco callus tissue was 45–50°C using the standard assay conditions with temperatures between 10° and 70°C [Fig. 3(a)].

The effect of pH was examined in four different buffers at 12 pH values over the range 4.5–10.0. The enzyme preparation exhibited a pH optimum between 8.5 and 9.0 when assayed in 100 mM H_3BO_3-KCl-NaOH buffer (Fig. 4a). The maximum cytokinin oxidase activity was also found at pH 8.5–9.0 with 100 mM TAPS-NaOH buffer in the reaction mixture (data not shown).

A kinetic study of the enzyme was carried out at pH 8.5 and 37°C. Under these conditions, the apparent K_m value for iP was 4.3 μM (Fig. 5a).

Substrate specificity was investigated by testing

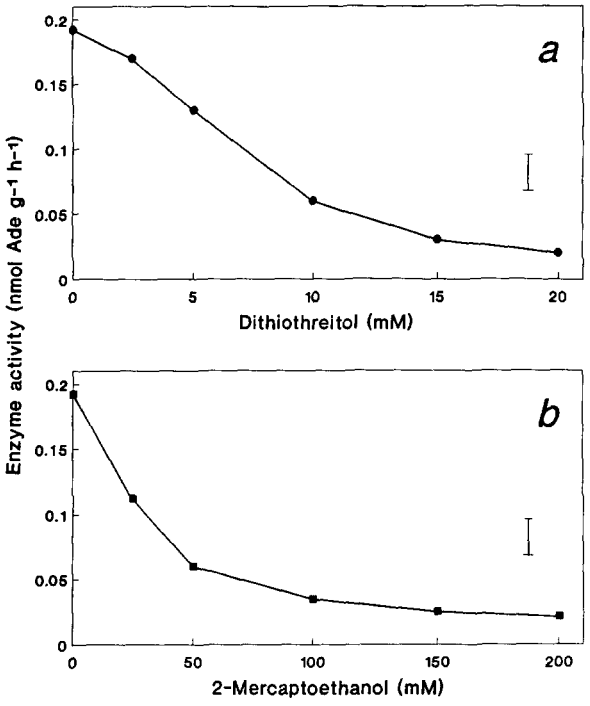

Fig. 2. The effect of antioxidants (**a**) dithiothreitol and (**b**) 2-mercaptoethanol on the in vitro activity of cytokinin oxidase from tobacco callus tissue. The cytokinin oxidase assays were performed in the presence of indicated concentrations of antioxidants in 100 mM H_3BO_3-KCl-NaOH buffer (pH 8.5); other details are described in Materials and Methods. Vertical bars represent L.S.D. (three replicates).

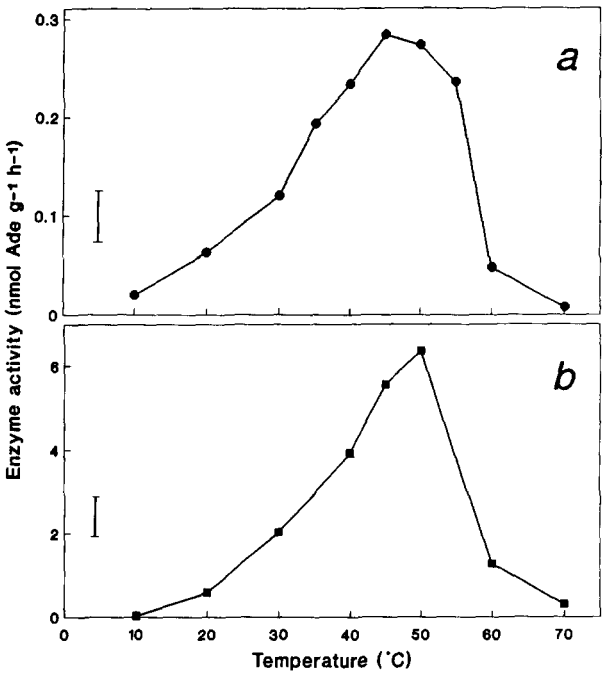

Fig. 3. The effect of temperature on the in vitro activity of cytokinin oxidase from tobacco callus tissue. The cytokinin oxidase assays were performed (**a**) in 100 mM H_3BO_3-KCl-NaOH buffer, pH 8.5 and (**b**) in 100 mM imidazole buffer containing sodium acetate (25 mM) and $CuCl_2$ (15 mM), pH 6.0; other details are described in Materials and Methods. Vertical bars represent L.S.D. (three replicates).

the effects of unlabeled cytokinins (10 μM, 100 μM) on the *in vitro* degradation of [2,8-^{3}H]iP in the standard cytokinin oxidase assay. The results are summarized in Table 1. As expected, unlabeled iP was a powerful inhibitor of the degradation of the labeled substrate. Zeatin only weakly inhibited the degradation, when applied at a concentration of 100 μM, that is, tenfold higher than that of a substrate cytokinin. Both *cis*- and *trans*-isomers of zeatin displayed the same effectiveness in the inhibition.

Cytokinins bearing saturated isoprenoid side chains {(diH)Z, (diH)iP}, and aromatic structures in side chains {BAP, (*o*OH)[9R]BAP, (*m*OH)[9R]BAP} did not inhibit the conversion of [2,8-^{3}H]iP to adenine. In accordance with the results of substrate competition experiments, cytokinins bearing aromatic side chain structure BAP and (*m*OH)[9R]BAP tritiated on the purine ring were not degraded to adenine by the cytokinin oxidase activity from tobacco calli (data not shown).

As with iP, the cytokinin-active urea derivative thidiazuron strongly inhibited the degradation of [2,8-^{3}H]iP in the standard cytokinin oxidase assay (Table 1). Auxins (IAA, IBA, NAA) and abscisic acid did not inhibit the conversion of iP to adenine (Table 2).

Effects of Copper–Imidazole Complex on Cytokinin Oxidase Activity

The cell-free preparations of cytokinin oxidase from tobacco calli exhibited increased activity in the presence of copper–imidazole complex. The effects of copper–imidazole were assayed in 100 mM imidazole buffer containing 25 mM sodium acetate (for stabilizing pH) and $CuCl_2$. The optimal $CuCl_2$ concentration was 15 mM (Fig. 6), and the corresponding pH optimum 6.0 (Fig. 4b). Under these conditions the activity of cytokinin oxidase was enhanced ca. 40-fold. The temperature optimum of the tobacco enzyme preparation (45–50°C), as well as the apparent K_m value (4.5 μM for iP), were not significantly altered by the presence of copper–imidazole complex in the reaction mixture (Figs. 3b, 5b).

The stimulatory effect of copper on the enzyme activity was observed only with imidazole as the assay buffer. The structurally similar amino acid

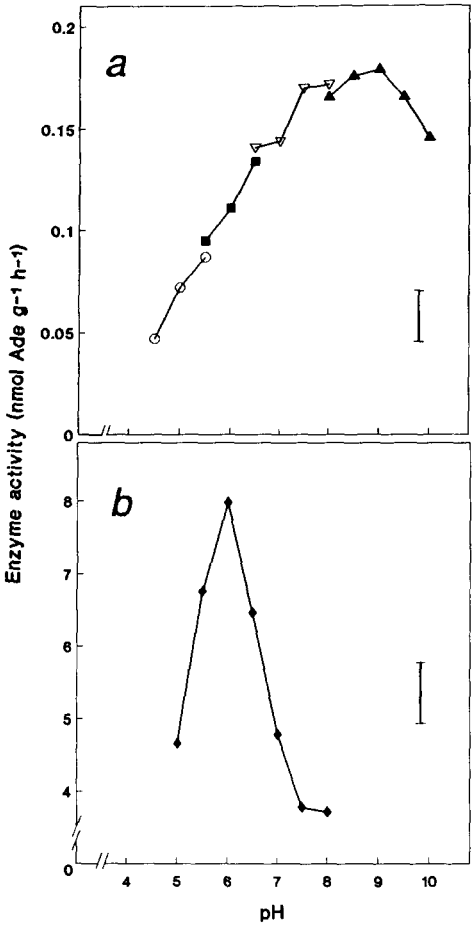

Fig. 4. The effect of pH on the in vitro activity of cytokinin oxidase from tobacco callus tissue. The effect of pH was investigated (**a**) in 100 mM CH_3COOH-NaOH (○—○), MES-NaOH (■—■), Hepes-NaOH (▽—▽), and H_3BO_3-KCl-NaOH (▲—▲) buffers and (**b**) in 100 mM imidazole buffer containing 25 mM sodium acetate and 15 mM $CuCl_2$ (◆—◆); other details are described in Materials and Methods. Vertical bars represent L.S.D. (four replicates).

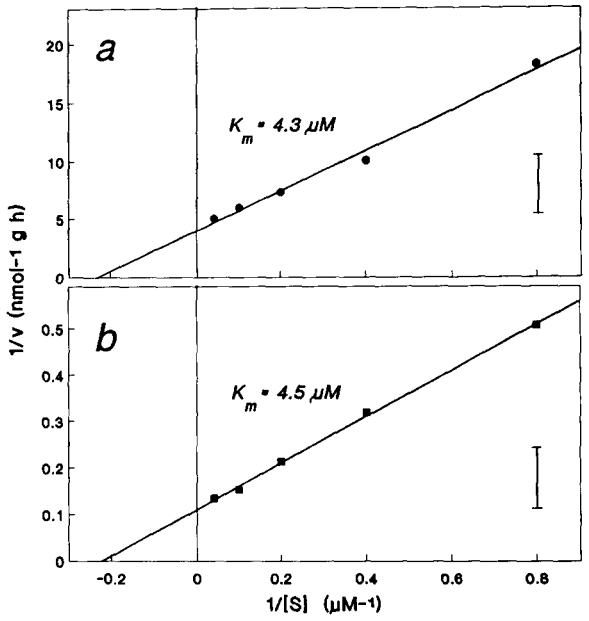

Fig. 5. Double reciprocal plot of cytokinin oxidase activity as a function of iP concentration. The cytokinin oxidase assays were performed (**a**) in 100 mM H_3BO_3-KCl-NaOH buffer, pH 8.5 and (**b**) in 100 mM imidazole buffer containing sodium acetate (25 mM) and $CuCl_2$ (15 mM), pH 6.0; other details are described in Materials and Methods. Vertical bars represent L.S.D. (three replicates).

histidine did not substitute for imidazole in stimulating the activity of the enzyme (data not shown).

The enhanced degradation of iP observed in the presence of copper–imidazole complex was inhibited by the chelating agent Na_2EDTA. The addition of 20 mM Na_2EDTA to the reaction mixture containing copper and imidazole reduced the activity of cytokinin oxidase to that of control without copper (Fig. 7).

As determined by substrate competition assays, the substrate specificity of cytokinin oxidase in copper–imidazole complex did not differ from that found in the absence of copper and imidazole. The inhibition of [2,8-^{3}H]iP degradation was observed by the naturally occurring cytokinins iP and zeatin (both *cis* and *trans* isomers) as well as the cytokinin-active urea derivative thidiazuron. Cytokinins bearing saturated isoprenoid side chains {(diH)Z, (diH)iP}, or cyclic side chain structures {BAP, (*o*OH)[9R]BAP, (*m*OH)[9R]BAP} did not exhibit inhibitory interaction with the labeled substrate (Table 1).

Concanavalin A Affinity of Cytokinin Oxidase

The affinity of cytokinin oxidase from tobacco callus tissue for Con A-Sepharose 4B was determined (Fig. 8). Most of the cytokinin oxidase activity (90% of the total) did not bind to the lectin Con A. The rest of the enzyme activity (10% of the total) was retained on the column and eluted only after the addition of 200 mM methylmannose to the eluting buffer. Calibration with egg albumin showed that the capacity of the column was sufficient to bind all tobacco protein applied.

Discussion

Metabolism of cytokinins in tobacco tissues has been extensively examined (for review, see Letham and Palni 1983). Degradation resulting from the re-

Table 1. The effects of unlabeled cytokinins on the in vitro degradation of [2,8-^{3}H]iP by cytokinin oxidase from tobacco callus tissue.

Unlabeled cytokinin	Cytokinin oxidase activity (% of control)[a,b]			
	H_3BO_3-KCl-NaOH buffer		Cu^{2+}-imidazole	
	Concentration of unlabeled cytokinin			
	10 μM	100 μM	10 μM	100 μM
iP	45	11	49	10
trans-zeatin	94	79	97	78
cis-zeatin	95	75	94	61
(diH)Z	109	105	105	103
(diH)iP	103	89	103	95
BAP	98	89	99	98
(*o*OH) [9R]BAP	107	102	109	103
(*m*OH) [9R]BAP	109	98	109	100
thidiazuron	40	23	25	17

Note. Unlabeled cytokinins were added to standard assay mixtures containing 10 μM [2,8-^{3}H]iP and 100 mM H_3BO_3-KCl-NaOH buffer (pH 8.5) or 100 mM imidazole buffer with sodium acetate (25 mM) and $CuCl_2$ (15 mM), pH 6.0; other details are described in Materials and Methods.

[a] The cytokinin oxidase activity of control incubated without unlabeled cytokinins in H_3BO_3-KCl-NaOH buffer was 0.16 nmol $g^{-1} h^{-1}$ (= 100%). Standard deviation (four replicates) averaged 5% of the means and did not exceed 11% of the means.

[b] The cytokinin oxidase activity of control incubated without unlabeled cytokinins in Cu^{2+}-imidazole complex was 6.55 nmol $g^{-1} h^{-1}$ (= 100%). Standard deviation (four replicates) averaged 4% of the means and did not exceed 9% of the means.

Table 2. The effects of auxins and abscisic acid on the in vitro degradation of [2,8-^{3}H]iP by cytokinin oxidase from tobacco callus tissue

Unlabeled compound	Cytokinin oxidase activity (% of control)[a]	
	Concentration of unlabeled compound	
	10 μM	100 μM
IAA	96	98
IBA	94	97
NAA	98	95
ABA	97	97

Note. Auxins or abscisic acid were added to standard assay mixtures containing 10 μM [2,8-^{3}H]iP and 100 mM H_3BO_3-KCl-NaOH buffer, pH 8.5; other details are described in Materials and Methods.

[a] The cytokinin oxidase activity of control incubated without auxins and abscisic acid was 0.16 nmol $g^{-1} h^{-1}$ (= 100%). Standard deviation (three replicates) averaged 4% of the means and did not exceed 10% of the means.

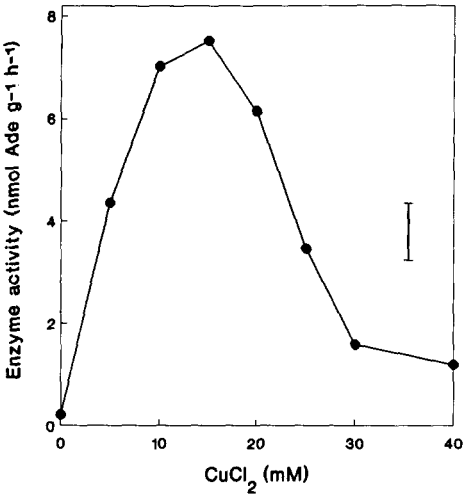

Fig. 6. The effect of $CuCl_2$ concentration on the in vitro activity of cytokinin oxidase from tobacco callus tissue. The cytokinin oxidase assays were performed at pH 6.0 in 100 mM imidazole buffer containing sodium acetate (25 mM) and $CuCl_2$ at the indicated concentrations; other details are described in Materials and Methods. Vertical bar represents LSD (four replicates).

moval of the isopentenyl side chain giving adenylic products was found to be a common metabolic pathway of externally applied isoprenoid cytokinins in cell suspensions of tobacco (Laloue et al. 1977, Terrine and Laloue 1980). Although the presence of an enzyme activity catalyzing the N^6-side chain cleavage of cytokinins was originally detected in cultured tobacco cells (Pačes et al. 1971), the characterization of the degradative enzyme(s) in this material has not been given until this report.

The cytokinin oxidase from auxin- and cytokinin-dependent tobacco callus cultures exhibits a high temperature optimum (45–50°C) with short-term incubation, indicating a thermal stability of the enzyme. The pH optimum (pH 8.5–9.0) is also surprisingly high and differs from that established for cytokinin oxidases in most plant tissues (Whitty and Hall 1974, McGaw and Horgan 1983b, Chatfield and Armstrong 1986, Laloue and Fox 1989). The similarly high-pH optimum (pH 8.4) has been reported so far only for the cytokinin oxidase in *Phaseolus lunatus* callus cultures (Kamínek and Armstrong 1990). A minor peak of cytokinin oxidase activity at pH 8.5 in addition to the major peak at pH 6–7 was also found for *Zea mays* kernel enzyme (Whitty and Hall 1974), suggesting the existence of two molecular forms of the oxidase in this tissue.

The apparent K_m value of tobacco enzyme (4–5 μM for iP) is one order of magnitude lower than those reported for cytokinin oxidases from corn kernels and *Vinca rosea* crown galls (McGaw and Horgan 1983b). On the other hand, the K_m value reported here is more than 10 times higher than that

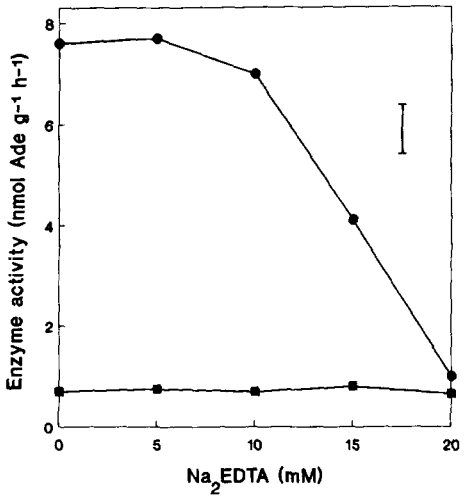

Fig. 7. The effect of Na_2EDTA on the in vitro activity of cytokinin oxidase from tobacco callus tissue in the presence (●—●) and absence (■—■) of Cu^{2+}-imidazole complex in the reaction mixture. The cytokinin oxidase assays were performed in 100 mM imidazole buffer containing sodium acetate (25 mM) ±15 mM $CuCl_2$ (pH 6.0); other details are described in Materials and Methods. Vertical bar represents L.S.D. (three replicates).

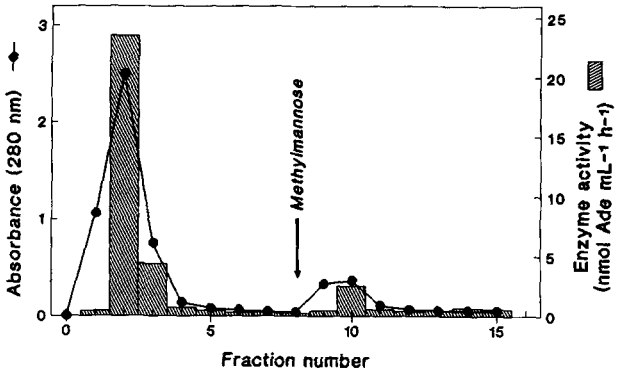

Fig. 8. Concanavalin A-Sepharose 4B chromatography of cytokinin oxidase activity from tobacco callus tissue. The protein preparation (obtained as described in Materials and Methods) equivalent to 15 g of callus tissue fresh weight was dissolved in 3 ml of 25 mM bisTris-HCl (pH 6.5). The sample was applied to Con A—Sepharose 4B column (0.75 × 7 cm, 3 ml bed volume) equilibrated with 25 mM bisTris-HCl (pH 6.5) containing $(NH_4)_2SO_4$ (200 mM), $CaCl_2$ (1 mM), and $MnCl_2$ (1 mM). The column was washed with 24 ml (eight bed volumes) of 25 mM bisTris-HCl (pH 6.5) containing $(NH_4)_2SO_4$ (200 mM) and eluted with the same solution containing methylmannose (200 mM). Fractions of 3 ml were collected and assayed for cytokinin oxidase activity using the copper–imidazole-enhanced assay described in Materials and Methods.

of the wheat germ enzyme determined for [9R]iP (Laloue and Fox 1989). The present kinetic constants may be affected by the use of partially purified enzyme and specific assay conditions (a 4-h incubation and 20–25% substrate conversion).

Details of the reaction mechanism and the nature of the prosthetic group for cytokinin oxidase have yet to be established. However, it has been shown that an imine intermediate is formed during the reaction catalyzed by the enzyme (Laloue and Fox 1985); thus, cytokinin oxidase seems to be a copper-dependent amine oxidase (Burch and Horgan 1989). As described previously, a molecular oxygen is required for the activity of the enzyme (Whitty and Hall 1974, Chatfield and Armstrong 1987, Laloue and Fox 1989). A strong inhibition of cytokinin oxidase activity in the presence of antioxidants dithiothreitol and 2-mercaptoethanol, reported here, confirms an oxidative mechanism for cytokinin degradation.

The substrate specificity of cytokinin oxidase from tobacco callus tissues does not significantly differ from that observed in previous studies with cytokinin oxidase preparations from other plant sources (Whitty and Hall 1974, McGaw and Horgan 1983b, Chatfield and Armstrong 1986, Kamínek and Armstrong 1990). The preferred substrate of the tobacco callus enzyme is iP. Zeatin also appears to be a substrate of cytokinin oxidase from tobacco tissue. However, in accordance with the literature (Whitty and Hall 1974, Chatfield and Armstrong 1986, Kamínek and Armstrong 1990), its inhibitory effect in substrate competition assays is much weaker than that of iP, indicating that cytokinin content in plant cells is regulated by cytokinin oxidase at the iP pool level. *Cis*-zeatin is as effective a substrate for the tobacco enzyme as is the *trans* isomer. In the previous study of Pačes and Kamínek (1976), the *trans* isomer of ribosylzeatin was reported to be more effective than the *cis* isomer in the inhibition of the degradation of labeled [9R]iP by cytokinin oxidase from a cytokinin-autonomous strain of tobacco callus. This difference may be caused by the examination of tissues differing in cytokinin requirements and the use of different labeled substrates and tested cytokinins (bases versus ribosides).

In agreement with previously published data (Whitty and Hall 1974, McGaw and Horgan 1983b, Chatfield and Armstrong 1986, Kamínek and Armstrong 1990), cytokinins bearing saturated isoprenoid side chains {(diH)Z and (diH)iP} and cyclic side chain structures {BAP, BAP derivatives, and kinetin} are resistant to attack by the oxidase from tobacco callus. The reported degradation of kinetin by the enzyme preparation from moss protonema (Gerhäuser and Bopp 1990) and a slight conversion of BAP to adenine by the oxidase from wheat germ (Laloue and Fox 1989) demonstrate the possibility

that some plant cells may contain cytokinin oxidases that degrade a wider range of cytokinins. A somewhat broader substrate specificity than that of higher plant enzymes has been reported for cytokinin oxidase from the cellular slime mold, *Dictyostelium discoideum* (Armstrong and Firtel 1989).

The cytokinin-active urea derivative thidiazuron is a highly effective inhibitor of iP degradation by the tobacco callus enzyme. A strong inhibitory effect of urea derivatives on cytokinin degradation reported for cytokinin oxidases from different plant tissues (Chatfield and Armstrong 1986, Laloue and Fox 1989, Kamínek and Armstrong 1990, Abdelnour-Esquivel et al. 1992) may indirectly explain the high cytokinin-like activity of these compounds by the inhibition of endogenous cytokinin breakdown. This is also indicated by a reported accumulation of natural purine-type cytokinins in soybean callus cultures grown on media containing thidiazuron (Thomas and Katterman 1986). The mechanism of action of urea derivatives on cytokinin degradation is still unclear, but kinetic data obtained by Burch and Horgan (1989) for cytokinin oxidase from *Zea mays* kernels suggest that N-(2-chloro-4-pyridyl)-N′-phenylurea acts as a noncompetitive inhibitor of the enzyme.

Auxins and abscisic acid do not significantly affect the activity of cytokinin oxidase from the tobacco callus tissue. Although NAA and IBA have been reported to exert a stimulatory influence upon iP degradation by the corn kernel enzyme (Palni et al. 1988), this stimulatory effect was not found in the case of tobacco callus enzyme assayed *in vitro*.

A significant enhancement of cytokinin oxidase activity *in vitro* in reaction mixtures containing copper and imidazole, as reported for *Phaseolus* callus enzymes (Chatfield and Armstrong 1987, Kamínek and Armstrong 1990), was also observed in enzyme preparations from auxin- and cytokinin-dependent tobacco callus tissue. The optimal $CuCl_2$ concentration for the increase of tobacco enzyme activity (15 mM) and the corresponding pH optimum (pH 6.0) are the same as that found for the oxidase in *Phaseolus lunatus* calli (Kamínek and Armstrong 1990). The substrate specificity of the copper-imidazole enhanced reaction is identical to that observed in the absence of copper and imidazole.

The principle behind the enhancement of cytokinin oxidase activity by copper and imidazole is not clear. The absence of inhibition of the enzyme activity in vitro by the chelating agent Na_2EDTA in the imidazole buffer without copper suggests that the copper is not present in the active site of the enzyme molecule. It has been reported that the copper–imidazole complex probably substitutes for oxygen in the reaction mechanism of the enzyme (Chatfield and Armstrong 1987). A described interaction of copper with cytokinin bases in the regulation of horseradish peroxidase activity (Miller 1985) and a superoxide dismutase mimetic effect of cytokinin-Cu^{2+} complexes (Inoue and Hirobe 1986) support a possible role for cytokinins and copper in oxidative processes. An increase in the activity of cytokinin oxidase cell-free preparations from broad bean and soybean callus cultures, corn kernels, and wheat and oat leaves in the presence of cupric ions and imidazole (V. Motyka and M. Kamínek, unpublished results) suggests that the stimulatory effect of copper–imidazole complexes on enzyme activity is not species specific and is not limited to dicotyledonous plants.

Cupric ions applied to the cultivation media of tobacco callus cultures in the form of chloride or nitrate enhanced *in vivo* activity of cytokinin oxidase in cultured tissues (V. Motyka, unpublished results). The increase of cytokinin oxidase activity is proportional to the concentration of cupric salts in the medium and associated with a decrease in the level of the principal substrate cytokinin iP in the tissue. This knowledge may be of great practical importance with respect to varying copper contents in different commercial agar preparations used in plant biotechnology.

Most of the cytokinin oxidase activity from auxin- and cytokinin-dependent tobacco callus does not bind to the lectin Con A, which suggests the occurrence of an unglycosylated form of the enzyme in this tissue. A similar distribution of cytokinin oxidase activity in elution profiles from Con A-Sepharose 4B columns has been found for *Phaseolus lunatus* callus enzyme (Kamínek and Armstrong 1990). The cytokinin oxidase activity from *Phaseolus vulgaris* callus (Chatfield and Armstrong 1988, Kamínek and Armstrong 1990), from immature corn kernels and wheat germ (Burch and Horgan 1989) have been reported to bind to the lectin. A relatively strong affinity for Con A was also observed for the cytokinin oxidase from cytokinin-autonomous cell suspensions of tobacco cv. Virginia Bright Italia (preliminary results). These data indicate that plant tissues contain at least two molecular forms of cytokinin oxidase, differing in glycosylation of the protein molecule and probably localization within plant cell. Both molecular forms may occur in the tissue simultaneously as two distinct (major and minor) isozymes. Protein glycosylation probably affects the enzyme properties, such as enzyme activity in vivo, pH optima, sensitivity to copper–imidazole complex, affinity for Con A, and its localization in plant cells.

These results indicate that the molecular form of the enzyme previously identified and characterized

in *Phaseolus lunatus* callus cultures (Kamínek and Armstrong 1990) also occurs in auxin- and cytokinin-dependent cultured tobacco tissue. Variations in glycosylation and pH optima for the two cytokinin oxidase isozymes suggest that a metabolic inactivation of cytokinins may be influenced differently depending on the compartmentation of the particular form of the enzyme, and thus on the availability of cytokinin substrates. It makes the problem of cytokinin degradation in plant cells more complex, but also more interesting.

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