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Zeatin Glycosylation Enzymes in *Phaseolus*[1]

Isolation of *O*-Glucosyltransferase from *P. lunatus* and Comparison to *O*-Xylosyltransferase from *P. vulgaris*

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ABSTRACT

An enzyme catalyzing the formation of *O*-glucosylzeatin in immature embryos of *Phaseolus lunatus* was purified 2500-fold using ammonium sulfate precipitation followed by affinity and anion exchange chromatography. The enzyme uses *trans*-zeatin as substrate (K_m 28 micromolar) but not *cis*-zeatin, ribosylzeatin, or dihydrozeatin. Both UDP-glucose and UDP-xylose can serve as glycosyl donors, with K_ms of 0.2 and 2.7 millimolar, respectively, for the formation of *O*-glucosylzeatin and *O*-xylosylzeatin. In comparison, the UDPxylose-zeatin:*O*-xylosyltransferase (JE Turner, DWS Mok, MC Mok, G Shaw [1987] Proc Natl Acad Sci USA 84: 3714–3717) isolated by the same procedures from *P. vulgaris* embryos uses only UDP-xylose as donor substrate and the K_ms for both zeatin and UDP-xylose are much lower (2 and 3 micromolar, respectively). The chromatographic behavior on affinity columns and molecular weights (approximate M_r 44,000 daltons) of the two enzymes are similar. Results from substrate competition experiments and enzyme separation by anion exchange HPLC indicate a single, distinct, zeatin *O*-glycosylation enzyme occurs in embryos of each of these *Phaseolus* species.

Zeatin is a highly active, naturally occurring cytokinin. Interconversion between zeatin and its derivatives may be important in regulating the level of active cytokinins in plant tissues. Interspecific variations in the metabolism of zeatin have been detected in immature embryos of *Phaseolus* (7). One of the genetic differences between *P. vulgaris* and *P. lunatus* concerns the conversion of zeatin to *O*-glycosyl derivatives. Incubation with [^{14}C]zeatin[2] resulted in recovery of *O*-xylosylzeatin in *P. vulgaris* embryos but led to the formation of *O*-glucosylzeatin in *P. lunatus* embryos. The enzyme involved in the former conversion, UDPxylose-zeatin:*O*-xylosyltransferase, was isolated from *P. vulgaris* embryos and partially purified (16). The enzyme did not catalyze the formation of *O*-glucosylzeatin (11, 16). These studies suggest that the occurrence of different *O*-glycosyl derivatives of zeatin in the two species is most likely the result of the presence of distinct metabolic enzymes rather than substrate availability. To provide further support for this interpretation, we have isolated the enzyme mediating the formation of *O*-glucosylzeatin from *P. lunatus* embryos. In this paper we describe the procedures for purification of the enzyme and compare its properties to those of the *O*-xylosyltransferase of *P. vulgaris*.

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[2] Abbreviations: [^{14}C]zeatin, *trans*-[8-^{14}C]zeatin; Z, *trans*-zeatin; DHZ, dihydrozeatin; *c*-Z, *cis*-zeatin; OGZ, *O*-glucosylzeatin, *O*-β-D-glucopyranosyl-*trans*-zeatin; 9GZ, *trans*-zeatin 9-glucoside, 9-β-D-glucopyranosyl-*trans*-zeatin; OXZ, *O*-xylosylzeatin, *O*-β-D-xylopyranosyl-*trans*-zeatin; UDPG, uridine diphosphate glucose; UDPX, uridine diphosphate xylose; UDP-galactose, uridine diphosphate galactose; ADPG, adenosine diphosphate glucose; TEA, triethylamine.

MATERIALS AND METHODS

Plant Materials

Immature embryos, 5 to 10 mm in length, of *Phaseolus lunatus* cv Kingston and *P. vulgaris* cv Great Northern were used for the isolation of *O*-glucosyltransferase and *O*-xylosyltransferase, respectively. The embryos were obtained from plants grown in the field or in the greenhouse at 25°C (day) and 20°C (night) and a photoperiod of 14 h.

Chemicals

trans-Zeatin, *trans*-ribosylzeatin, *cis*-zeatin, dihydrozeatin, UDPG, UDPX, ADPG, and UDP-galactose were obtained from Sigma. Column materials for affinity chromatography, Blue Sepharose CL-6B, and AgAMP agarose, were obtained from Pharmacia and Sigma, respectively. [8-^{14}C]Zeatin and its labeled *cis*-isomer (24 mCi/mmol) were synthesized from 6-chloro[8-^{14}C]purine (Amersham) following procedures reported earlier (6). [^{14}C]-Labeled *trans*-ribosylzeatin and dihydrozeatin were obtained by incubating [^{14}C]zeatin with crude extracts of PRPP-ribosyltransferase (1) and zeatin reductase (recently identified in our laboratories [11]), respectively.

Enzyme Isolation

For the isolation of *O*-glucosyltransferase, immature embryos of *P. lunatus* were homogenized in one part (v/w) of extraction buffer (55 mM Tris-HCl [pH 7.2], containing 0.5

mM EDTA and 5 mM DTT) with a Tissuemizer equipped with a Microprobe Shaft (Tekmar). The homogenate was centrifuged at 27,000*g* for 20 min. Cell debris was removed, and the enzyme was precipitated from the supernatant by ammonium sulfate (30–60%). After centrifugation at 12,000*g* for 15 min, the pellet was redissolved in extraction buffer (v/w) and centrifuged at 27,000*g* for 20 min. The supernatant was transferred to Centriprep 30 (Amicon) filtration tubes and centrifuged at 3,000*g*, rinsed three times with the extraction buffer, and concentrated to approximately 0.5 mL/g of embryo.

Affinity Chromatography

AgAMP-agarose (1 mL/2 g of embryo) was packed in a 10 mL syringe and equilibrated with the extraction buffer. Enzyme extracts were loaded onto the column and washed with 2 bed volumes of the same buffer. The eluate was passed onto a Blue Sepharose column (mL/g of tissue) and washed with two bed volumes of extraction buffer. The enzyme was eluted with four bed volumes of buffer containing UDPG (2.5 mM). The eluate was concentrated to 150 μL by centrifugation using Centriprep 30 (at 3000*g*) and then Centricon 30 (at 4360*g*) ultrafiltration tubes.

Anion Exchange HPLC

A Beckman model 110B dual-pump HPLC system with an anion exchange column (Aquapore AX-300, 10 μm particle size, 30 nm pore size, 4.6 × 220 mm; Brownlee) was used. The mobile phase consisted of buffers A (0.02 M Tris-HCl [pH 7.2], containing 0.5 mM EDTA and 5 mM DTT) and B (buffer A with 0.5 M KCl added). Enzyme samples purified by affinity columns were applied to the anion exchange column and eluted with a linear gradient of buffer B (10–100%) in buffer A over 45 min. The flow rate was 1 mL/min and 1 mL fractions were collected.

Enzyme Assays

Enzyme activity was determined under the following conditions: enzyme extract (100 μL), UDPG (3 mM), ATP (0.5 mM), $MgCl_2$ (0.05 M), and 0.025 μCi of [^{14}C]zeatin (0.001 μmol) in 200 μL at pH 8 (buffered with 1 mM Tris) and 27°C. One mL of cold ethanol was added after 1 h and the mixture was placed at 4°C for 15 min and then centrifuged at 27,000*g* for 20 min. The supernatant was concentrated to 100 μL *in vacuo* (Speed Vac Concentrator, Savant) and analyzed by HPLC using a reversed phase C_{18} column (see below). The amount of protein was determined using a Bio-Rad protein assay kit following procedures recommended by the manufacturer.

Cytokinin Analysis

To detect and quantify cytokinins resulting from enzymic reactions, a Beckman model 110A dual-pump HPLC system with a reversed-phase column (Ultrasphere ODS C_{18}, 5 μm particle size, 4.6 × 250 mm; Altex) was used. The aqueous buffer consisted of 0.2 M acetic acid adjusted to pH 3.5 with TEA. Samples were eluted with a linear gradient of methanol (5–50% over 90 min) in TEA buffer. The flow rate was 1 mL/min and 0.5 mL fractions were collected. A combination of a Beckman model 117 flow-through isotope detector and Isco UV monitor allowed the initial identification of fractions of interest. Radioactivity in these fractions was determined in Ready-Gel scintillation fluid (Beckman) with a Beckman LS 7000 scintillation counter.

Confirmation of Reaction Products

HPLC fractions containing the reaction product were treated with β-glucosidase, following the procedures reported earlier (7), and rechromatographed by HPLC at pH 4.8. In addition, standards of *O*-glucosylzeatin (synthesized in the laboratory of G. S.), *O*-xylosylzeatin and [^{14}C]-*O*-xylosylzeatin (synthesized earlier [15]), and 9-glucosylzeatin (provided by Dr. R. Durley, Monsanto, St. Louis) were used to confirm the identity of cytokinins obtained from enzyme reactions.

Enzyme Characterization

The pH optimum was determined using enzymes purified by ammonium sulfate precipitation and anion exchange chromatography. Standard assays were performed at pH 6 to 10 with 0.5 increments. Phosphate and Tris-HCl buffers were used for pH 6.0 to 7.5 and pH 7.5 to 10, respectively. The reaction velocity was determined by taking aliquots at 10 min intervals up to 1 h. The mol wt of the enzyme was determined by Sephadex G-100 gel filtration using a 0.9 × 90 cm column and a flow rate of 0.25 mL/min. Mol wt standards used were bovine serum albumin (M_r 66,000), carbonic anhydrase (M_r 29,000), Cyt *c* (M_r 12,400), and aprotinin (M_r 6,500).

Substrate Specificity and K_m Determinations

The following compounds were tested as substrates of the enzyme: [^{14}C]zeatin, [^{14}C]dihydrozeatin, [^{14}C]ribosylzeatin, and [^{14}C]*cis*-zeatin. The K_m value for [^{14}C]zeatin was determined using enzyme preparations purified with both affinity and anion exchange columns. The concentrations of [^{14}C]zeatin ranged from 0.8 to 7.2 μM, with 0.4 μM increments, at 2.6 mM UDPG. K_ms for UDPG and UDPX were determined using concentration ranges from 0.05 to 4 mM with 0.15 mM increments, at 5 μM [^{14}C]zeatin.

Comparison with Zeatin O-Xylosyltransferase

The isolation procedures described above were also used to isolate the *O*-xylosyltransferase from *P. vulgaris* embryos, with the exception that UDPX (in place of UDPG) was used to elute this enzyme from the Blue Sepharose affinity column. Substrate specificities of both enzymes for a number of cytokinins were compared after each purification step. Other specific experimental conditions are noted in the "Results" section.

RESULTS

Enzymic Reaction and Identification of Reaction Products

Enzyme activity was detected only in the soluble fraction of the tissue extract. Incubation of purified enzyme preparations with [^{14}C]zeatin and UDPG resulted in one radioactively labeled product. The reaction required ATP and $MgCl_2$. A typical profile of the reaction mixture after HPLC analysis is presented in Figure 1. The product coeluted with authentic samples of *O*-glucosylzeatin (Fig. 1B), and after treatment with β-glucosidase, the radioactivity shifted to the position of zeatin (Fig. 1C). No other glycosyl derivatives, such as *N*-glucosides of zeatin, could be detected.

Enzyme Purification

A major consideration in the purification protocol was the removal of other enzyme activities, primarily ribosyl- and phosphoribosyl transferases, kinases, and cytokinin oxidases which interfere with the assays. AgAMP agarose columns effectively retained the ribosyltransferases and kinases but had only low affinity for the *O*-glucosyltransferase. The majority of the enzyme activity (74%) was recovered in the eluate (Table I), with a 2-fold purification over the fraction obtained after the ammonium sulfate precipitation. Blue Sepharose,

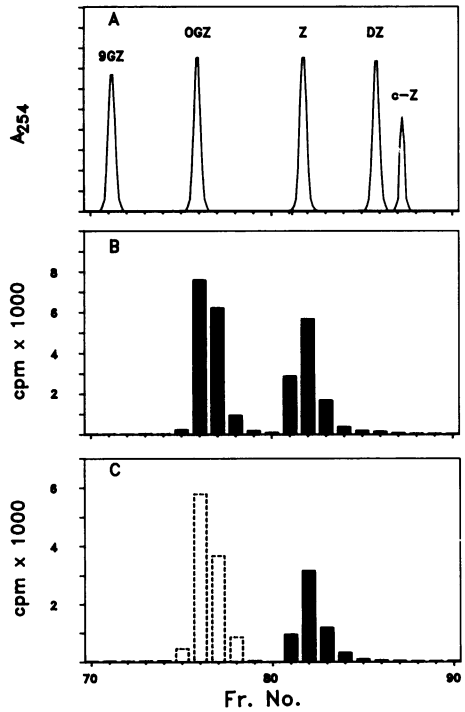

Figure 1. Separation of cytokinin standards and analyses of products of enzymic reactions by HPLC. A, Cytokinin standards; B, product of enzymic reaction after incubation with labeled zeatin (Z), UDPG, and purified enzyme (extracted from 100 mg of embryos); C, products (approximately 11,000 cpm) obtained from (B) treated with β-glucosidase. Bars with discontinuous border indicate elution position of radioactivity before treatment. The HPLC analyses were performed at pH 3.5. Samples were eluted with a gradient of methanol (5–50% over 90 min) and 0.5 mL fractions were collected.

Table I. *Purification of O-Glucosyltransferase from Embryos of P. lunatus cv Kingston*

Sample[a]	Protein	Activity	Specific Activity	Enrichment	Recovery
	μg/assay	*cpm of product*	*cpm/μg protein*	*-fold*	%
Crude	1260	924	0.73	1	
$(NH_4)_2SO_4$	752	6486	9	12	100
AgAMP	325	4791	15	20	74
BS-6B	70	10123	145	197	38
AX-300[b]	8.6	15948	1854	2528	6

[a] 55,000 cpm = 1 nmol of product (*O*-glucosylzeatin). [b] Based on one fraction with the highest enzyme activity.

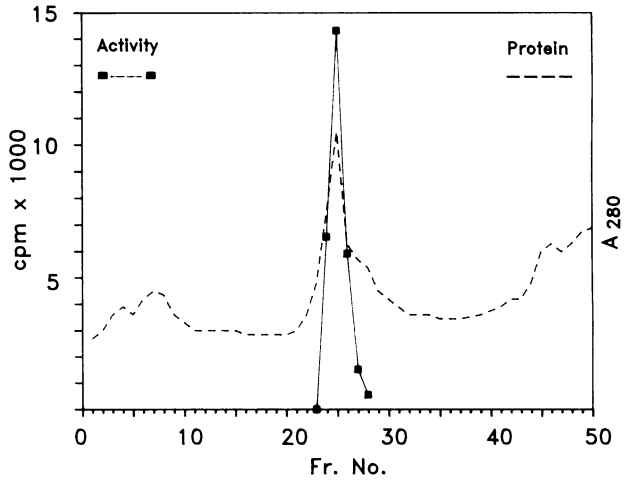

Figure 2. Distribution of enzyme activity after AX-300 anion exchange HPLC. Enzymes were eluted with KCl (10–100% over 45 min) at pH 7.2 and 1 mL fractions were collected. Each assay contained the equivalent of enzyme extracted from 500 mg of embryos. Extracts were purified by ammonium sulfate precipitation and affinity columns prior to anion exchange HPLC.

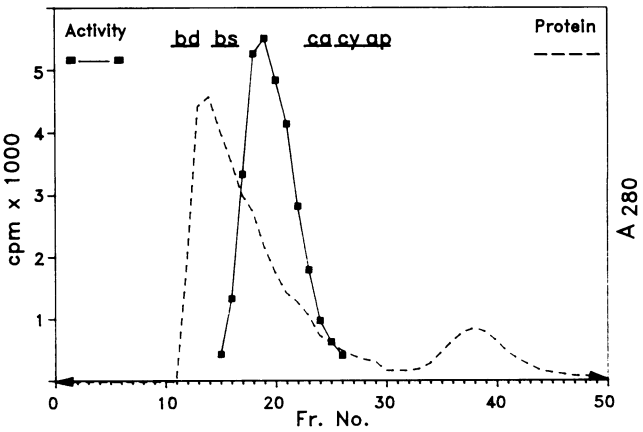

Figure 3. Distribution of *O*-glucosyltransferase activity eluted from Sephadex G-100 gel filtration column. Enzyme was extracted from 2 g of *P. lunatus* embryos. The flow rate was 0.25 mL/min and 1 mL fractions were collected. (bd, blue dextran; bs, bovine serum albumin; ca, carbonic anhydrase; cy, Cyt *c*; ap, aprotinin.)

Table II. *Substrate Specificity and K_ms of O-Glucosyltransferase Isolated from Embryos of P. lunatus cv Kingston*

Substrate	Reaction	K_m
		μM
trans-Zeatin	+	28
Ribosylzeatin	−	
Dihydrozeatin	−	
cis-Zeatin	−	
UDP-glucose	+	216
UDP-xylose	+	2,700
ADP-glucose	−	
UDP-galactose	+	ND[a]

[a] ND, not determined.

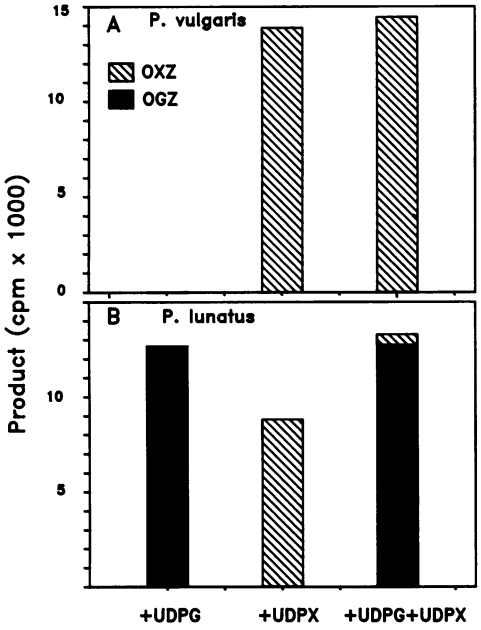

Figure 4. Products from incubating *O*-xylosyltransferase from *P. vulgaris* (A) and *O*-glucosyltransferase from *P. lunatus* (B) with UDPG, UDPX, and UDPG plus UDPX, in the presence of labeled zeatin. Enzymes were purified by affinity and anion exchange chromatography. Each assay contained the equivalent of enzyme extracted from 120 mg of *P. lunatus* or 500 mg of *P. vulgaris* embryos.

which has high affinity for a wide range of enzymes using dinucleotides as substrates or cofactors, retained the *O*-glucosyltransferase, whereas cytokinin oxidases were removed in the initial wash. The enzyme was selectively eluted from the Blue Sepharose column by four bed volumes of Tris buffer containing 2.5 mM of UDPG. Approximately 50% of the activity was recovered, with a 10-fold purification over the previous step (Table I). The enzyme was further purified by anion exchange HPLC. Enzyme activity was detected in fractions 24 to 28 (Fig. 2). The fraction with the highest activity represented a further 12-fold purification. The specific activity of the purified enzyme was 34 pmol/μg protein/h. The recovery of enzyme activity based on an HPLC fraction with the highest activity was 6%. The amount of protein after this purification sequence was approximately 0.12% of the total protein in the crude extract.

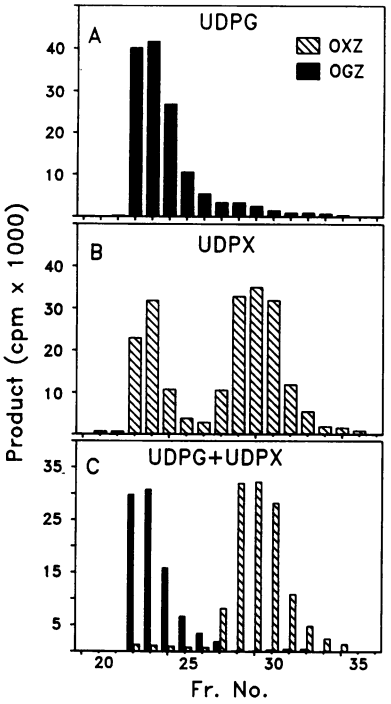

Figure 5. Distribution of enzyme activity, of combined samples, eluted from anion exchange chromatography. A, Incubation with UDPG (3 mM) and labeled zeatin (50,000 cpm); B, incubation with UDPX (3 mM) and labeled zeatin; C, incubation with UDPG (3 mM) plus UDPX (3 mM) and labeled zeatin. *O*-glucosyltransferase and *O*-xylosyltransferase were isolated from 8 g of *P. lunatus* and *P. vulgaris* embryos, respectively. Preparations were purified separately by ammonium sulfate precipitation, AgAMP, and Blue Sepharose affinity columns. The preparations were then combined and purified on an AX-300 column. Fractions of 1 mL were collected and assayed for enzyme activity by incubating with the glycosyl donors.

Characterization of the Enzyme

The conversion of zeatin to *O*-glucosylzeatin was linear up to 1 h. The pH optimum of the reaction was approximately 8. From the elution positions of the enzymes and the protein standards in relation to that of blue dextran (V_o) after gel filtration, the mol wt of the enzyme was 44,000 D ±4,000 (Fig. 3). Of the four cytokinins tested, zeatin was the only substrate for the enzyme (Table II). The K_m of zeatin was 28 μM. A number of compounds were tested as glycosyl donors in the enzymic reaction (Table II). UDPG served efficiently as the sugar donor with a K_m of 216 μM. In addition, UDPX was found to be a substrate, but the K_m (2.7 mM) for this compound was 10-fold higher than for UDPG. ADPG did not serve as a substrate but UDP-galactose was used by the enzyme to form an *O*-galactosyl derivative of zeatin.

Comparison to UDPX:Zeatin O-Xylosyltransferase from *P. vulgaris*

It was somewhat unexpected that partially purified *O*-glucosyltransferase isolated from *P. lunatus* could mediate the formation of *O*-xylosylzeatin (in the presence of zeatin and UDPX) although the activity was much lower than for *O*-xylosyltransferase of *P. vulgaris* (16). These observations

prompted a series of experiments to compare the *O*-glucosylation enzyme from *P. lunatus* with the *O*-xylosyltransferase of *P. vulgaris*.

To examine the possibility that additional glycosylation enzymes were lost from embryos during Blue Sepharose affinity chromatography, UDPX and UDPG were applied separately to affinity columns to elute enzyme preparations of *P. vulgaris* or *P. lunatus*. Interestingly, enzymes of both species could be eluted from the affinity column using either sugar dinucleotide. These observations indicate that the relative recognition, to the glycosyl donors, of the enzymes isolated from the two species was not the result of differential recovery after affinity chromatography.

Competition experiments using UDPG, UDPX, or equal amounts of both substrates, in the presence of labeled zeatin, were carried out with enzymes obtained from embryos of both species. The *P. vulgaris* enzyme did not recognize UDPG as a substrate and no interference with the formation of *O*-xylosylzeatin occurred by the addition of UDPG (Fig. 4A). The enzyme preparation isolated from *P. lunatus* recognized both donor substrates and in the competition experiment *O*-glucosylzeatin was formed almost exclusively (Fig. 4B). These results suggest that there are two distinct *O*-glycosylation enzymes, one occurring in *P. lunatus* and the other in *P. vulgaris* embryos.

With the objective of testing this interpretation, experiments were designed based on the different elution positions of the two enzymes from anion exchange columns. Enzyme extracts from *P. vulgaris* and *P. lunatus* were purified separately by ammonium sulfate precipitation and the two types of affinity columns. The preparations were then combined and chromatographed using the AX-300 anion exchange column. Aliquots of each fraction eluted were incubated with UDPG, UDPX, or UDPG plus UDPX. Enzyme eluted in fractions 22 to 25 catalyzed the formation of *O*-glucosylzeatin (Fig. 5A) as well as *O*-xylosylzeatin (Fig. 5B), characteristic of the glucosyltransferase. Enzyme contained in fractions 27 to 31 catalyzed only the formation of *O*-xylosylzeatin (Fig. 5B), indicating the presence of *O*-xylosyltransferase. Incubation with both UDPG and UDPX resulted in the formation primarily of *O*-glucosylzeatin by the early fractions and exclusively of *O*-xylosylzeatin by the later fractions (Fig. 5C), again illustrating that the two enzyme activities can be separated. These results provide further evidence in favor of the occurrence of only a single zeatin *O*-glycosylation enzyme in embryos of each of the two *Phaseolus* species.

DISCUSSION

The results presented above indicate the occurrence of a second *O*-glycosylation enzyme in *Phaseolus* embryos. This enzyme, UDPG:zeatin-*O*-glucosyltransferase, found in immature embryos of *P. lunatus*, has several characteristics in common with the *O*-xylosyltransferase isolated from *P. vulgaris* embryos. The mol wt of the enzyme as determined by gel filtration is similar to that of the *P. vulgaris* enzyme. Both enzymes recognize zeatin but not ribosylzeatin or *cis*-zeatin as substrates. Moreover, the enzymes possess similar chromatographic properties, with the exception of the elution position from the anion exchange column, indicating different charges of the enzymes.

The major distinction between the two enzymes is the broader substrate specificity of the *O*-glucosyltransferase. This enzyme is capable of mediating the transfer of glucose as well as xylose moieties, while the *O*-xylosyltransferase is more specific and only catalyses the transfer of the xylose moiety. In addition, the affinities of the enzymes for other substrates are also widely different. It may be speculated that the enzyme of *P. vulgaris* represents adaptation to a more specialized function. The findings also confirm our earlier interpretation that the occurrence of species-specific metabolites is related to the presence of distinct metabolic enzymes (11).

O-Glucosylzeatin has been identified in many other plant species (2, 12, 14), but reports of purification of the enzyme(s) catalyzing the glycosyl transfer have not appeared from any of these sources. However, two *N*-glucosyl transferases that catalyze the formation of 7- and 9-glucosyl derivatives of several cytokinins as well as other purines have been isolated (3). As expected, the *O*-glucosyltransferase described here does not mediate formation of *N*-glucosides. We have not been able to detect any *N*-glucosyl derivatives in either *in vivo* or *in vitro* metabolism studies using *Phaseolus* embryos. It appears that *N*-glucosylation of zeatin (4, 5, 13) may not be pronounced in this genus.

We have identified a number of genotypic differences in cytokinin metabolism in *Phaseolus* embryos as well as in callus tissues (7–9). Many of the differences are related to differential side chain removal or conjugation of naturally occurring cytokinins. Recently, we have also identified genetic differences in side chain reduction of zeatin (10, 11), and the enzyme, zeatin reductase, has been partially purified. These results suggest that the genetic differences in cytokinin metabolism can be associated with distinct enzymes. The isolation and purification of these enzymes will be useful in identifying the mechanisms regulating the expression of these enzymes and thereby the levels of cytokinin metabolites in *Phaseolus* tissues. The purified *O*-glycosylation enzymes are being used for production of mono-specific antibodies as probes for gene cloning experiments and to further characterize the interspecific differences in zeatin metabolism.

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