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Genotypic Variation in Cytokinin Oxidase from *Phaseolus* Callus Cultures[1]

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ABSTRACT

Genotypic variation in cytokinin oxidase has been detected in enzyme preparations from *Phaseolus vulgaris* L. cv Great Northern and *Phaseolus lunatus* L. cv Kingston callus cultures. Although cytokinin oxidase preparations from Great Northern and Kingston callus tissues appear to have very similar substrate specificities, the cytokinin oxidase activities from the two callus tissues were found to differ in a number of other properties. The cytokinin oxidase from *P. vulgaris* cv Great Northern callus tissue exhibited a pH optimum of 6.5 (bisTris) and had a strong affinity for the lectin concanavalin A. The cytokinin oxidase from *P. lunatus* cv Kingston callus tissue exhibited a pH optimum of 8.4 (Taps) and did not bind to concanavalin A. The two enzymes also differed in position of elution when chromatographed on DEAE-cellulose. Both cytokinin oxidase activities exhibited enhanced activity and lower pH optima in the presence of copper-imidazole complexes, but the optimum copper-imidazole ratio and the magnitude of enhancement differed for the two activities. In both callus tissues, transient increases in the supply of exogenous cytokinins induced increases in cytokinin oxidase activity. The differences in pH optima and in glycosylation (as evidenced by the observed difference in lectin affinity) of the cytokinin oxidases from Great Northern and Kingston callus tissues suggest that the compartmentation of cytokinin oxidase may differ in the two callus tissues. The possibility that enzyme compartmentation and isozyme variation in cytokinin oxidase may play a role in the regulation of cytokinin degradation in plant tissues is discussed in relation to known differences in the rates of cytokinin degradation in Great Northern and Kingston callus tissues.

The regulatory networks that control cytokinin levels in plant tissues include mechanisms for regulating cytokinin degradation. Cytokinin oxidase activities that specifically catalyze the degradation of cytokinins bearing unsaturated isoprenoid sidechains have been isolated from a number of plant tissues (5, 11, 15, 22, 28), and the enzyme from *Zea mays* kernels has been purified to homogeneity (4). The naturally occurring substrates of cytokinin oxidase are i^{6}Ade[3], io^{6}Ade, and their ribonucleosides (i^{6}Ado and io^{6}Ado). The products of the reaction catalyzed by the enzyme are Ade or Ado and an aldehyde derived from the N^6-sidechain of the cytokinin substrate (3, 14). Molecular oxygen is required (6, 11, 28), and an iminopurine intermediate postulated by Whitty and Hall (28) has been isolated from reaction mixtures containing 2-mercaptoethanol (10). Cytokinins bearing saturated sidechains (hi^{6}Ade and hio^{6}Ade) are resistant to attack by the cytokinin oxidase preparations isolated to date from higher plant sources, as are the cytokinins f^{6}Ade and b^{6}Ade (5, 11, 15, 21, 22, 28).

The regulation of cytokinin degradation appears to be an important feature of the control of cytokinin levels in *Phaseolus* callus cultures (5, 17, 19). In these cultures, rates of cytokinin degradation are conditioned by the genotype of the callus. Thus, callus tissues derived from *P. vulgaris* cv Great Northern and *P. lunatus* cv Kingston differ in their ability to degrade exogenous cytokinins bearing unsaturated isoprenoid sidechains (17, 19). Great Northern callus tissues rapidly degrade exogenously supplied i^{6}Ado to Ado (19) and require high levels of exogenous i^{6}Ade or io^{6}Ade to support growth (17). Kingston callus tissues respond to low levels of either i^{6}Ade or io^{6}Ade, and the major product of the short-term metabolism of i^{6}Ado by tissues of this genotype is the nucleotide i^{6}AMP rather than Ado (17, 19).

The cytokinin oxidase from *P. vulgaris* cv Great Northern callus tissues has been investigated by Chatfield and Armstrong (5–7). The substrate specificity of the Great Northern enzyme is similar to that reported for cytokinin oxidase activities from other plant sources (5). Cell-free preparations of the enzyme exhibit greatly enhanced activity in the presence of copper-imidazole complexes (6). Under these conditions, substrate specificity is unaltered, but the oxygen requirement of the cytokinin oxidase reaction is reduced or eliminated (6). Transient increases in the supply of exogenous cytokinins

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[3] Abbreviations: i^{6}Ade, N^6-(Δ^2-isopentenyl)adenine; Ade, adenine; Ado, adenosine; b^{6}Ade, N^6-benzyladenine; f^{6}Ade, kinetin, N^6-furfuryladenine; hi^{6}Ade, N^6-isopentyladenine; hio^{6}Ade, dihydrozeatin, N^6-(4-hydroxy-3-methylbutyl)adenine; i^{6}Ade-2,8-^{3}H, N^6-(Δ^2-isopentenyl)adenine-2,8-[^{3}H]; i^{6}Ado, N^6-(Δ^2-isopentenyl)adenosine; i^{6}AMP, N^6-(Δ^2-isopentenyl)adenosine-5′-monophosphate; io^{6}Ade, zeatin, N^6-(4-hydroxy-3-methyl-2-butenyl)adenine; io^{6}Ado, zeatin riboside, N^6-(4-hydroxy-3-methyl-2-butenyl)adenosine; PVPP, polyvinylpolypyrrolidone; thidiazuron, N-phenyl-N′-1,2,3-thidiazol-5-ylurea; Con A, concanavalin A.

induce relatively rapid increases in *in vivo* levels of cytokinin oxidase activity in Great Northern callus tissue (5). The increase in activity is sensitive to inhibitors of RNA and protein synthesis and is induced by all cytokinin-active compounds tested, including cytokinins that are inactive as substrates for cytokinin oxidase. The Great Northern enzyme appears to be a glycoprotein, as evidenced by its affinity for the lectin Con A (7).

The cytokinin oxidase activities from *P. vulgaris* cv Great Northern and *P. lunatus* cv Kingston callus tissues have been compared in the present study. The results reported here provide evidence of genotypic variation in the properties of cytokinin oxidase. Although the cytokinin oxidases from Great Northern and Kingston callus tissues have similar substrate specificities, the two enzymes are chromatographically distinct and exhibit marked differences in pH optima and in glycosylation. The latter observations suggest that the compartmentation of cytokinin oxidase may differ in the two callus tissues and raise the possibility that isozyme variation in cytokinin oxidase may play a role in the regulation of cytokinin degradation in plant tissues.

MATERIALS AND METHODS

Chemicals

Picloram (4-amino-3,5,6-trichloropicolinic acid) was purchased from Aldrich. The i^6Ade-2,8-^{3}H used to assay for cytokinin oxidase activity was synthesized as described by Chatfield and Armstrong (6). The cytokinin hi^6Ade was synthesized as described by Leonard *et al.* (12). All other cytokinin-active purine derivatives were purchased from Sigma. Thidiazuron (N-phenyl-N′-1,2,3-thidiazol-5-ylurea, DROPP, SN 49537) was a gift from NOR-AM Chemical Co.

Polymin P (polyethyleneimine, 50% w/v) was obtained from Miles Laboratories. PVPP (purchased from Sigma) and Porapak Q (100–120 mesh, purchased from Waters Associates) were prepared for use as previously described (5). Ammonium sulfate (enzyme grade) was obtained from Schwarz/Mann. Methylmannose (methyl α-D-mannopyrannoside), Sephadex LH-20 and Con A Sepharose 4B were purchased from Sigma. DE52-cellulose is a product of Whatman. Reverse phase Si-C_{18} TLC plates (without fluorescent indicator) were purchased from J. T. Baker Co. Standard silica gel TLC plates were purchased from Eastman (Eastman No. 13179).

Plant Materials

Seeds of *Phaseolus vulgaris* cv Great Northern and *P. lunatus* cv Kingston were obtained from Atlee Burpee Co. Callus cultures of the two genotypes were established from hypocotyl tissue as described previously (6).

The medium used to culture *Phaseolus* callus tissues contained the inorganic nutrients specified by Murashige and Skoog (20) and the following organics: sucrose (30 g/L), *myo*-inositol (100 mg/L), thiamine-HCl (1 mg/L), pyridoxine-HCl (0.5 mg/L), picloram (2.5 μM), kinetin (5 μM), and Difco Bacto-agar (10 g/L). The pH of the medium was adjusted to 5.7 prior to adding the agar. The medium was dispensed into 125 mL Erlenmeyer flasks (50 mL/flask) and autoclaved at 120°C for 15 min.

The *Phaseolus* callus cultures were grown in the dark at 28°C and subcultured at 21-d intervals. The tissues were harvested for enzyme extraction, or used for enzyme induction experiments, when the fresh weight of the cultures had reached approximately 5 to 6 g/flask (16 or 17 d following transfer of Great Northern and Kingston tissues, respectively).

Extraction of Cytokinin Oxidase Activity from *Phaseolus* Callus Tissues

Cytokinin oxidase activity was extracted from *Phaseolus* callus tissues by methods similar to those described by Chatfield and Armstrong (5). The callus tissues were homogenized (Sorvall Omnimixer, 1 min, setting 6) with an equal volume of cold 100 mM bisTris-HCl (pH 6.5) or (as indicated) 100 mM Tris-HCl (pH 7.5), or 100 mM Tris-HCl (pH 8.4). All subsequent operations were performed at 4°C. Homogenate volumes equivalent to tissue fresh weights of 5 g were each mixed with 1.5 g (dry weight) of PVPP hydrated with 50 mM homogenization buffer (see above). The resulting suspensions were filtered under pressure (N_2 at 2 lb/in^2) through one layer of Miracloth. For each homogenate volume, the solids retained by the Miracloth filter were washed with two 5 mL volumes of 50 mM homogenization buffer. The combined filtrates were centrifuged (10,000*g*; 10 min), and Polymin P (1% v/v, adjusted to the pH of the homogenization buffer) was added dropwise with stirring to the supernatants. The volume of 1% Polymin P added to the supernatants varied with the pH of the extraction buffer, from 40 μL/mL (pH 6.5) to 50 μL/mL (pH 7.5) to 60 μL/mL (pH 8.4). In each case, the volume of Polymin P used was determined in preliminary experiments to be optimal for precipitation of nucleic acids and associated proteins without loss of cytokinin oxidase activity. (Representative Polymin P precipitation data for Kingston callus tissue homogenized in pH 8.4 buffer are shown in Fig. 1.) After 20 min, the precipitates were removed by centrifugation (10,000*g*, 10 min). Solid ammonium sulfate was added to the supernatants to give 80% saturation. (The pH was maintained at the pH of the homogenization buffer.) After standing in the cold for at least 30 min, the ammonium sulfate precipitates were collected by centrifugation (20,000*g*; 20 min) and stored at −20°C.

In experiments involving the induction of increases in cytokinin oxidase activity by application of solutions of exogenous cytokinins to the surfaces of callus tissues, the above protocol was modified to include passage of the enzyme extract through Porapak Q columns to remove cytokinins (9). The supernatants from Polymin P precipitation were adjusted to 10% saturation with solid ammonium sulfate and applied to 0.5 mL Porapak Q columns equilibrated with 50 mM homogenization buffer containing ammonium sulfate at 10% saturation. The columns were each rinsed with the two 1 mL volumes of the same buffer, and solid ammonium sulfate was added to the eluates to give 80% saturation. The precipitated protein was recovered by centrifugation as described above.

Assays of Cytokinin Oxidase Activity

The enzyme preparations from above were dissolved in buffer appropriate to the enzyme assay conditions (usually 1

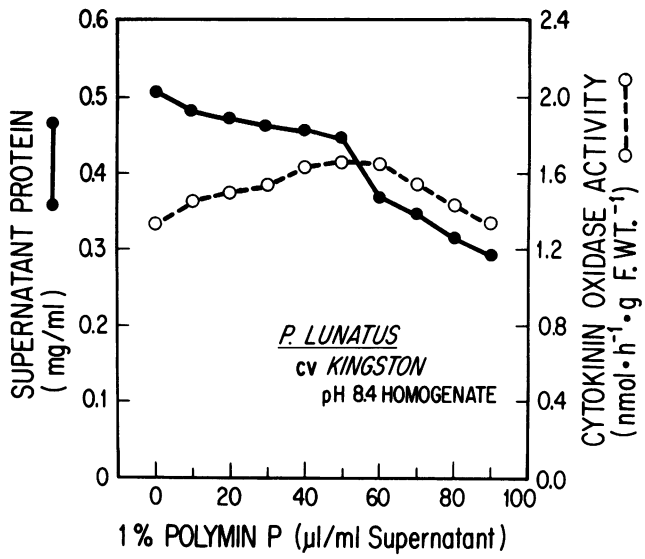

Figure 1. Effect of Polymin P on the recovery of protein and cytokinin oxidase activity from homogenates of *P. lunatus* cv Kingston callus tissues. Callus tissue was homogenized with 100 mM Tris-HCl (pH 8.4) and treated with PVPP as described in "Materials and Methods." Polymin P (pH 8.4, 1% v/v) was added to 10 mL aliquots of the PVPP-treated homogenate supernatant. Precipitated material was removed by centrifugation, and the supernatants were made to 80% saturation with solid ammonium sulfate. Precipitated protein was recovered by centrifugation, dissolved in 100 mM Taps-NaOH (pH 8.4), and assayed for cytokinin oxidase activity in the standard assay.

mL buffer per enzyme preparation equivalent to 5 g callus fresh weight). For determination of pH optima, the enzyme preparations were dissolved in distilled water. The enzyme solutions were centrifuged (10,000*g*; 10 min) to remove any undissolved solids, and assayed for cytokinin oxidase activity using i^{6}Ade-2,8-^{3}H as a substrate.

The standard cytokinin oxidase assay mixtures contained 100 mM buffer of the composition indicated in individual experiments, 0.01 mM i^{6}Ade-2,8-^{3}H (0.05 μCi, specific activity 100 μCi/μmol), other additives as appropriate to the experiment, and enzyme in a total volume of 50 μL in minifuge tubes. The assays were incubated for 30 min at 37°C. The reactions were terminated by the addition of 100 μL of cold 95% (v/v) ethanol containing unlabeled Ade and i^{6}Ade (0.75 mM each). The precipitated protein was removed by centrifugation (minifuge). Samples (100 μL) of the supernatants were streaked on 5 cm wide Si-C_{18} TLC plates. The chromatograms were developed to a height of 5 cm in 40% (v/v) ethanol containing 100 mM Na_4EDTA. The locations of the i^{6}Ade and Ade standards were determined by inspection under UV light, and zones (1.3 cm wide) corresponding to the standards were removed and counted in 5 mL of Beckman Ready-Protein scintillation fluid in a Beckman Model 1801 scintillation counter.

Cytokinin oxidase activity in the fractions from DEAE-cellulose or Con A Sepharose 4B column chromatograhpy was determined using a copper-imidazole enhanced assay (6). The assay mixture contained the following constituents adjusted to pH 5.8: 100 mM imidazole-HCl, 25 mM sodium acetate, 20 mM $CuCl_2$, 0.01 mM i^{6}Ade-2,8-^{3}H (0.05 μCi, specific activity 100 μCi/μmol), and 20 μL of column fraction (pH unadjusted) in a total assay volume of 50 μL. The assay volumes were incubated for 30 min at 37°C. For assays involving fractions from Con A Sepharose 4B columns, the reactions were terminated by the addition of 10 μL of 200 mM Na_4EDTA followed by 120 μL of cold 95% (v/v) ethanol containing unlabeled Ade and i^{6}Ade (0.75 mM each). The distribution of label in i^{6}Ade and Ade was determined by chromatography on Si-C_{18} TLC plates as in the standard cytokinin oxidase assay procedure described above. For assays involving fractions from DEAE-cellulose columns, the reactions were terminated by the addition of 50 μL of 40 mM Na_4EDTA containing i^{6}Ado and Ade (each at 1 mM) followed by the addition of 300 μL of butyl acetate saturated with a 1 mM Ade solution. The mixture was vortexed, centrifuged (minifuge), and the organic phase (containing undegraded i^{6}Ade) was removed. The extraction with butyl acetate (saturated with 1 mM Ade) was repeated two times. Samples (72 μL) of the final aqueous phase (containing the Ade product of the enzyme reaction) were counted in 5 mL of Beckman Ready-Protein scintillation fluid.

Preparation of Materials for Column Chromatography

Con A Sepharose 4B was packed into 1 cm diameter chromatography columns to give 1 mL bed volumes. The columns were each washed with 30 mL of 25 mM bisTris-HCl (pH 6.5) containing 1 mM $MnCl_2$, 1 mM $CaCl_2$, and 200 mM $(NH_4)_2SO_4$.

DE52-cellulose was precycled and fines removed according to the manufacturer's instructions. The cellulose slurry was adjusted to pH 7.5 with Tris base, washed with 10 bed volumes of 20 mM Tris-HCl (pH 7.5) containing 1 M KCl, and packed into 1.5 cm diameter chromatography columns in the same buffer-salt solution using a pumped flow rate of greater than 1 mL/min. The packed columns (1.5 × 14 cm, 25 mL bed volume) were equilibrated in 20 mM Tris-HCl (pH 7.5) without KCl. The flow rates were reduced to 0.5 mL per minute for sample loading and chromatography.

Protein Assays

Samples of protein solutions were mixed with equal volumes of cold 20% (w/v) TCA and allowed to stand on ice for 30 min. The precipitated protein was collected by centrifugation (20,000*g*; 10 min) and dissolved in 0.1 N NaOH for protein determination by the Folin phenol method of Peterson (25). BSA was used as a standard.

RESULTS

Comparison of the pH Optima of the Cytokinin Oxidases from *Phaseolus vulgaris* cv Great Northern and *Phaseolus lunatus* cv Kingston Callus Tissues

The pH optima of the cytokinin oxidase activities extracted from Great Northern and Kingston callus tissues by homogenization in bisTris buffer at pH 6.5 were compared. The crude enzyme preparations used for this purpose were ob-

tained from the homogenates by treatment with PVPP and Polymin P followed by ammonium sulfate precipitation, as described in "Materials and Methods." The cytokinin oxidase activities extracted from the two callus tissues exhibited pronounced differences in pH optima (Fig. 2). The enzyme from *P. lunatus* cv Kingston callus tissue exhibited a pH optimum of 8.4. As reported by Chatfield and Armstrong (5), the cytokinin oxidase activity from *P. vulgaris* cv Great Northern callus tissue exhibited a pH optimum of 6.5 (bisTris). However, in imidazole buffer, the pH optimum of the Great Northern enzyme was shifted to a slightly higher value (pH 7.0).

The levels of cytokinin oxidase activity extracted from the two *Phaseolus* callus tissues differed significantly when assayed at pH values optimal for the respective enzymes. Under these conditions (as shown in Fig. 2), the cytokinin oxidase activity recovered from Kingston callus tissue exceeded that recovered from an equal fresh weight of Great Northern callus tissue by more than twofold. The levels of cytokinin oxidase activity recovered from the two callus tissues were not altered by changing the pH of homogenization over the range from pH 6.5 to pH 8.4

Substrate Specificity and Reaction Characteristics of the Cytokinin Oxidase from *P. lunatus* cv Kingston Callus Tissue

The substrate specificity of the cytokinin oxidase from Kingston callus tissue was examined by testing the effects of unlabeled cytokinins on the degradation of i^6Ade-2,8-^{3}H in the standard cytokinin oxidase assay at pH 8.4 (Taps). Unlabeled cytokinins were added to the assay mixture at concentrations equal to that of the labeled i^6Ade substrate (10 μM) or in 10-fold excess (100 μM). The results are summarized in Table I. The substrate specificity of the Kingston enzyme was very similar to that previously reported for the cytokinin oxidase from *P. vulgaris* cv Great Northern callus tissue (5). On the basis of these tests, i^6Ade appears to be the preferred substrate of the enzyme. Substrate competition was observed with the naturally occurring cytokinins i^6Ado and io^6Ade and with the cytokinin-active urea derivative thidiazuron. Unmodified Ade, cytokinins bearing saturated isoprenoid sidechains (hi^6Ade and hio^6Ade), and the cytokinins f^6Ade and b^6Ade did not exhibit competitive interaction with the labeled substrate.

A single radioactive degradation product was detected by TLC following incubation of the Kingston enzyme preparations with i^6Ade-2,8-^{3}H at the optimum pH of 8.4. This product chromatographed with Ade standards in both the reverse-phase TLC system used for cytokinin oxidase assays and on silica gel TLC plates developed with the upper phase of a mixture of ethyl acetate:*n*-butanol:water (4:1:2). The degradation of i^6Ade by the Kingston enzyme was inhibited over 80% when cytokinin oxidase activity was assayed in sealed tubes using reaction mixtures purged with nitrogen (data not shown).

Table I. *Effects of Unlabeled Cytokinins on the Degradation of i^6Ade-2,8-^{3}H by Cytokinin Oxidase from P. lunatus cv Kingston Callus Tissue*

Unlabeled cytokinins were added to 50 μL assay mixtures containing 100 mM Taps (pH 8.4), 10 μM i^6Ade-2,8-^{3}H (100 μCi/μmol), and enzyme equivalent to 100 mg callus (fresh weight). Enzyme preparations were obtained from callus homogenized in Tris-HCl (pH 8.4) as described in "Materials and Methods."

Unlabeled Cytokinin	Cytokinin Oxidase Activity[a]	
	10 μM unlabeled cytokinin	100 μM unlabeled cytokinin
	% of control	
Ade	105	100
i^6Ade	51	16
i^6Ado	89	70
hi^6Ade	102	89
hio^6Ade	104	92
Zeatin (*t*-io^6Ade)	93	78
cis-Zeatin (*c*-io^6Ade)	90	78
f^6Ade	104	99
b^6Ade	98	92
Thidiazuron	52	14

[a] The cytokinin oxidase activity of the controls incubated without the addition of unlabeled cytokinins was equal to 0.13 nmol/h. All values are the average of two experiments, each involving two replicate assays.

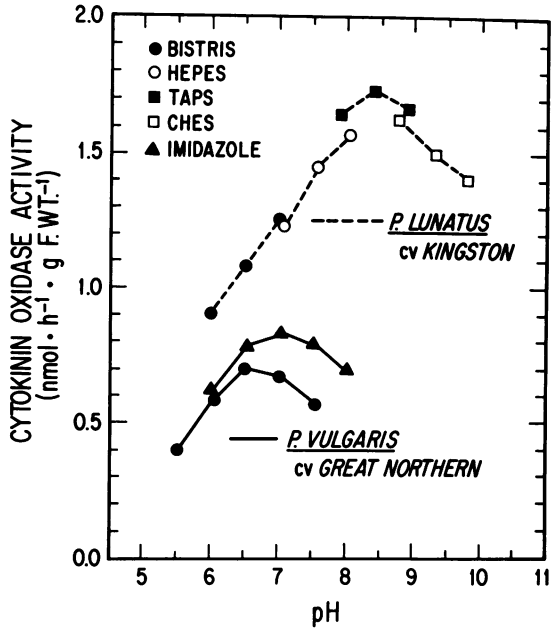

Figure 2. Effect of pH on the activity of cytokinin oxidase preparations from *P. lunatus* cv Kingston and *P. vulgaris* cv Great Northern callus tissues. Cytokinin oxidase preparations were obtained from Kingston and Great Northern callus homogenized with 100 mM bisTris-HCl at pH 6.5 as described in "Materials and Methods." Cytokinin oxidase activity was determined in 50 μL assay volumes containing 100 mM buffer, 10 μM i^6Ade-2,8-^{3}H (100 μCi/μmol), and enzyme equivalent to 100 mg tissue fresh weight.

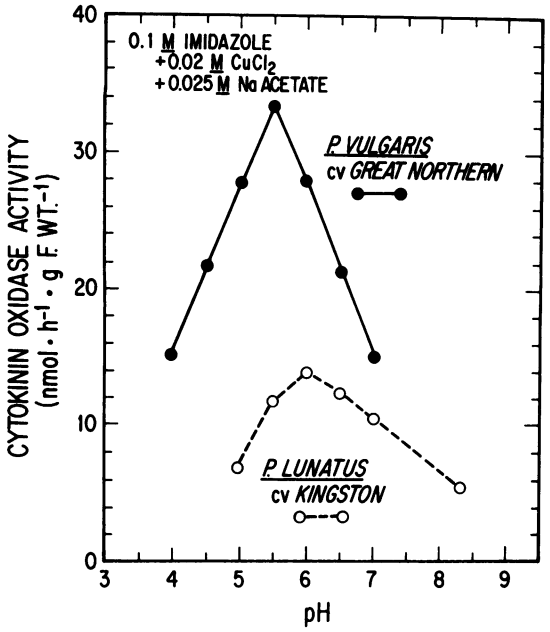

Figure 3. Effect of copper-imidazole complexes on cell-free cytokinin oxidase preparations from *P. lunatus* cv Kingston and *P. vulgaris* cv Great Northern callus tissues. Cytokinin oxidase preparations were obtained from Kingston and Great Northern callus homogenized with 100 mM bisTris-HCl at pH 6.5 as described in "Materials and Methods." Cytokinin oxidase activity was determined at the pH values indicated, using assay mixtures containing 100 mM imidazole-HCl, 20 mM $CuCl_2$, 25 mM sodium acetate, 10 μM i^6Ade-2,8-^{3}H (100 μCi/μmol), and enzyme equivalent to 3.3 mg tissue fresh weight per 50 μL assay volumes.

Comparison of the Effects of Copper-Imidazole Complexes on the Cytokinin Oxidase Activities from *P. lunatus* cv Kingston and *P. vulgaris* cv Great Northern Callus Tissues

The ability of copper-imidazole complexes to enhance the activity of cell-free preparations of the cytokinin oxidase from Great Northern callus tissue was reported by Chatfield and Armstrong (6), but the pH dependence of the reaction was not investigated. The pH optima and relative activities of the cytokinin oxidase activities extracted from Kingston and Great Northern callus tissues were compared here in reaction mixtures containing 100 mM imidazole, 25 mM sodium acetate, and 20 mM $CuCl_2$. The results are shown in Figure 3. Compared to the standard assay conditions (Fig. 2), the activity of both enzymes were considerably enhanced in the presence of copper-imidazole complexes. In both cases, the pH optimum of the cytokinin oxidase reaction was shifted to a lower value in the presence of copper-imidazole (pH 6.0 and 5.6 for the enzymes from Kingston and Great Northern callus tissues, respectively). The shifts in pH optima probably reflect both an altered reaction mechanism and the pH dependence of the formatino of copper-imidazole complexes.

The response of the two cytokinin oxidase activities to copper-imidazole complexes was further investigated by varying the $CuCl_2$ concentration in reaction mixtures containing 100 mM imidazole buffer at the pH value optimal for each enzyme. The results are shown in Figure 4. The activity of the enzyme from Great Northern callus tissues increased with increasing copper concentration, reaching values more than 50-fold those observed in the standard cytokinin oxidase assay, and plateaued near the limits of solubility of the copper-imidazole complex. The activity of the Kingston enzyme was enhanced about 10-fold at an optimal $CuCl_2$ concentration of approximately 15 to 20 mM. Higher concentrations of $CuCl_2$ resulted in decreasing activity of the Kingston enzyme. The $CuCl_2$ concentrations optimal for the Great Northern and Kingston enzymes were not altered if the pH values used to assay the two cytokinin oxidase activities were reversed (data not shown).

Regulation of Cytokinin Oxidase Activity in *P. lunatus* cv Kingston Callus Tissues Treated with Exogenous Cytokinins

Chatfield and Armstrong (5) demonstrated that transient increases in the supply of exogenous cytokinins induced relatively rapid increased in the cytokinin oxidase activity that could be extracted from callus tissues of *P. vulgaris* cv Great Northern. To determine whether cytokinin oxidase activity in callus tissues of *P. lunatus* cv Kingston was regulated in a similar manner, solutions of exogenous cytokinins were ap-

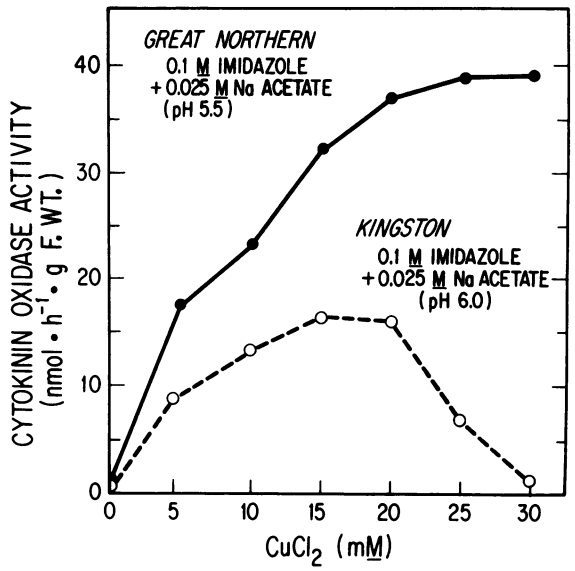

Figure 4. Effect of $CuCl_2$ concentration on the copper-imidazole enhanced activity of cell-free preparations of cytokinin oxidase from callus tissues of *P. lunatus* cv Kingston and *P. vulgaris* cv Great Northern. Cytokinin oxidase preparations were obtained from Great Northern callus tissues homogenized in bisTris-HCl (pH 6.5) and from Kingston callus tissues homogenized in Tris-HCl (pH 8.4) as described in "Materials and Methods." Cytokinin oxidase activity was assayed using reaction mixtures containing 100 mM imidazole-HCl, 25 mM sodium acetate buffer, 10 μM i^6Ade-2,8-^{3}H (100 μCi/μmol), the indicated concentrations of $CuCl_2$, and enzyme equivalent to 2.5 mg (Great Northern) or 10 mg (Kingston) tissue fresh weight per 50 μL assay volumes. Assays were performed at the pH value optimal for each enzyme (pH 5.5 and 6.0 for the Great Northern and Kingston enzymes, respectively).

Table II. *Effects of Exogenously Applied Cytokinins on Cytokinin Oxidase Activity in P. lunatus cv Kingston Callus Tissue*

Cytokinin solutions (100 μM) were applied to the surface of 17-d-old callus (average fresh weight: 5.7 g/flask) at a rate of 0.1 mL/g fresh weight. Treated tissues were incubated for 5 h in the dark at 28°C. Each cytokinin was tested using three replicate flasks of callus per each of two experiments. Cytokinin oxidase activity was assayed using enzyme preparations obtained from tissue homogenized in Tris-HCl at pH 8.4 as described in "Materials and Methods." Assay mixtures contained 100 mM Taps (pH 8.4), 10 μM i^6Ade-2,8-^{3}H (100 μCi/μmol), and enzyme equivalent to 75 mg tissue (fresh weight) per 50 μL assay volume.

Cytokinin Treatment	Cytokinin Oxidase Activity after 5 h Incubation[a]
	% of time zero control
Ade	105[b]
i^6Ade	110
hi^6Ade	159
hio^6Ade	147
Zeatin (*t*-io^6Ade)	144
cis-Zeatin (*c*-io^6Ade)	125
Thidiazuron	177

[a] Cytokinin oxidase activity in untreated control tissue averaged 1.3 nmol/h per g fresh weight. [b] All values are the average of two experiments involving two replicate assays per experiment.

plied to the surface of Kingston callus tissues, and cytokinin oxidase activity was assayed at the end of a 5-h incubation period. The results are shown in Table II. Most of the cytokinins tested induced an increase in the cytokinin oxidase activity extracted from the tissue. Thidiazuron and hi^6Ade were the most effective of the compounds tested. As in the tests with Great Northern callus tissues (5), i^6Ade itself and Ade had little effect on the cytokinin oxidase activity in the tissue.

Con A Affinity of the Cytokinin Oxidase Activities from *P. lunatus* cv Kingston and *P. vulgaris* cv Great Northern Callus Tissues

The cytokinin oxidase activity from Great Northern callus tissue has been shown previously to exhibit a pronounced affinity for the lectin Con A (7). To compare the Con A affinities of the cytokinin oxidase activities from Great Northern and Kingston callus tissues, samples of each tissue were homogenized in bisTris-HCl (pH 6.5) and in Tris-HCl (pH 8.4). The duplicate enzyme preparations were applied to columns of Con A Sepharose 4B. The results obtained with the enzyme preparations from callus tissues homogenized in bisTris-HCl (pH 6.5) are shown in Figure 5. Similar results were obtained using the enzyme preparations from callus tissues homogenized in Tris-HCl (pH 8.4) (data not shown). As expected (7), most of the cytokinin oxidase activity (about 75% of the total) from Great Northern callus tissue bound to the Con A Sepharose 4B column and was not eluted from the column until 0.2 M methylmannose was added to the elution buffer. In contrast to these results, most of the cytokinin oxidase activity from Kingston callus tissue (85% of the total) was not retained on the Con A Sepharose 4B column. The marked difference in affinity for Con A indicates that the two cytokinin oxidase enzymes differ in glycosylation.

DEAE-Cellulose Fractionation of the Cytokinin Oxidase Activities from *P. lunatus* cv Kingston and *P. vulgaris* cv Great Northern Callus Tissues

The cytokinin oxidase activities from Kingston and Great Northern callus tissues were further compared by chromatographic fractionation on DEAE-cellulose. For this purpose, cytokinin oxidase activity was extracted from each of the two callus tissues, and from a mixture containing equal fresh weights of both tissues, by homogenization in Tris-HCl (pH 7.5). The crude enzyme preparations obtained from the three homogenates as described in "Materials and Methods" were each applied to a DEAE-cellulose column. The elution profiles for cytokinin oxidase activity were determined using the copper-imidazole enhanced assay. The results are shown in Figure 6. The cytokinin oxidase activity from Great Northern

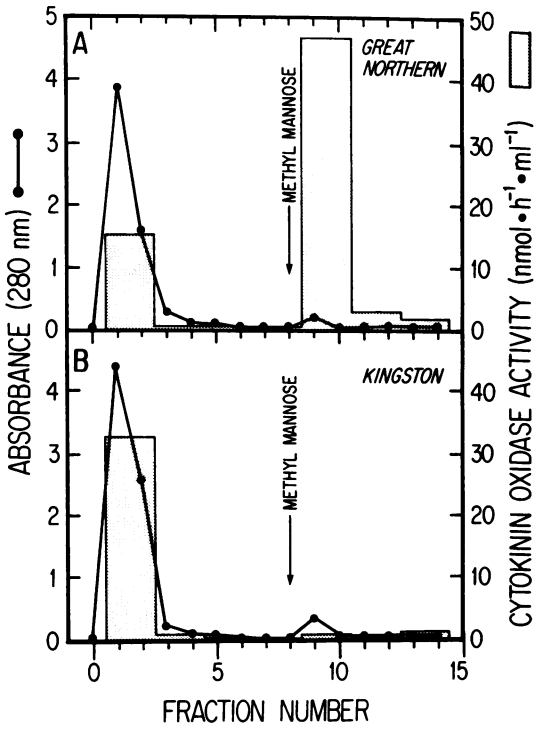

Figure 5. Con A Sepharose-4B chromatography of cytokinin oxidases from *P. lunatus* cv Kingston and *P. vulgaris* cv Great Northern callus tissues. Cytokinin oxidase preparations were obtained from Kingston and Great Northern callus tissues homogenized in bisTris-HCl (pH 6.5) as described in "Materials and Methods." Protein samples equivalent to 20 g (fresh weight) of callus tissue were each dissolved in 4 mL of 25 mM bisTris-HCl (pH 6.5). Undissolved particulate matter was removed by centrifugation, and 3.7 mL of the supernatants were applied to Con A:Sepharose 4B columns (1 × 1.3 cm, 1 mL bed volume) equilibrated in 25 mM bisTris-HCl (pH 6.5) containing 200 mM $(NH_4)_2SO_4$, 1 mM $CaCl_2$, and 1 mM $MnCl_2$. The columns were each washed with 11 mL of 25 mM bisTris-HCl (pH 6.5) containing 200 mM $(NH_4)_2SO_4$ and eluted with the same solution containing 200 mM methylmannose. Fractions (3.7 mL) were collected and assayed for cytokinin oxidase activity using the copper-imidazole enhanced assay described in "Materials and Methods."

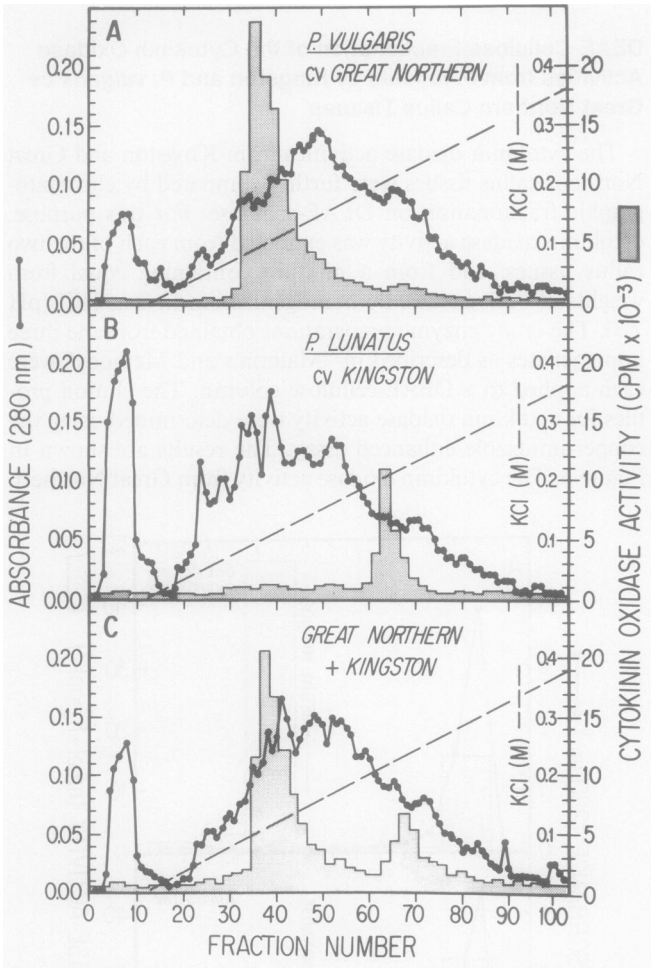

Figure 6. DEAE-cellulose chromatography of cytokinin oxidase preparations from *P. lunatus* cv Kingston and *P. vulgaris* cv Great Northern callus tissues. Cytokinin oxidase preparations, each equivalent to 40 g (fresh weight) of callus tissue, were obtained from Kingston callus tissues, from Great Northern callus tissues, and from a mixture of equal fresh weights of the two callus tissues, as described in "Materials and Methods" and using 100 mM Tris-HCl (pH 7.5) as the homogenization buffer. Following ammonium sulfate precipitation, each cytokinin oxidase preparation was dissolved in 8 mL of 20 mM Tris-HCl (pH 7.5). Undissolved particulate matter was removed by centrifugation, and each protein solution was desalted on a Sephadex G-25 column (150 mL bed volume) equilibrated in the same buffer. Each desalted protein preparation (30 mL per preparation) was applied to a DEAE-cellulose column (1.5 × 14 cm, 25 mL bed volume) that had been equilibrated with 20 mM Tris-HCl (pH 7.5). Each DEAE-cellulose column was washed with 100 mL of 20 mM Tris-HCl (pH 7.5) and then eluted with a linear gradient of KCl (0–400 mM, over a total volume of 500 mL) in the same buffer. Fractions of 5 mL were collected at flow rate of approximately 0.5 mL/min. Cytokinin oxidase activity was assayed using the copper-imidazole enhanced assay procedure described in "Materials and Methods" and is expressed as counts per minute of Ade formed per assay. The apparent difference in peak heights of the two enzyme activities may be attributed to the difference in their response to the copper-imidazole reagent.

callus tissue eluted as a single peak early in the salt gradient. The cytokinin oxidase activity from Kingston callus tissue also eluted as a single peak but much later in the salt gradient than the Great Northern enzyme. (It should be noted that the difference in peak height observed in the two chromatographic profiles is the result of the use of the copper-imidazole enhanced assay and the difference in sensitivity of the two enzyme activities to this reagent.) The enzyme preparation obtained by homogenizing the two callus tissues together gave two peaks of cytokinin activity that eluted at positions corresponding to the enzymes from the respective callus tissues.

DISCUSSION

The cytokinin oxidase activities from *Phaseolus vulgaris* cv Great Northern and *Phaseolus lunatus* cv Kingston callus tissues have been compared in the present study. The substrate specificities of the two enzymes appear to be identical, and the activities of both enzymes are enhanced (although to different degrees) in the presence of copper-imidazole complexes. However, the enzymes exhibit marked differences in their pH optima (pH 6.0 and 8.4, respectively), in their affinity for Con A, and in their chromatographic behavior on DEAE-cellulose. The affinity of the cytokinin oxidase from Great Northern callus cultures for Con A indicates that the enzyme must be present in the callus tissue as a glycoprotein (7). Burch and Horgan (4) have recently reported that the cytokinin oxidase activities from immature corn kernels and from wheat germ also bind to Con A. The cytokinin oxidase from Kingston callus tissue does not bind to this lectin and must be unglycosylated or have a pattern of glycosylation that differs significantly from that of the other enzymes.

The differences in glycosylation and pH optima of the cytokinin oxidases from Great Northern and Kingston callus are correlated with pronounced differences in the rates of cytokinin degradation in these tissues (17, 19). This correlation suggests that the compartmentation of cytokinin oxidase may differ in the two callus tissues. For example, the rapid degradation of exogenously supplied i^6Ado-8-^{14}C by Great Northern callus tissues (19) could be explained if the glycosylated cytokinin oxidase present in this tissue is secreted to cell exteriors. Conversely, retention of the Kingston enzyme (which has the higher pH optimum and exhibits no affinity for Con A) in an internal cell compartment could restrict the access of cytokinin oxidase to exogenous cytokinins and account for the lower rate of cytokinin degradation in these callus cultures. A precedent for such a model exists in the distribution of peroxidase isozymes in cell walls and internal compartments of plant cells (8, 13). However, other explanations for the difference in rates of cytokinin degradation in *Phaseolus* callus tissues may be hypothesized. Laloue and Fox (11) have reported that the nucleotide form of i^6Ade is resistant to attack by the cytokinin oxidase from wheat germ, and it is possible that a rapid conversion of exogenous cytokinins to nucleotides (or other differences in cytokinin metabolism or uptake) may protect i^6Ade and io^6Ade against the attack of cytokinin oxidase in Kingston callus tissue.

The contrasting properties of the cytokinin oxidases from Kingston and Great Northern callus tissues suggest the pos-

sibility of developmental, as well as genotypic, variation in the structural and catalytic properties of the enzyme. Additional evidence of structural variation in cytokinin oxidase is provided by the wide range of molecular masses reported for the enzyme from different plant sources. Published estimates of the molecular masses of cytokinin oxidase range from 25,000 (*Vinca rosea* crown-gall tissue) (15) to 40,000 (wheat germ) (11) to 78,000 D (immature corn kernals) (4). Isozyme variation has yet to be unequivocally demonstrated in cytokinin oxidase preparations from a single source, but Laloue and Fox (11) have observed that the cytokinin oxidase from wheat germ is separated into two peaks of activity by chromatofocusing.

The structural variation in cytokinin oxidase from higher plant sources does not appear to be related to differences in substrate specificity. The cytokinin i^6Ade is the preferred substrate of all cytokinin oxidase activities for which data are available (1, 5, 11, 15, 21, 22, 28). Zeatin (io^6Ade) and the ribonucleosides io^6Ado and i^6Ado also appear to be universal substrates of cytokinin oxidase. Cytokinins with saturated isoprenoid sidechains (hio^6Ade and hi^6Ade) or cyclic sidechain structures (b^6Ade and f^6Ade) are relatively resistant to attack by all of the cytokinin oxidases that have been isolated from higher plant sources (5, 11, 15, 21, 22, 28). However, a cytokinin oxidase activity from the cellular slime mold, *Dictyostelium discoideum*, appears to have somewhat broader specificity (1), and the reported degradation of f^6Ade to Ade by an enzyme preparation from moss protonema (2) suggests that some plant cells may contain cytokinin oxidase activities that attack a wider range of structures than do the plant enzymes characterized to date.

All cytokinin oxidase activities for which data are available, including the enzymes examined in the present study, interact with cytokinin-active urea derivatives. In substrate competition tests, the cytokinin thidiazuron (18) was highly effective in inhibiting the degradation of i^6Ade by cytokinin oxidase activities from *P. vulgaris* cv Great Northern callus tissue (5), *P. lunatus* cv Kingston callus tissue (Table I of the present study), and vegetative amoeba of *D. discoideum* (1). Similar results were obtained by Laloue and Fox (11) using other cytokinin-active urea derivatives and the cytokinin oxidase from wheat germ. Burch and Horgan (4) have obtained kinetic data that suggest that cytokinin *N*-(2-chloro-4-pyridyl)-*N'*-phenylurea acts as a noncompetitive inhibitor of the cytokinin oxidase from *Zea mays* kernels. The slow metabolism of thidiazuron in callus tissues of *P. lunatus* cv Kingston (16) and the increases in endogenous cytokinin levels observed in tobacco callus cultures following treatment with thidiazuron are consistent with this interpretation (27).

Cytokinin degradation appears to be tightly regulated in plant systems. In both Kingston and Great Northern callus tissues, the levels of cytokinin oxidase activity are controlled by a mechanism that is sensitive to cytokinin supply and capable of inducing relatively rapid changes in the levels of enzyme activity in the callus tissues (Table II and Chatfield and Armstrong [5]). Evidence is available that similar mechanisms exist in tobacco (26) and soy bean callus (23) and in protonemal cells of the moss *Funaria hygrometrica* (2). Regulatory effects of auxins on the *in vivo* degradation of cytokinins in tobacco callus and on the activity of cell-free preparations of cytokinin oxidase from the same tissue have been reported (24). The regulation of cytokinin degradation may be even more complex than is currently perceived if there are *in vivo* processes that mimic the effect of copper-imidazole complexes on the activity of cell-free preparations of cytokinin oxidase (6).

The present study has demonstrated that genotypic differences in the rates of cytokinin degradation in *Phaseolus* callus tissues are correlated with variations in the structure and catalytic properties of the cytokinin oxidase activities present in these tissues. It will be of interest to determine whether the observed variations in glycosylation and pH optima of cytokinin oxidase are linked to tissue-specific differences in the compartmentation of the enzyme and to establish whether isozyme variation in cytokinin oxidase plays a role in the developmental regulation of cytokinin degradation in plant tissues.

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