Plant Physiol. (1989) 90, 1630–1635
0032-0889/89/90/1630$06/$01.00/0

Received for publication March 13, 1989
and in revised form April 15, 1989

# An Enzyme Mediating the Conversion of Zeatin to Dihydrozeatin in *Phaseolus* Embryos[1]

**Ruth C. Martin, Machteld C. Mok, Gordon Shaw, and David W. S. Mok***

*Department of Horticulture, Oregon State University, Corvallis, Oregon 97331* (R.C.M., M.C.M., D.W.S.M.): *and Department of Chemistry, University of Bradford, Yorkshire, BD7 1DP, United Kingdom* (G.S.)

## ABSTRACT

**A reductase catalyzing the conversion of zeatin to dihydrozeatin was detected in soluble fractions of immature *Phaseolus vulgaris* embryos. The enzyme was partially purified by ammonium sulfate fractionation and affinity, gel filtration, and anion exchange chromatography. NADPH was the only cofactor required for enzyme activity, and the pH optimum was 7.5 to 8.0. The enzyme did not recognize compounds closely related to zeatin, such as ribosylzeatin, *cis*-zeatin, *O*-xylosylzeatin, $N^6$-($\Delta^2$-isopentenyl)adenine, or $N^6$-($\Delta^2$-isopentenyl)adenosine. No conversion of dihydrozeatin to zeatin by the enzyme was observed. Two forms of the reductase could be separated by either gel filtration or anion exchange high performance liquid chromatography. The high molecular weight isozyme ($M_r$ 55,000 ± 5,000) eluted as the second peak from the anion exchange column, while the low molecular weight isozyme ($M_r$ 25,000± 5000) was less negatively charged. The results suggest that side chain reduction occurs at the free base level. In addition, *Phaseolus* embryos are useful for the detection of zeatin-specific metabolic enzymes.**

Zeatin and dihydrozeatin are highly active cytokinins. Conversion of [$^{14}$C]zeatin to [$^{14}$C]dihydrozeatin was first observed in embryo axes of *Phaseolus vulgaris* by Sondheimer and Tzou (17). Our earlier studies on the metabolism of [$^{14}$C]zeatin in *Phaseolus* embryos led to the discovery of *O*-xylosylzeatin and *O*-xylosyldihydrozeatin (7, 11), and subsequently, *O*-xylosyldihydrozeatin was identified as a naturally occurring cytokinin (our unpublished data). Dihydrozeatin derivatives have also been found in vegetative parts of *P. vulgaris* (19). These findings suggest the presence of a cytokinin reductase in this genus. Reduction of the $N^6$-side chain may play a role in the regulation of active cytokinin levels, especially in plant species such as *P. vulgaris* in which saturated cytokinins display higher activity than their unsaturated counterparts in callus bioassays (9, 10).

We have isolated two enzymes, zeatin *O*-xylosyltransferase and zeatin *O*-glucosyltransferase, from immature embryos of *P. vulgaris* and *P. lunatus*, respectively (3, 12, 18). These results indicate that developing embryos of this genus are ideal for the identification of cytokinin specific enzymes. Recently, we have isolated an enzyme catalyzing the conversion of zeatin to dihydrozeatin. This paper describes the procedures of enzyme purification and some important characteristics of zeatin reductase.

## MATERIALS AND METHODS

### Plant Materials

Immature embryos, 5 to 10 mm in length, of *Phaseolus vulgaris* cv Great Northern were used for enzyme isolation. Embryos were obtained from plants grown in the field or greenhouse (at 25/20°C, day/night and a photoperiod of 14 h).

### Chemicals

Z,[2] *trans*-ribosylzeatin, c-Z, DHZ, i$^6$Ade, i$^6$Ado, NADH, NADPH, and mol wt markers were obtained from Sigma. ipn$^6$Ade and ipn$^6$Ado were synthesized in our laboratories (9). [$^{14}$C]Zeatin, its labeled *cis*-isomer, [$^{14}$C]dihydrozeatin, and *O*-xylosyl[$^{14}$C]zeatin (24 mCi/mmol) were synthesized from 6-chloro[8-$^{14}$C]purine (Amersham) following procedures reported earlier (6, 15). $^{14}$C-Labeled i$^6$Ade and i$^6$Ado (24 mCi/mmol) were synthesized previously (10). $^{14}$C-Labeled *trans*-ribosylzeatin was obtained by incubating [$^{14}$C]zeatin with crude extracts of PRPP-ribosyltransferase (2). Column materials for affinity chromatography, Blue Sepharose CL-6B, and AgAMP agarose, were obtained from Pharmacia and Sigma, respectively.

### Enzyme Isolation

Immature embryos of *P. vulgaris* were homogenized in one part (v/w) of extraction buffer (0.1 M phosphate [pH 7.3], containing 5 mM DTT and 0.5 mM EDTA) with a Tissuemizer equipped with a Microprobe Shaft (Tekmar). The homogenate was centrifuged at 27,000*g* for 20 min. Proteins in the supernatant were fractionated by ammonium sulfate. The fraction precipitating between 30 and 60% saturation of ammonium sulfate was centrifuged at 12,000*g*. The pellet was redissolved in extraction buffer and centrifuged at 27,000*g* for 20 min. The supernatant was concentrated by centrifugation

[1] Research was supported by the Oregon Agricultural Experiment Station and by grants from National Science Foundation (DCB-8818620 and US-UK Cooperative Sciences INT-8513026). This is technical paper No. 8789 of the Oregon Agricultural Experiment Station.

[2] Abbreviations: [$^{14}$C]zeatin, *trans*-[8-$^{14}$C]zeatin; Z, *trans*-zeatin; c-Z, *cis*-zeatin; DHZ, dihydrozeatin; i$^6$Ade, $N^6$-($\Delta^2$-isopentyl)adenine; i$^6$Ado, $N^6$-($\Delta^2$-isopentenyl)adenosine; ipn$^6$Ade, $N^6$-isopentyladenine; ipn$^6$Ado, $N^6$-isopentyladenosine.

at 3,000*g* using Centriprep 10 or 30 (Amicon) filtration tubes and desalted with three rinses of extraction buffer.

### Affinity Chromatography

AgAMP-agarose (0.5 mL/g of embryos) was packed in glass columns and equilibrated with phosphate buffer (0.05 M [pH 7.3] containing 5 mM DTT and 0.5 mM EDTA). Enzyme extracts were loaded onto the column and washed with three bed volumes of the same buffer. The eluate was passed directly onto a Blue Sepharose column (1 mL/g of embryos) which was then washed with two additional bed volumes of the same buffer. The enzyme was eluted from the Blue Sepharose column with three bed volumes of phosphate buffer (0.05 M [pH 8] containing 5 mM DTT, 0.5 mM EDTA, 5 mM NADPH, and 0.5 M KCl). The eluate was concentrated using Centriprep 10 or 30 (at 3000*g*) and then Centricon 10 or 30 (at 4000*g*). When preparations were not immediately processed, glycerol (20%) was added and the preparation was stored at −20°C.

### Gel Filtration

Enzyme extracts purified by affinity chromatography were concentrated to 400 μL and loaded onto a Sephadex G-100 gel filtration column (0.9 × 60 cm) equilibrated with extraction buffer. The flow rate was 0.184 mL/min and fractions of 0.85 mL were collected.

### 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 phosphate [pH 7.2] containing 10 mM DTT, 0.5 mM EDTA, and 20% glycerol) and B (buffer A with 1 M KCl added). Enzyme preparations purified by affinity columns and gel filtration were applied to the anion exchange column and eluted with a linear gradient of buffer B (5–95%) in buffer A over 45 min. The flow rate was 1 mL/min and 1 mL fractions were collected.

### Enzyme Assays

The composition of the standard enzyme assay was as follows: enzyme extract (100 μL), NADPH (0.25 mM), and [$^{14}$C]zeatin (0.6 nmol, 0.014 μCi) in 200 μL phosphate buffer (0.1 M [pH 8]). The mixture was incubated for 1 h at 27°C and the reaction was stopped by adding 1 mL of cold ethanol. After allowing the mixture to stand at 4°C for 15 min, it was 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 separate 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 4.8 with triethylamine (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 an Isco UV monitor allowed the initial identification of fractions of interest. Radioactivity in these fractions was determined in Ready-Gel scintillation fluid (Beckman) using a Beckman LS 7000 scintillation counter.

### Enzyme Characterization

The effect of cofactors on the conversion of zeatin to dihydrozeatin has been determined previously (12). The reaction was NADPH dependent and did not require ATP or cations. The optimal concentration of NADPH was confirmed in the present study using enzyme extracts obtained from ammonium sulfate precipitation. The pH optimum was determined using enzymes purified by ammonium sulfate precipitation and affinity columns. Preparations obtained after gel filtration were also used to confirm the pH requirement of the isozymes. Standard assays were performed at pH 6 to 9 with 0.5 increments. Phosphate buffers were used for the assays, while duplicate assays using Tris-HCl were included for the higher pH values (8–9). 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 the elution position of enzyme activity from the gel filtration column as described above. 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) were used as molecular weight standards.

### Substrate Specificity and $K_m$ Determination

The following compounds were tested as substrates of the enzyme: [$^{14}$C]zeatin, *cis*-[$^{14}$C]zeatin, [$^{14}$C]dihydrozeatin, [$^{14}$C]ribosylzeatin, *O*-xylosyl[$^{14}$C]zeatin, [$^{14}$C]i$^6$Ade, and [$^{14}$C]i$^6$Ado. The $K_m$ for [$^{14}$C]zeatin of the isozymes was determined using preparations purified by ammonium sulfate, affinity chromatography, and gel filtration. The concentrations of [$^{14}$C]zeatin ranged from 4 to 21 μM.

## RESULTS

### Identification of Primary Substrate and Cofactor

The origin of *O*-xylosyldihydrozeatin (11), either via a reduction of zeatin to dihydrozeatin followed by *O*-xylosylation or by a direct reduction of *O*-xylosylzeatin, was determined by incubating enzyme extracts with labeled compounds. No labeled reduced product was recovered when enzymes were incubated with *O*-xylosyl[$^{14}$C]zeatin *in vitro* whereas [$^{14}$C]zeatin was converted to a labeled product which coeluted with dihydrozeatin at both pH 4.8 (Fig. 1) and 3.5. The reaction was NADPH dependent but higher concentrations (0.5 or 1 mM) were inhibitory (Table I). NADH could not substitute for NADPH. Extraction with phosphate buffer increased enzyme activity approximately 3-fold over extraction with Tris-HCl buffer.

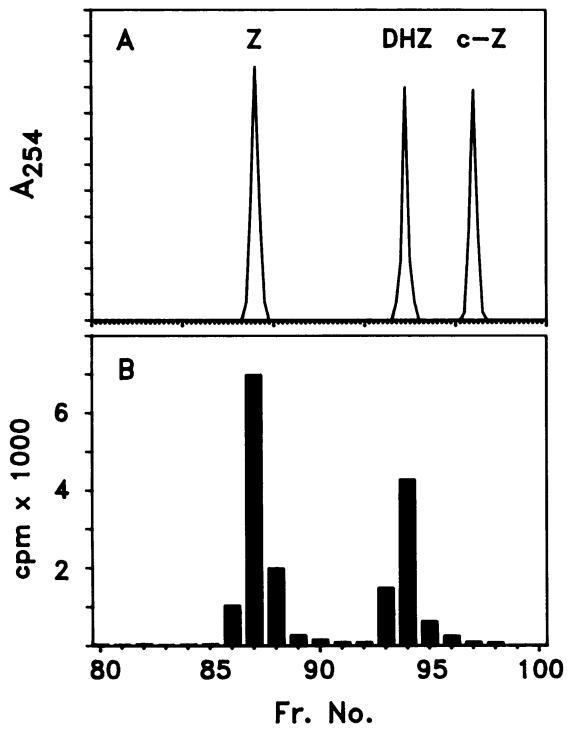


**Figure 1.** Conversion of zeatin to dihydrozeatin by zeatin reductase. A, Cytokinin standards separated by HPLC on a $C_{18}$ reversed phase column; B, separation of reaction mixture after incubation of labeled zeatin with reductase. The assay contained enzyme extracted from 600 mg of GN embryos and purified by ammonium sulfate precipitation and AgAMP and Blue Sepharose affinity columns. Standards and reaction mixture were eluted from a reversed phase $C_{18}$ column at pH 4.8 by increasing concentration of MeOH (5–50% over 90 min). Fractions of 0.5 mL were collected.

**Table I.** *Conversion of Zeatin to Dihydrozeatin at Various Concentrations of NADPH*

Each assay contained enzyme extracted from 1.2 g of embryos, purified by ammonium sulfate precipitation. Labeled zeatin (50,000 cpm, 1 nmol) was incubated with enzyme in a reaction mixture (200 μL, pH 8) for 1 h at 27°C.

| NADPH | Product |
|---|---|
| *mM* | *% of DHZ/DHZ + Z* |
| 0.0312 | 36 |
| 0.0625 | 54 |
| 0.125 | 55 |
| 0.25 | 45 |
| 0.5 | 25 |
| 1.0 | 14 |

### Initial Purification by Affinity Chromatography

An important consideration in the purification procedure was the removal of interfering enzymes, such as ribosyl- and phosphoribosyl transferases, kinases, and cytokinin oxidases. AgAMP columns effectively retained the ribosyltransferases and kinases but had low affinity for the reductase. Blue Sepharose, which has high affinity for a wide range of enzymes that use dinucleotides as a substrate or a cofactor, retained the reductase but allowed cytokinin oxidases to be removed in the initial wash. The reductase was eluted from the affinity column using 5 mM NADPH and 0.5 M KCl. Due to the high affinity of the reductase to Blue Sepharose, when either NADPH (up to 20 mM) or KCl (up to 2 M) was used, only about one-tenth of the enzyme activity obtained by the combined use of both compounds was recovered.

### Isozymes of Zeatin Reductase

Enzyme preparations precipitated by ammonium sulfate and eluted from affinity columns were further purified by gel filtration chromatography. The distribution of enzyme activity was bimodal with an early peak of activity at fractions 18 to 20 and a later peak at fractions 28 to 30 (Fig. 2). This observation suggested the existence of isozymes. To confirm the results obtained with gel filtration, enzyme preparations purified by ammonium sulfate precipitation and affinity columns were loaded directly onto an anion exchange column. Again, reductase activity was detected in two regions, centering around fractions 25 and 28 (Fig. 3).

By comparing the elution positions of the isozymes relative to mol wt standards, the mol wt of the two isozymes were estimated as 55,000 ± 5,000 and 25,000 ± 5,000. The two forms of the reductase were designated as HMW (high mol wt) and LMW isozymes. Gel filtration fractions containing the HMW and LMW isozymes were applied separately to the anion exchange column. The HMW isozyme was recovered in fractions 30 to 32 (Fig. 4A), while the LMW isozyme eluted in fractions 24 to 26 (Fig. 4B), indicating a less negative charge of this isozyme.

### Purification and Stability of Zeatin Reductase

The sequence of purification steps consisted of ammonium sulfate precipitation, affinity columns, gel filtration, and anion exchange HPLC. After gel filtration, the two isozymes were purified separately. The reductase was enriched 11-fold after affinity columns (Table II) with the recovery of 58% of the initial enzyme activity. The final enrichment after anion exchange chromatography was 31-fold for the HMW and 12-fold for the LMW isozymes.

The specific activity of the zeatin reductase after the five purification steps was low. This was not entirely unexpected, since many dinucleotide-requiring reductases have been reported to be unstable (4, 5, 14). Our preliminary studies indicated that the reductase became unstable when diluted. Gel filtration and anion exchange HPLC in particular resulted in substantial losses of activity. These losses were indicated by the protein concentration of samples after each purification step (Table II). For example, after anion exchange HPLC, the protein content was only 0.14% (HMW isozyme) and 0.07% (LMW isozyme) of the original extract, suggesting a high degree of purification. The specific activity should have been much higher had there been no substantial loss of enzyme activity. The decrease in specific activity of the LMW isozyme after anion exchange HPLC as compared with gel filtration (Table II) further supports this interpretation. Addition of glycerol (20%) to concentrated preparations prevented the loss of activity during short-term storage (Table III); however,

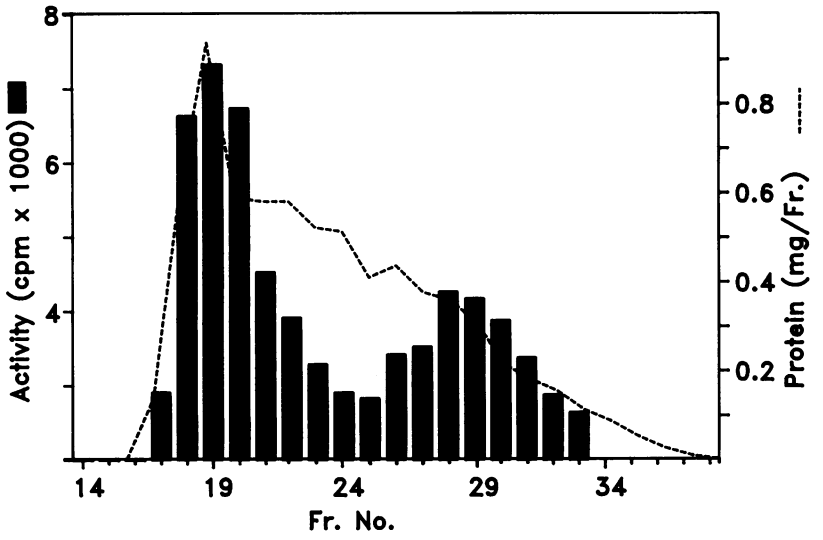


**Figure 2.** Distribution of reductase activity after elution from Sephadex G-100 gel filtration column. Enzyme was extracted from 8 g of GN embryos and purified by ammonium sulfate precipitation and AgAMP and Blue Sepharose affinity columns. The enzyme preparation was concentrated to 400 μL and loaded onto a Sephadex G-100 column (0.9 × 60 cm). Enzyme was eluted with phosphate buffer (0.1 M) at a flow rate of 0.184 mL/min. The fraction size was 0.85 mL; 0.75 mL of each fraction was concentrated further and used for the activity assay with the remainder used for protein determination.

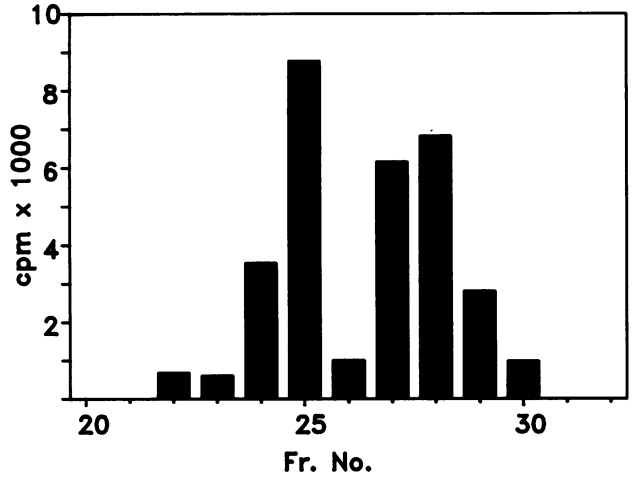


**Figure 3.** Distribution of reductase activity after anion exchange HPLC. Enzyme was extracted from 10 g of GN embryos and purified by ammonium sulfate precipitation and AgAMP and Blue Sepharose affinity columns. The preparation was concentrated to 170 μL and loaded on an anion exchange HPLC column (AX-300, 0.46 × 22 cm). Enzymes were eluted with increasing concentration of KCl (5–95% over 45 min) dissolved in 0.02 M phosphate buffer at pH 7.2. Fractions of 1 mL were collected; 500 μL of each fraction was concentrated to 50 μL and assayed for enzyme activity.

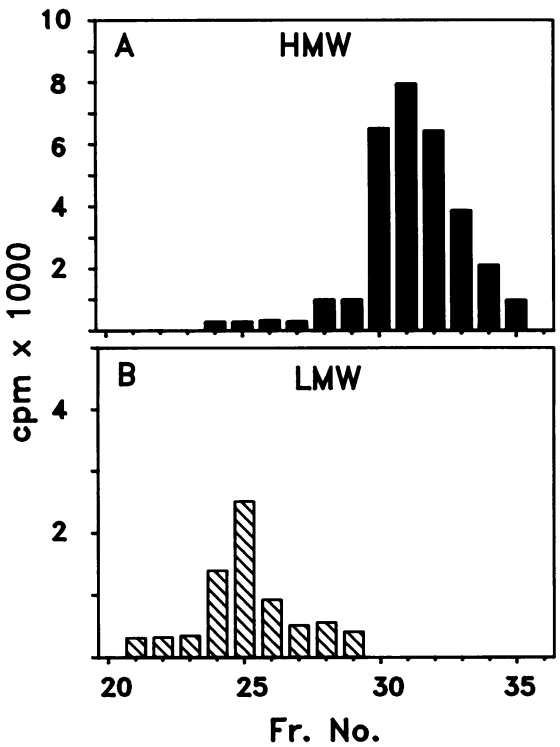


**Figure 4.** Distribution of reductase activity of HMW and LMW isozymes separated by gel filtration after elution from anion exchange HPLC. Enzyme was extracted from 30 g of GN embryos and purified by ammonium sulfate precipitation and affinity columns. Eluant was concentrated and purified by gel filtration. Fractions containing high and low mol wt isozymes were collected separately. Pooled fractions of each isozyme were combined, concentrated, and subjected to anion exchange HPLC. Fractions of 1 mL were collected, concentrated, and assayed for activity.

glycerol in the anion exchange buffers was less effective in preventing the loss of enzyme activity (Table II).

### Characterization of Zeatin Reductase

The conversion of zeatin to dihydrozeatin was linear up to 1 h with a pH optimum between 7.5 and 8 (Fig. 5). These properties were identical for enzyme preparations containing either or both isozymes. Of the four zeatin-related compounds (*trans*-zeatin, *cis*-zeatin, *trans*-ribosylzeatin, and *O*-xylosylzeatin) tested, *trans*-zeatin was the only substrate of the reductase. Incubation of the enzyme with labeled $i^6$Ade and $i^6$Ado did not result in the formation of $ipn^6$Ade or $ipn^6$Ado (Fig. 6). In addition, the possibility of the enzyme mediating the reverse reaction was examined by incubation of zeatin reductase with [$^{14}$C]dihydrozeatin in the presence of NADP. No formation of [$^{14}$C]zeatin was detected. The $K_m$ for *trans*-zeatin of the HMW isozyme was 70 ± 10 μM. The estimated

**Table II.** *Purification of Zeatin Reductase from Embryos of Phaseolus*

Enzymes were extracted from 30 g of embryos and purified by the steps listed. Fractions containing the high mol wt (HMW) and low mol wt (LMW) isozymes eluted from the gel filtration column were collected separately and purified by an AX-300 column. Fractions containing the highest specific activity were used to compare the degree of purification (1 pmol = 55 cpm).

| Sample | Activity | Specific Activity | Enrichment | Protein Recovery | |
|---|---|---|---|---|---|
| | *cpm of DHZ* | *pmol/μg protein/h* | *-fold* | *μg/g fresh weight* | % |
| Crude | 1,977 | 0.014 | 1 | 13,034 | 100 |
| $(NH_4)_2SO_4$ | 2,587 | 0.036 | 2.59 | 8,797 | 67 |
| Affinity columns | 7,936 | 0.156 | 11.33 | 1,156 | 8.8 |
| Gel filtration (HMW) | 6,287 | 0.057 | 4.12 | 503 | 3.8 |
| Gel filtration (LMW) | 10,407 | 0.200 | 14.54 | 236 | 1.8 |
| AX-300 (HMW) | 7,847 | 0.438 | 31.74 | 20 | 0.14 |
| AX-300 (LMW) | 1,495 | 0.168 | 12.17 | 10 | 0.07 |

**Table III.** *Effect of Glycerol on the Stability of Zeatin Reductase Stored at −20°C*

Each assay contained enzyme extracted from 600 mg of embryos, purified by ammonium sulfate precipitation and affinity columns. Glycerol was added to the eluate.

| Treatment | Enzyme Activity | |
|---|---|---|
| | Day 1 | Day 3 |
| | *cpm of DHZ* | |
| Phosphate buffer | 6943 | 1652 |
| Phosphate buffer with 20% glycerol | 6803 | 6242 |

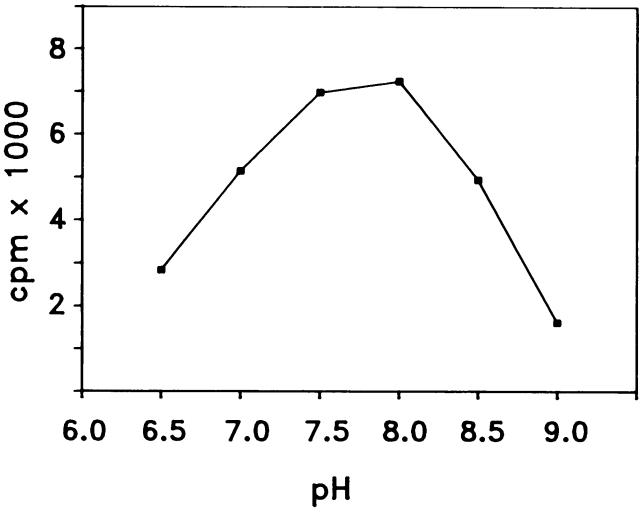


**Figure 5.** Effect of pH on the conversion of zeatin to dihydrozeatin. Each assay contained enzyme extracted from 500 mg of GN embryos and purified by affinity columns. Values obtained using phosphate buffers were presented. Tris buffer gave similar results.

$K_m$ for the LMW isozyme varied greatly between experiments with values ranging from 100 to 230 μM. Instability of the isozyme after gel filtration may have contributed to the variation.

## DISCUSSION

We have shown that a NADPH-dependent zeatin reductase can be isolated from *Phaseolus* embryos. The two most interesting features of the enzyme reside in its high substrate specificity and the presence of isozymes with different mol wt. The enzyme recognizes *trans*-zeatin, but not *cis*-zeatin. Neither ribosylzeatin nor cytokinins such as $i^6$Ade which lack

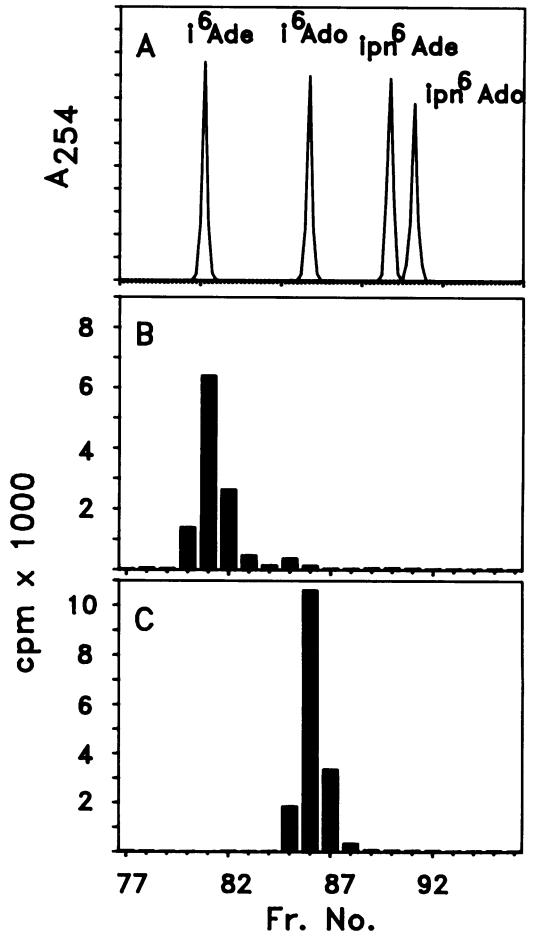


**Figure 6.** Incubation of $N^6$-($\Delta^2$-isopentenyl)adenine) and $N^6$-($\Delta^2$-isopentenyl)adenosine with zeatin reductase. Each assay contained enzyme extracted from 0.5 g of GN embryos assayed under the standard conditions described. The reaction mixture was analyzed by reversed phase $C_{18}$ column at pH 3.5. Samples were eluted with increasing gradient of MeOH (15–100% over 85 min). Fractions of 0.5 mL were collected.

a hydroxyl group in the side chain were substrates for the enzyme. The high affinity for zeatin exhibited by this enzyme and the two *O*-glycosylation enzymes isolated previously from *Phaseolus* embryos (3, 12, 18) supports the importance of zeatin as a cytokinin in plant tissues.

The two isozymes could be clearly distinguished by gel filtration and anion exchange chromatography. The molecular mass of the HMW isozyme was approximately twofold greater than the LMW isozyme, suggesting a difference in the number of subunits between these two isozymes. Preliminary studies indicate that reductase activity also differs among *Phaseolus* species. It will be of interest to characterize further the isozymes and to determine their relative amounts in embryos of these species.

The reductase is less stable than the zeatin *O*-xylosyl- and *O*-glucosyltransferases isolated earlier. Although the enzyme retains its activity when stored in the presence of glycerol, dilution of the enzyme preparation during purification by gel filtration and ion exchange chromatography resulted in significant losses of activity. However, these steps are necessary to separate the isozymes. Instability *in vitro* may be a general feature of this type of enzyme since activity losses of reductases and oxidoreductases under these circumstances have been reported previously (4, 5, 14).

The function of the enzyme may be related to the regulation of cytokinin levels in plant tissues (13). Cytokinins with a saturated $N^6$-side chain are much more active than their unsaturated counterparts in callus bioassays of *P. vulgaris*. Moreover, the saturated cytokinins are resistant to attack by degradative enzymes (1, 8, 20), therefore, rapid conversion of zeatin to dihydrozeatin could preserve high cytokinin activity.

Three metabolic enzymes which are specific for zeatin have now been identified in *Phaseolus* embryos. The high activities of these enzymes in embryos, together with indications that cytokinins in embryos are produced endogenously rather than being transported from the maternal tissues (16), suggest that the regulation of cytokinin levels in embryos may be autonomous. Some of our future studies will focus on the genetic mechanisms regulating these enzymes and their effects on the availability of cytokinins for growth and development.

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