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Communication

Cytokinin Oxidase from *Phaseolus vulgaris* Callus Cultures[1]

AFFINITY FOR CONCANAVALIN A

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ABSTRACT

Cytokinin oxidase activity from *Phaseolus vulgaris* cv Great Northern callus cultures exhibited affinity for the lectin concanavalin A. Over 80% of the activity extracted from the callus tissue bound to a concanavalin A-Sepharose 4B column. The bound activity was eluted from the column by the addition of methylmannose to the eluting buffer. On the basis of this result, it appears that most of the cyokinin oxidase activity present in Great Northern callus cultures exists in the form of a glycoprotein. The apparent pI of this enzyme, as estimated by chromatofocusing, is approximately 5.0.

Cytokinin oxidase activity is widely distributed in plant tissues (2, 4–8, 10, 11). The enzyme catalyzes the oxidative degradation of cytokinins bearing unsaturated isoprenoid side chains. The products of the reaction are Ade[3] or Ado and a side chain fragment bearing an aldehyde group (1). Naturally occurring substrates for the enzyme include i^{6}Ade io^{6}Ade, and the corresponding ribonucleosides, i^{6}Ado and io^{6}Ado.

The regulation of cytokinin oxidase activity in callus cultures of *Phaseolus vulgaris* cv Great Northern was examined in earlier studies by this laboratory (2, 3). Transient increases in exogenous cytokinin supply were observed to induce relatively rapid increases in the levels of cytokinin oxidase activity present in the Great Northern callus tissues (2). This increase in enzyme activity was sensitive to inhibitors of RNA and protein synthesis and was induced by all cytokinin-active compounds tested, including a number of cytokinins that were not substrates for the enzyme (2). Cell-free preparations of the Great Northern enzyme exhibited greatly enhanced activity in the presence of copper-imidazole complexes (3). These complexes appeared to substitute for oxygen in the reaction catalyzed by cytokinin oxidase (3).

We report here that cytokinin oxidase activity extracted from callus tissues of *P. vulgaris* cv Great Northern is bound by concanavalin A-Sepharose 4B. The affinity of the enzyme for this lectin suggests that the cytokinin oxidase activity extracted from Great Northern callus tissues is present in the form of a glycoprotein.

MATERIALS AND METHODS

Chemicals. Picloram (4-amino-3,5,6-trichloropicolinic acid) was purchased from Aldrich. Polymin P (polyethyleneimine, 50% w/v) was obtained from Miles Laboratories. Polyvinylpolypyrrolidone (PVPP) was purchased from Sigma and prepared for use as previously described (2). Ammonium sulfate (enzyme grade) was purchased from Schwarz/Mann. The i^{6}Ade-2,8-^{3}H used to assay for cytokinin oxidase activity was synthesized as previously described (3). Concanavalin A-Sepharose 4B (10–14 mg of concanavalin A per mL of settled gel) and methylmannose were purchased from Sigma. Polyexchanger PBE94 and Polybuffer 74 are products of Pharmacia.

Extraction of Cytokinin Oxidase Activity from *Phaseolus* Callus Tissues. Callus tissues of *Phaseolus vulgaris* cv Great Northern were grown as previously described (2) and were harvested at 19 to 22 days of age (5–7.5 g/flask). The callus tissues were homogenized, and protein preparations were obtained from the homogenates by treatment with PVPP and Polymin P followed by ammonium sulfate precipitation as described by Chatfield and Armstrong (2). The precipitated protein was recovered by centrifugation and stored at −20°C prior to chromatographic fractionation.

Preparation of Chromatography Columns. Concanavalin A-Sepharose 4B was packed into a 1 × 6 cm column (5 mL bed volume) and washed with 50 bed volumes of 0.05 M bisTris-HCl (pH 6.5) containing 0.25 M ammonium sulfate, 0.1 M methylmannose, 0.001 M $CaCl_2$, and 0.001 M $MnCl_2$. This initial wash was followed by 50 bed volumes of the same buffer without methylmannose, $CaCl_2$, or $MnCl_2$.

Polyexchanger PBE94 was washed with 25 bed volumes of 0.025 M imidazole-HCl (pH 7.4) and packed into a 0.9 × 32 cm column (20 mL bed volume). The packed column was equilibrated with 25 bed volumes of the same buffer. The Polybuffer 74 used to elute the column was titrated to pH 4.0 with HCl and diluted 8-fold from the original concentration. Throughout the equilibration and elution of the chromatofocusing column, the Polyexchanger and all buffers were degassed under vacuum and protected from carbon dioxide by 75-mL column of Ascarite.

Details of chromatographic procedures are given in the legends to the figures.

Assay of Cytokinin Oxidase Activity. Cytokinin oxidase activity was assayed by the copper-imidazole-enhanced reaction described by Chatfield and Armstrong (3). The reaction mixtures contained 100 mM imidazole-HCl (pH 6.5), 0.01 mM i^{6}Ade-2,8-^{3}H (0.05 μCi, specific radioactivity 100 μCi/μmol), 10 mM $CuCl_2$, and either 10 μL of column fraction or an appropriately diluted protein preparation in a total volume of 50 μL. The assays were

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[3] Abbreviations: Ade, adenine; Ado, adenosine; i^{6}Ade, N^6-(Δ^2-isopentenyl)adenine; io^{6}Ade, zeatin; i^{6}Ado, N^6-(Δ^2-isopentenyladenosine; io^{6}Ado, ribosylzeatin.

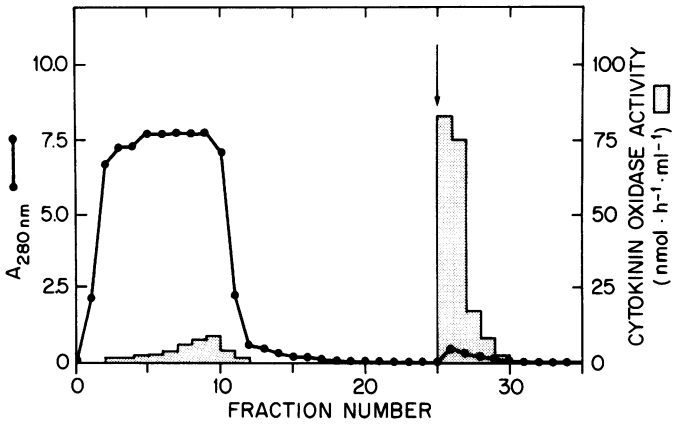

FIG. 1. Concanavalin A-Sepharose 4B chromatography of cytokinin oxidase activity from *P. vulgaris* cv Great Northern callus tissue. The protein recovered from 400 g of callus tissue (following homogenization, Polymin P treatment, and ammonium sulfate precipitation as described under "Materials and Methods") was dissolved in sufficient 50 mM bisTris-HCl (pH 6.5) to give a conductivity equivalent to that of 0.25 M ammonium sulfate. The sample was loaded on a 1 × 6-cm column (5 mL bed volume) of concanavalin A-Sepharose 4B equilibrated with 50 mM bisTris (pH 6.5) containing 0.25 M ammonium sulfate. The column was rinsed with 150 mL of the equilibration solution (to remove unbound protein) and then eluted with 100 mL of the same solution containing 0.1 M methylmannose. Fractions of 10 mL were collected at a flow rate of 25 mL/h. The arrow indicates the point at which methylmannose elution was begun.

incubated at 37°C for 30 min. The reactions were terminated, and cytokinin oxidase activity was determined by fractionating the reaction products by butyl acetate partitioning (column fractions) or by thin layer chromatography (all other enzyme preparations) as previously described (3).

Assay for Peroxidase Activity. Peroxidase activity was determined by a modification of the assay procedure described in the *Worthington Enzyme Manual* (12). Aliquots of column fractions were assayed in reaction mixtures containing 100 mM KH_2PO_4 (pH 7.0), 1 mM 4-aminoantipyrene, 80 mM phenol, and 1 mM H_2O_2. The reaction mixtures were incubated at 25°C, and the enzyme activity was determined from the rate of increase in *A* at 510 nm.

Protein Determinations. Aliquots 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 using the Folin phenol method of Peterson (9). BSA was used as a standard.

RESULTS

Affinity of Cytokinin Oxidase for Concanavalin A-Sepharose 4B. The protein preparation obtained from *P. vulgaris* cv Great Northern callus tissue as described in "Materials and Methods" was applied to a concanavalin A-Sepharose 4B column. The absorbance of the column eluate was monitored at 280 nm. Following sample application, the column was washed with the sample buffer until the A_{280} of the eluate had returned to baseline. The column was then eluted with the same buffer containing 0.1 M methylmannose. All column fractions were assayed for cytokinin oxidase activity using the copper-imidazole-enhanced assay described by Chatfield and Armstrong (3). The results are shown in Figure 1. Over 80% of the total cytokinin oxidase activity extracted from the Great Northern callus tissue bound to the concanavalin A affinity column and was eluted only after the addition of methylmannose to the eluting buffer. The protein fraction eluted by methylmannose was more than 20-fold enriched in cytokinin oxidase activity relative to the material applied to the column (Table I).

Fractionation of Partially Purified Cytokinin Oxidase Activity By Chromatofocusing. The composition of the protein fraction recovered from the concanavalin A-Sepharose 4B column by elution with methylmannose was examined by chromatofocusing on a Polyexchanger PBE94 column using a pH range from pH 7–pH 4. The fractions recovered from the column were assayed for both cytokinin oxidase activity and peroxidase activity. The results are shown in Figure 2. Several peaks of protein eluted from the column. The cytokinin oxidase activity eluted as a single peak in the pH range 4.8–5.1. The activity peaked at pH 5.0 and was separated from early and late peaks of peroxidase activity. The pooled cytokinin oxidase activity recovered from the column was enriched approximately 15-fold relative to the material applied to the column and approximately 455-fold relative to the crude homogenate (Table I). Native polyacrylamide gel electrophoresis of the active material recovered from the chromatofocusing column gave several bands of protein (data not shown). Cytokinin oxidase activity was associated with one band that was rather diffuse.

DISCUSSION

Most of the cytokinin activity recovered from homogenates of *P. vulgaris* cv Great Northern callus tissue was observed here to bind to concanavalin A-Sepharose 4B and to be eluted from this affinity column in the presence of methylmannose. This property

Table I. *Partial Purification of Cytokinin Oxidase Activity from P. vulgaris cv Great Northern Callus Tissue*

Fraction	Total Protein	Total Activity[a]	Specific Activity[a]	Purification	Yield
	mg	*μmol/h*	*μmol/h·mg*	*fold*	%
Crude homogenate from 1 kg tissue	1180.0	33.7	0.028	1	100
Polymin P: $(NH_4)_2SO_4$-treated fraction	755.0	28.6	0.038	1.3	85
Concanavalin A-Sepharose 4B (fractions 26–29)	28.7	22.8	0.796	28	68
Chromatofocusing (fractions 39–43)	1.4	18.3	13.1	455	54

[a] All values for cytokinin oxidase activity were obtained using the copper-imidazole-enhanced assay, with a copper concentration of 10 mM and an imidazole concentration of 100 mM, as described under "Materials and Methods." To approximate the values expected in assays performed using bisTris buffer in the absence of both copper and imidazole, the activities in this table should be divided by 30.

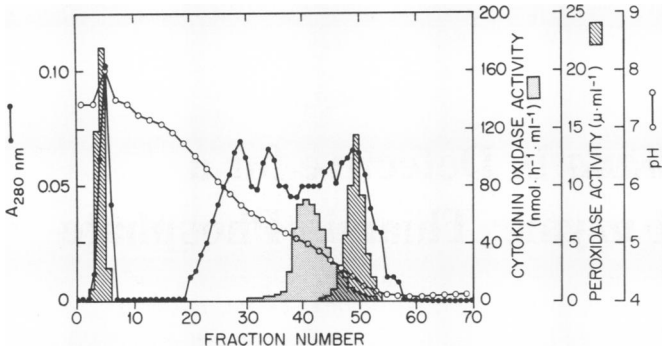

FIG. 2. Chromatofocusing of cytokinin oxidase activity from *P. vulgaris* cv Great Northern callus tissue. Fractions 26–29 from the concanavalin A-Sepharose 4B fractionation shown in Figure 1, containing the cytokinin oxidase activity eluted by methylmannose, were pooled. The pooled fractions were concentrated, and the buffer was exchanged for 25 mM imidazole-HCl (pH 7.5) using an Amicon ultrafiltration cell equipped with a YM10 membrane. An 8-mL aliquot of the concentrate, containing 8 mg of protein derived from the equivalent of 280 g of callus tissue, was loaded on a 0.9 × 32-cm column (20 mL bed volume) of Polyexchanger PBE94 equilibrated with the same buffer. The column was eluted with Polybuffer 74 (adjusted to pH 4.0 with HCl and diluted 1:8). Fractions of 4 mL were collected at a flow rate of 20 mL/h. The pH and A_{280} of the eluant were monitored, and the fractions were assayed for cytokinin oxidase activity (using the copper-imidazole-enhanced assay) and for peroxidase activity as described under "Materials and Methods."

of the enzyme provides a convenient and rapid method for the partial purification of cytokinin oxidase activity from Great Northern callus tissue. Moreover, the affinity of the enzyme for the lectin concanavalin A indicates that the cytokinin oxidase from Great Northern callus tissues is a glycoprotein.

The small fraction of cytokinin oxidase activity that failed to bind to the concanavalin A column in the experiments described here appears to be chromatographically distinct from the major activity (data not shown). However, we are not certain whether this fraction represents a distinct isozyme, a small amount of unglycosylated protein related to the major peak of activity, or a degradation product produced during the isolation procedure.

The ability of concanavalin A to bind cytokinin oxidase activities from other plant sources has not yet been examined. Evidence for heterogeneity in cytokinin oxidase activity is provided by the range of molecular weight estimates obtained for the enzyme in preparations from different plant tissues (4, 6, 10, 11), and it will be of interest to determine whether any of this apparent heterogeneity is related to glycosylation. The possibility that glycosylated and unglycosylated forms of cytokinin oxidase activity may exist in plant tissues could have a number of interesting implications for the compartmentalization and regulation of the enzyme.

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