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Title: Identification of Cytokinin Reductase in Phaseolus Embryos

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A critical balance of hormones is necessary for normal plant development. Therefore, precise mechanisms must exist to maintain proper levels of cytokinins, a group of hormones regulating cell division and differentiation. Structural modifications of the naturally occurring cytokinin, zeatin; including the oxidation, O-glycosylation and reduction of the N^6-side chain; may represent such control mechanisms to regulate active cytokinin levels. This study concerns the identification and partial purification of the enzyme responsible for the reduction of zeatin in Phaseolus embryos.

The presence of zeatin reductase was indicated by the occurrence of dihydrozeatin derivatives and O-xylosyldihydrozeatin in vegetative and embryonic tissues, respectively, in this genus. A cytokinin reductase catalyzing the conversion of zeatin to dihydrozeatin was initially detected in soluble fractions of immature embryos of Phaseolus. Subsequently, the enzyme was partially purified by ammonium sulfate fractionation and affinity, gel filtration and anion exchange chromatography. NADPH was required for enzyme activity, but was found to be inhibitory at high (0.5-1.0 mM) concentrations. The reaction had

a pH optimum of 7.5-8.0 and was linear up to one hour. The enzyme did not recognize compounds closely related to zeatin, such as ribosylzeatin, cis-zeatin, O-xylosylzeatin, N^6-(Δ^2-isopentenyl)adenine or N^6-(Δ^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 HPLC. 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 and Phaseolus embryos are useful for the detection of zeatin specific metabolic enzymes. Preliminary studies also indicate that quantitative differences in reductase exist in Phaseolus species.

As dihydrozeatin (and possibly its derivatives) is more active than zeatin in most of the Phaseolus bioassays, the zeatin reductase identified in this study may be representative of enzymatic processes converting zeatin to a more active compound in selected tissues. The significance of qualitative and quantitative variations of this enzyme between species and tissues will be examined further to study this hypothesis.

Identification of Cytokinin Reductase in *Phaseolus* Embryos

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

ABBREVIATIONS

DHZ	dihydrozeatin
AMP	adenosine-5'-monophosphate
Ade	adenine
Ado	adenosine
i^6Ade	N^6-(Δ^2-isopentenyl)adenine
i^6AMP	N^6-(Δ^2-isopentenyl) adenosine-5'-monophosphate
i^6Ado	N^6-(Δ^2-isopentenyl)adenosine
ipn^6Ade	N^6-isopentyladenine
ipn^6Ado	N^6-isopentyladenosine
ATP	adenosine-5'-triphosphate
$MgCl_2$	Magnesium chloride
NADH	nicotinamide-adenine dinucleotide
NADPH	nicotinamide-adenine dinucleotide phosphate
[^{14}C]zeatin	trans-[8-^{14}C]zeatin
Z	trans-zeatin
c-Z	cis-zeatin
ZMP	zeatin riboside-5'-monophosphate
ZR	zeatin riboside

IDENTIFICATION OF CYTOKININ REDUCTASE IN PHASEOLUS EMBRYOS

INTRODUCTION

Hormones play an important role in plant growth and development. Cytokinins, a group of plant hormones, mediate cell division and differentiation. Most cytokinins are isoprenoid derivatives with zeatin being the most active naturally occurring cytokinin. A critical balance of hormones is necessary for normal plant development; therefore, precise mechanisms must exist to maintain proper cytokinin levels. Such mechanisms have not been elucidated, partly because very few genetic mutations in cytokinin biosynthesis and metabolism are available. A mutant which has been studied is the cytokinin overproducer (ove) in moss (Ashton et al., 1979). Other abnormalities in plant development associated with altered cytokinin levels include the tumorous interspecific hybrids of Nicotiana (Smith, 1972) and the crown gall tumors produced by Agrobacterium tumefaciens (Braun, 1958). A paucity of cytokinin mutants may indicate that severe derangements in cytokinin biosynthesis and metabolism are likely to be lethal in higher plants.

As an alternative to mutant analysis, the possibility of utilizing intrinsic variations to study the regulation of cytokinin metabolism has been investigated in Phaseolus. This approach consisted of detecting intrinsic variations followed by genetic and biochemical characterization. Several genetic variations have been identified in cell cultures. Differences in structure activity relationship occur between P. lunatus and P. vulgaris callus tissues (Mok et al., 1978).

Cytokinins with a saturated side chain (dihydrozeatin and ipn^{6}Ade) were more active than their unsaturated counterparts (zeatin and i^{6}Ade) in P. vulgaris; whereas in P. lunatus, cytokinins with an unsaturated side chain showed either equal or greater activity than the saturated analogues. As the activities of cytokinins with a saturated side chain were comparable in the both species, the inactivation of cytokinins with an unsaturated side chain in P. vulgaris was responsible for the differences observed (Mok et al., 1982b). The presence of cytokinin oxidase, which selectively cleaves the unsaturated isoprenoid side chain, may be related to the differential structure-activity relationships. Another intrinsic variation was differential cytokinin requirements (dependent vs. independent growth) in tissue culture. Genetic analysis indicated that cytokinin autonomy was conditioned by a single locus in P. vulgaris (Mok et al., 1980).

The metabolism of [^{14}C]zeatin in immature embryos of various Phaseolus species was compared to determine if genetic differences in cytokinin metabolism also occurred in organized tissue (Lee et al., 1985; Mok and Mok, 1987). Qualitative differences in the O-glycosylation of zeatin between P. vulgaris and P. lunatus led to the identification of novel zeatin metabolites (O-xylosylzeatin and its riboside) which occur only in P. vulgaris. Zeatin was converted to O-glucosylzeatin in P. lunatus embryos. These interspecific differences were associated with the occurrence of a distinct enzyme in each of the species. The O-xylosyl- and O-glucosyl- zeatin transferases have been isolated from P. vulgaris and P. lunatus, respectively. The O-glycosylation of zeatin may represent functions other than storage or transport, as O-xylosylzeatin was biologically more active (100 fold)

than zeatin in P. vulgaris callus bioassays (Mok & Mok, 1987).

Recently dihydro-derivatives of zeatin (O-xylosyldihydrozeatin and its riboside) were also identified in embryos of Phaseolus (Mok and Mok, 1987). The reduction of the zeatin side chain must be a naturally occurring process, as the conversion of zeatin to dihydrozeatin has also been reported in embryo axes of P. vulgaris (Sondheimer and Tzou, 1971), and the saturated derivatives also occur in vegetative parts (Wang et al., 1977). The enzymatic conversion, however, has not been characterized. It is important to isolate the enzyme(s) responsible for the reduction of zeatin because the reaction may represent a method of converting zeatin to a more active form, especially in species where dihydrozeatin is more active or where zeatin is susceptible to side chain degradation. Genotypic differences in the reduction enzymes could also be used in conjunction with the two zeatin specific O-glycosylation enzymes to examine cytokinin gene structure and expression using molecular and immunological techniques. This thesis describes the methods of enzyme purification as well as the preliminary characterization of the zeatin reductase.

LITERATURE REVIEW

Kinetin, the first "cell division promoter" identified, was isolated from autoclaved herring sperm DNA, and characterized as 6-furfurylaminopurine (Miller et al., 1955, 1956). The term "cytokinin" was proposed for compounds which exhibit kinetin-like biological activity (Skoog et al., 1965). Cytokinins are important in cell division, morphogenesis in tissue culture, release of lateral buds from apical dominance, delay of leaf senescence, and chloroplast development. Zeatin (6-(4-hydroxy-3-methylbut-trans-2amylamino) purine), the first naturally occurring cytokinin identified, was isolated from immature kernels of Zea mays (Letham, 1963; Letham et al., 1964; Miller, 1961). Since the discovery of kinetin, many compounds have been synthesized to determine the structural components necessary for cytokinin-like activity in bioassay systems (Matsubara, 1980; Skoog and Armstrong, 1970). Structural requirement for high activity include an adenine molecule having an intact purine ring with an N^6-substituent of moderate size. Activity was proportional to the length of side chain with the optimum of 5 carbons. Side chain planarity has also been shown as an important factor in imparting high levels of cytokinin activity (Hecht et al., 1970). Most of the naturally occurring cytokinins, which have been isolated and identified, are structurally related to i^6Ade, although derivatives of N^6-benzyladenine have also been reported as naturally occurring cytokinins (Horgan et al., 1975; Ernst et al., 1983). Certain compounds which are not N^6-substituted adenine derivatives also exhibit cytokinin-like activity in bioassay systems, most notably the phenyl ureas and their derivatives (Shantz and Steward,

1955; Bruce et al., 1965; Bruce and Zwar, 1966). The precise mechanism of action of adenine and phenylurea types of cytokinins is unknown.

Metabolism of Cytokinins

Several recent reviews on cytokinin biosynthesis and metabolism have been published (Letham and Palni, 1983; McGaw, 1987; Morris, 1986). The range of naturally occurring cytokinins and metabolites formed as a result of exogenous feeding experiments are represented by the following conversions: (1) formation of nucleosides and nucleotides (Parker & Letham, 1974; Sondheimer and Tzou, 1971), (2) N-glucoslyation at the 9-, 7-, or 3- position of the purine ring (Cowley et al.,1978; Fox et al., 1973; Paces and Kaminek, 1976), (3) attachment of the amino acid alanine to the 9-position of the purine ring to form lupinic acid (Duke et al., 1978; Parker et al., 1978), (4) O-glycosylation (Duke et al., 1979; Lee et al., 1985; Letham et al., 1977; Mok and Mok, 1987; Morris, 1977; Parker et al., 1973; Turner et al., 1987), (5) degradation of the N^6-side chain (Chatfield and Armstrong, 1987; Mok et al., 1982b; Paces and Kaminek, 1976; Terrine and Laloue, 1980; Whitty and Hall, 1974) and (6) reduction of the N^6-side chain of zeatin (Palmer et al., 1981b; Parker et al., 1978; Sondheimer and Tzou, 1971).

While the exact function of cytokinin derivatives is obscure, possible roles for the various metabolites may include: elicitation of a specific physiological response (active forms of cytokinin), translocation, storage, detoxification, cytokinin inactivation (products formed to lower endogenous cytokinin levels) and protection from

degradative processes. Although the active form(s) of cytokinin remains to be identified, the free base is generally more active than the ribonucleosides and ribonucleotides in bioassay systems (Hecht et al., 1971, 1975). Growth studies on low density tobacco cell cultures using i^6Ade, benzyladenine and their corresponding ribosides also indicated that conversion of cytokinin riboside to the free base is necessary for activity; therefore, cytokinin free base may be the active form (Laloue and Perthe, 1982). While cytokinin nucleotides are abundant in nature and are the predominant metabolites formed during short term feeding experiments, their function remains unknown.

Alanine conjugates and N-glucosides are stable compounds with much lower activity than their parent cytokinins (Letham et al., 1983). It has been suggested that N-glucosides are involved in storage functions or in decreasing the level of active cytokinins (Letham et al., 1982). Oxidative cleavage of the unsaturated N^6-side chain by cytokinin oxidase and other degradative enzymes results in irreversible loss of cytokinin activity. Saturation and O-glucosylation render the cytokinin resistant to enzymatic attack; therefore side chain reduction and the formation of O-glucosides may be important mechanisms in maintaining active levels of cytokinins. Dihydrozeatin and the O-glucosides of zeatin were shown to be stable compounds (Palmer et al., 1981b). The latter accumulated in decapitated P. vulgaris leaves, but decreased rapidly during lateral bud development and other phases of plant growth. These observations suggest that the O-glucosides may also function as storage compounds (Palmer et al., 1981a). The role of various other metabolites is rather uncertain, but should become more clear as the mode and site(s) of cytokinin action become better understood.

Biosynthesis of Free Cytokinins

Cytokinins occur as free components as well as modified bases in the anticodon region of specific t-RNAs. Elucidation of the cytokinin biosynthetic pathway is problematic since endogenous cytokinins are present in extremely low concentrations and the most likely precursors of cytokinins (adenine and its riboside and ribotides) are central to many other metabolic processes. There have been two pathways proposed for the biosynthesis of free cytokinins; the degradation of t-RNA molecules (Chen and Hall, 1969) and de novo biosynthesis (Miura and Miller, 1969).

While the degradation of t-RNA molecules may contribute to the pool of free cytokinins, there is strong evidence that this is not the sole pathway involved in free cytokinin biosynthesis (Letham and Palni, 1983). Several studies determined that the rate of t-RNA turnover is insufficient to account for the levels of endogenous cytokinins in plants (Hall, 1973; Trewavas, 1970). Free cytokinins were also found to be much more abundant than t-RNA bound cytokinins in pea roots (Short and Torrey, 1972). In order for t-RNA degradation to account for the high level of free cytokinins in this tissue, either a slow breakdown of free cytokinins or a very rapid turnover rate of t-RNA would be necessary. Furthermore, the cytokinins which are present in the t-RNA are often structurally distinct from the free cytokinins. For example, free zeatin is mainly in the trans isomer of zeatin, but the cis isomer is present in t-RNA. It is possible, however, that the cytokinins are rapidly metabolized upon release from the t-RNA. Further evidence for

an alternate pathway of cytokinin biosynthesis was obtained by tracing a labelled adenosine analogue in autotrophic tobacco tissue culture. While little or no label was incorporated into the t-RNA, label was recovered as the corresponding i^6Ado analogue (Chen and Eckert, 1976).

The second proposed pathway is de novo synthesis of cytokinins. Early efforts to elucidate the biosynthetic pathway were based on in vivo experiments using labelled adenine and adenosine. When Rhizopogon roseolus cultures were fed labelled adenine and hypoxanthine, labelled ribosylzeatin was recovered (Miura and Miller, 1969). If R. roseolus was incubated with labelled adenosine, the label was recovered as i^6Ado and i^6Ade. R. roseolus also converted i^6Ado to ribosylzeatin (Miura and Hall, 1973). These results suggest that the isopentenyl side chain is attached to the amino group of adenine or adenosine and subsequently hydroxylated to form zeatin or ribosylzeatin.

An enzyme isolated from cell-free preparations of the slime mold Dictyostelium discoideum catalyzed the synthesis of i^6AMP from AMP and isopentenylpyrophosphate (Taya et al, 1978). A similar enzyme has since been isolated (Chen and Melitz, 1979) and partially purified from cytokinin-autonomous tobacco callus tissue (Chen, 1982). Cytokinin biosynthesis has also been studied in crown gall tumor tissues which are known to have elevated cytokinin levels (Morris, 1986). The tmr gene of the T-DNA from Agrobacterium tumefaciens codes for the enzyme Δ^2-isopentenylpyrophosphate:5-AMP- Δ^2-isopentenyl transferase (ipt transferase). These enzymes did not use adenine or adenosine as substrates. The molecular weight of the plant enzyme is approximately twice the molecular weight of the bacterial enzyme. If the transfer of the isopentenyl group is the first step in cytokinin biosynthesis; then

the product, i^6AMP, must be trans-hydroxylated to form trans-zeatin derivatives. Microsomal enzymes which hydroxylate i^6Ade and i^6Ado to form zeatin and ribosylzeatin have been reported from cauliflower (Chen and Leisner, 1984). Studies of several woody species indicated that only certain species were able to hydroxylate i^6Ade to form zeatin suggesting that plants may differ in their biosynthetic pathways for zeatin (Einset, 1986). It is uncertain at what level (base, riboside or ribotide) hydroxylation actually occurs.

Enzymes Involved in Cytokinin Side Chain Modification

Several enzymes that modify the N^6-side chain of cytokinins have been characterized. Perhaps the most predominant and the most extensively studied of these enzymes are the oxidases which cleave the N^6-side chain resulting in inactive products. Enzymes that catalyze the removal of the isoprenoid side chain were isolated from tobacco tissue cultures (Paces et al., 1971), maize (Whitty and Hall, 1974), Vinca rosea tumor tissue (Scott et al., 1982; McGaw and Horgan, 1983), and Phaseolus (Chatfield and Armstrong; 1986, 1987). The substrate specificity of the cytokinin oxidases appears to be consistent regardless of the plant source. i^6Ade, i^6Ado, zeatin and ribosylzeatin were substrates for the various oxidases, while cytokinins with a saturated side chain (ipn^6Ade, ipn^6Ado, dihydrozeatin and dihydroribosylzeatin) and some other synthetic cytokinins (N^6-benzyladenine and kinetin) were not degraded by these oxidases. Additional cytokinins tested and recognized as substrates for oxidases in Vinca rosea tumor and Zea mays included 9-alanylzeatin and zeatin 7-

and 9- glucosides. O-glucosyl derivatives and benzyladenine derivatives were not degraded by these oxidases. Despite the similarities in substrate specificity, the molecular weight of these enzymes differed substantially. The molecular weights of the oxidases were estimated as follows: 88,000 (Whitty and Hall, 1974) and 94,400 (McGaw and Horgan, 1983) from Zea mays; 25,100 (McGaw and Horgan, 1983) from Vinca rosea tumor tissue; approximately 66,000 from P. vulgaris (Armstrong, pers. comm.).

Two enzymes responsible for the O-glycosylation of zeatin have been isolated and partially purified. An enzyme catalyzing the formation of O-xylosylzeatin was isolated from P. vulgaris embryos (Turner et al., 1987). The enzyme, UDP-xylose:zeatin O-xylosyl transferase, recognizes trans zeatin and dihydrozeatin but does not recognize cis- or ribosyl-zeatin. UDP-xylose serves as the pentose donor but UDP-glucose does not. A second O-glycosylation enzyme, which catalyzes the formation of O-glucosylzeatin, was isolated from P. lunatus embryos (Dixon et al., 1989). This enzyme could use both UDP-glucose and UDP-xylose as glycosyl donors, but favored UDP-glucose (K_m for UDP-glucose was .2 mM vs. 2.7 mM for UDP-xylose). O-glucosylzeatin was readily converted to zeatin by B-glucosidase while 7- and 9- glucosylzeatin could not be hydrolyzed to form zeatin (Letham et al., 1975). The interconversion between the various forms of metabolites with modified side chains and free bases may play an important role in regulating active cytokinin levels in plants.

Zeatin Metabolism in Phaseolus

The metabolism of zeatin has been investigated in various plant organs in Phaseolus including roots, stems, leaves, embryo axes, and embryos. In the roots of intact Phaseolus plants, the major cytokinin identified was O-glucosylzeatin and the minor cytokinins included zeatin, ribosylzeatin, and O-glucosylribosylzeatin (Scott and Horgan, 1984). In contrast, the major endogenous cytokinins identified in the stems of decapitated Phaseolus plants were zeatin riboside, dihydrozeatin riboside, and their corresponding nucleotides. These compounds may play a role in cytokinin transport. Minor cytokinins present in the stem included dihydrozeatin-O-glucoside and zeatin-O-glucoside ribotide (Palmer, Horgan, and Wareing, 1981). Dihydrozeatin-O-glucoside was identified as the major endogenous cytokinin in the leaves of decapitated debudded bean plants (Wang et al., 1977) and dihydrozeatin riboside was identified as a minor component (Wang and Horgan, 1978). Detached leaves of P. vulgaris were used to compare the stability and metabolism of labelled zeatin, zeatin-O-glucoside, and their dihydro-derivatives (Palmer et al., 1981b). The O-glucosides were more stable than the free bases, while the dihydrozeatin derivatives were more stable than the zeatin derivatives. The major metabolites of zeatin and dihydrozeatin were their O-glucosides, supporting the identification of dihydrozeatin-O-glucoside as the major endogenous cytokinin in the leaves of decapitated Phaseolus plants. Feedings of labelled zeatin resulted in a greater proportion of adenine and adenine derivatives than feedings with other cytokinins, probably due to the action of cytokinin

oxidase. It has been suggested that a concentration gradient exists; zeatin derivatives decrease and dihydrozeatin derivatives increase from the root to the leaves (Palmer, Horgan and Wareing, 1981) due to the relative activities of cytokinin oxidase and the interconversion of zeatin and dihydrozeatin. These processes could be important in the regulation of relative levels of active cytokinins in different plant parts during different stages of development.

Initial studies examining the endogenous cytokinins in P. vulgaris L. var. Pinto embryos suggested the presence of zeatin, dihydrozeatin, and their ribosides (Krasnuk et al., 1971). These compounds, in addition to their nucleotides, were later identified in immature seeds of P. coccineus L. (Sodi and Lorenzi, 1982). The metabolism of exogenously supplied ^{14}C-zeatin has also been investigated in Phaseolus embryo axes and embryos. When labelled zeatin was incubated with excised bean (P. vulgaris) axes, the radioactivity was recovered as ZR, ZMP, and their dihydro-derivatives (Sondheimer and Tzou, 1971). The metabolism of exogenously applied ^{14}C-zeatin has also been compared in embryos of several Phaseolus species (P. vulgaris, P. lunatus, P. acutifolius, and P. coccineus Lam.) (Lee et al., 1985; Mok and Mok, 1987). All species converted ^{14}C-zeatin to ZR, ZMP, and O-glucosyl-9-ZR. Several major differences in ^{14}C-zeatin metabolism between these species were detected. O-xylosylzeatin and O-xylosylzeatin-riboside were recovered from all species except P. lunatus. In P. lunatus, the major metabolite recovered was O-glucosylzeatin. The zeatin specific O-xylosyltransferase and the O-glucosyltransferase have been isolated from P. vulgaris and P. lunatus, respectively (Turner et al., 1987; Dixon et al., 1989). These

studies indicate that interspecific differences in the metabolism of ^{14}C-zeatin exist in embryos. The dihydro-derivatives of O-xylosylzeatin and O-xylosylribosylzeatin were were also detected in Phaseolus embryos.

Metabolites of ^{14}C-zeatin were also compared between embryos, seed coats and pod tissues of P. vulgaris and P. lunatus (Turner et al., 1985). The O-glucosyl and O-xylosyl derivatives of zeatin occurred in the seed coats and embryos of P. lunatus and P. vulgaris, respectively; but the level of breakdown products (Ade, Ado, & AMP) was much greater in the seed coat than in the embryos. Metabolism of zeatin in the pod tissue was very low, with ZMP being the major metabolite recovered in both species.

Cytokinin metabolism in Phaseolus is complex, differing between plant tissues as well as species. However, such complexity, especially the interspecific qualitative differences, could be useful in detecting intrinsic variations suitable for the characterization of regulatory mechanisms controlling cytokinin metabolism in this genus.

MATERIALS AND METHODS

Plant Materials

Immature embryos, 5-10 mm in length, of P. vulgaris cv. Great Northern were used for enzyme isolation. Embryos were obtained from plants grown in the field or greenhouse at 25/20 ^{o}C (day/night) and a 14 hour photoperiod.

Chemicals

trans-Zeatin, trans-ribosylzeatin, cis-zeatin, dihydrozeatin, i^6Ade, i^6Ado, NADH, NADPH and molecular weight markers were obtained from Sigma. ipn^6Ade and ipn^6Ado were synthesized in our laboratories (Mok et al., 1978). [^{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 (Kadir et al., 1984; Shaw et al., 1987). [^{14}C]-Labeled i^6Ade and i^6Ado (24 mCi/mmol) were synthesized previously (Mok et al., 1982). [^{14}C]-Labeled trans-ribosylzeatin was obtained by incubating [^{14}C]zeatin with crude extracts of PRPP-ribosyltransferase (Chen et al., 1982). 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 1 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 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 3 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 3,000 g) and then Centricon 10 or 30 (at 4,000 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 ul and loaded onto a Sephadex G-100 gel filtration column (0.9 x 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 um particle size, 30 nm pore size, 4.6 x 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 ul), NADPH (0.25 mM), [^{14}C]zeatin (0.6 nmol, 0.014 uCi) in 200 ul 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 ul *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 enzymatic reactions, a Beckman model 110A dual-pump HPLC system with a reversed-phase column (Ultrasphere ODS C_{18}, 5-um particle size, 4.6 x 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.

Confirmation of Reaction Products

HPLC fractions containing the reaction products were rechromatographed at a lower pH (3.5). Coelution with dihydrozeatin was used to confirm the identity of the product.

Enzyme Characterization

The effect of various cofactors (ATP, $MgCl_2$, NADH, & NADPH) on the conversion of zeatin to dihydrozeatin was determined using enzymes purified by ammonium sulfate precipitation. The optimal concentration of NADPH was also determined at this stage of purification. 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 to 9). The reaction velocity was determined by taking aliquots at 10 min intervals up to 1 h. The molecular weight 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), cytochrome C (M_r 12,400) and aprotinin (M_r 6,500) were used as molecular weight standards.

Substrate Specificity and Km Determination

The following compounds were tested as substrates of the enzyme: [^{14}C]zeatin, <u>cis</u>-[^{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 Km 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 uM to 21 uM.

Enzyme Quantification in Different Species

P. vulgaris, *P. lunatus*, and *P. coccineus* were compared for quantity of enzyme in immature embryos. Quantification of the enzyme was determined on extracts purified by ammonium sulfate precipitation and affinity chromatography.

RESULTS

Identification of Primary Substrate and Cofactor

The origin of O-xylosyldihydrozeatin (Mok and Mok, 1987), either via a reduction of zeatin to dihydrozeatin followed by O-xylosylation or by a direct reduction of O-xylosylzeatin (Fig. 1), 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 co-eluted with dihydrozeatin at both pH 4.8 (Fig. 2) and 3.5. This result indicated that side chain reduction preceeded O-xylosylation. The reaction was NADPH dependent but did not require ATP or cations (Table 1). NADH could not substitute for NADPH. High concentrations of NADPH (0.5 or 1 mM) were inhibitory (Table 2). Extraction with phosphate buffer increased enzyme activity approximately 3-fold over extraction with Tris-HCl buffer.

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

Figure 1. Alternative pathways for the formation of O-xylosyldihydrozeatin.

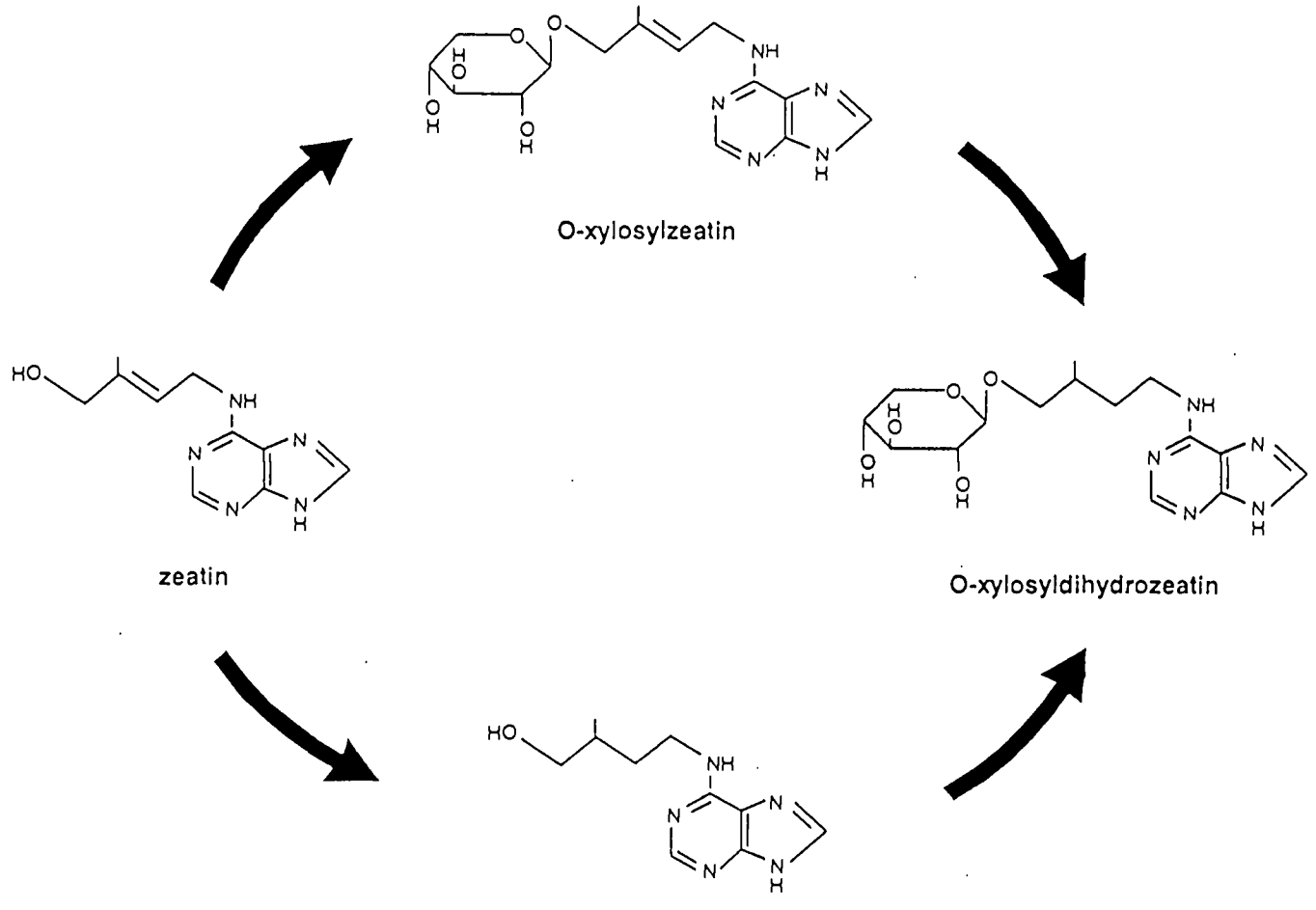
O-xylosylzeatin
zeatin
O-xylosyldihydrozeatin
dihydrozeatin

Figure 1

Figure 2. 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, 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 to 50% over 90 min). Fractions of 0.5 ml were collected.

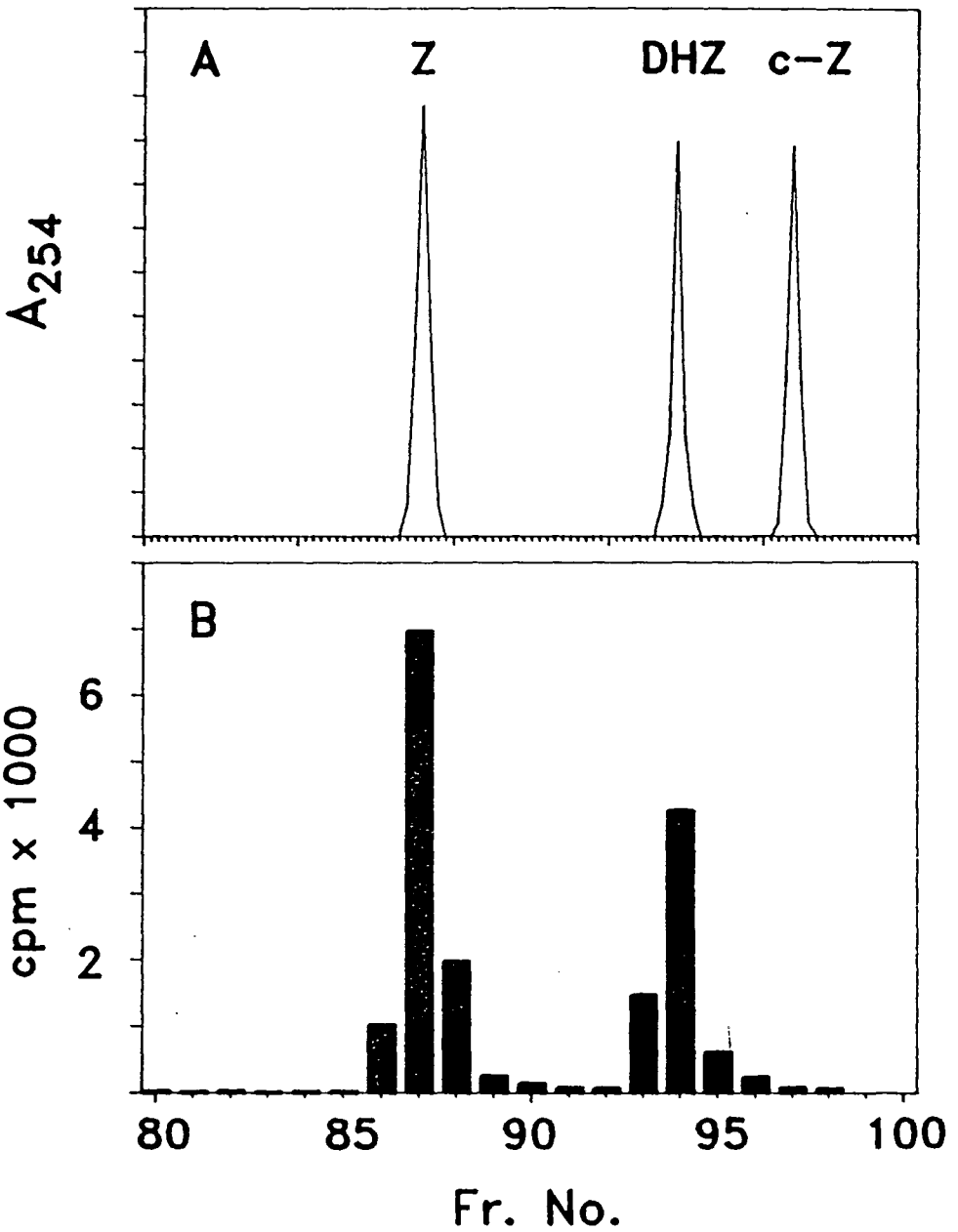

Figure 2

Table 1. Effects of co-factors on the conversion of zeatin to dihydrozeatin.

Co-factors	Distribution of radioactivity (% of Z + DHZ)* Z	DHZ
1. Phosphate buffer	100	0
2. Phosphate buffer + ATP + $MgCl_2$	100	0
3. Phosphate buffer + ATP + $MgCl_2$ + NADH	100	0
4. Phosphate buffer + ATP + $MgCl_2$ + NADPH	54	46
5. Phosphate buffer + NADPH	0	100

*Each assay contained enzyme extracted from 250 mg of embryos. Conditions: ATP (0.5 mM), $MgCl_2$ (0.05 M), NADPH or NADH (0.5 mM), enzyme (100 ul), reaction volume 200 ul by the addition of phosphate buffer (0.1 M, ph 7.3). Reaction time: 1 hr at 27°C.

Table 2. Conversion of zeatin to dihydrozeatin at various concentrations of NADPH.[a]

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

[a]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 ul, pH 8) for 1 h at 27°C.

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 bi-modal with an early peak of activity at fractions 18-20 and a later peak at fractions 28-30 (Fig. 3). This observation suggested the existence of iso-enzymes. In order 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. 4).

By comparing the elution positions of the iso-enzymes relative to molecular weight standards, the molecular weights 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 molecular weight) and LMW isozymes. Gel filtration fractions containing the HMW and LMW isozymes were applied separately to the anion exhange column. The HMW isozyme was recovered in fractions 30-32 (Fig. 5A), while the LMW isozyme eluted in fractions 24 to 26 (Fig. 5B), indicating a less negative charge of this isozyme.

Figure 3. Distribution of reductase activity after elution from Sephadex G-100 gel filtration column.

Enzyme was extracted from 8 g of GN embryos, purified by ammonium sulfate precipitation and AgAMP and Blue Sepharose affinity columns. The enzyme preparation was concentrated to 400 ul and loaded onto a Sephadex G-100 column (0.9 x 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.

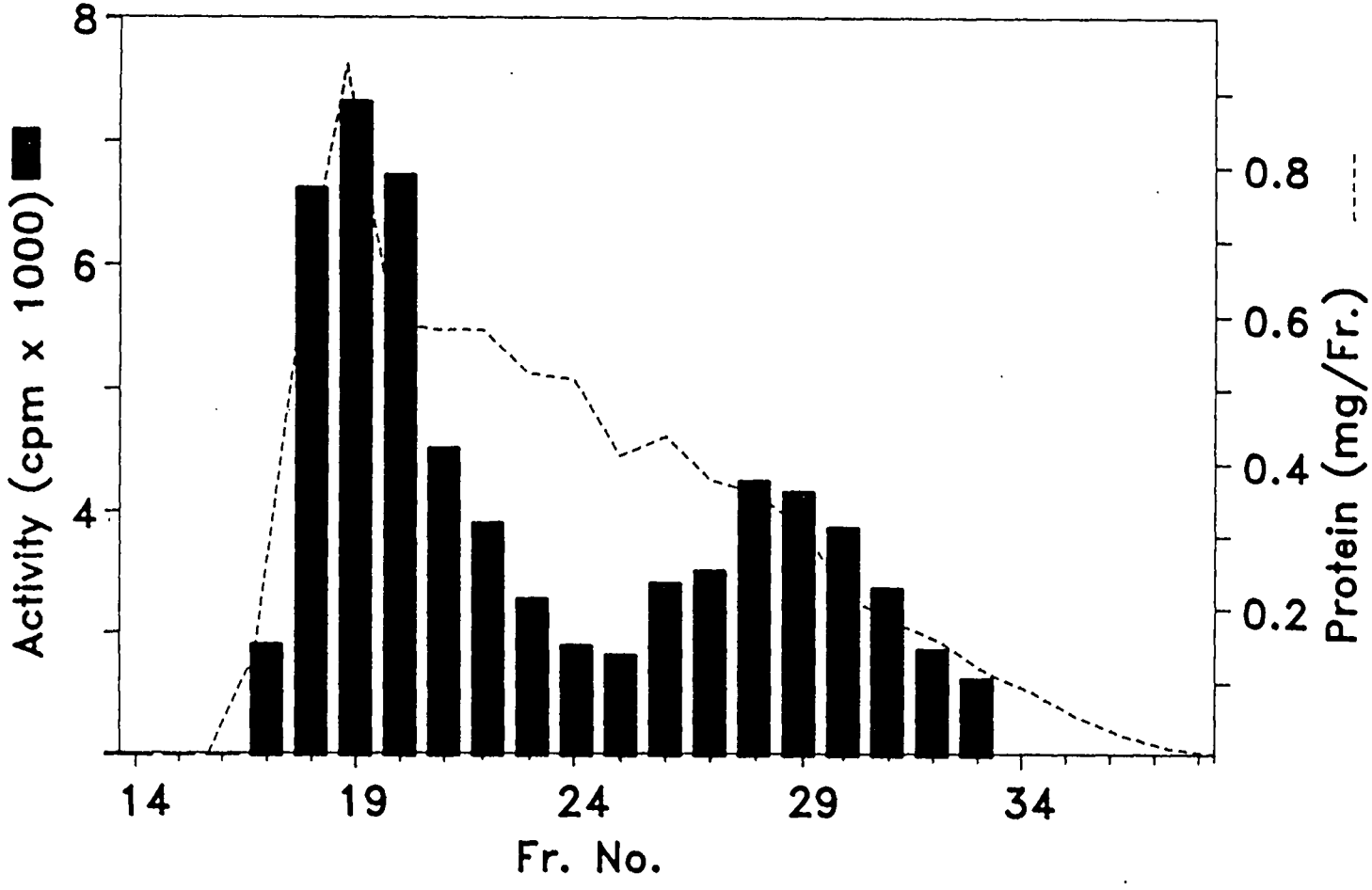
8
6
4
Activity (cpm x 1000)
0.8
0.6
0.4
0.2
Protein (mg/Fr.)
14
19
24
29
34
Fr. No.

Figure 3

Figure 4. 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 ul and loaded on an anion exchange HPLC column (AX-300, 0.46 x 22 cm). Enzymes were eluted with increasing concentration of KCl (5 to 95% over 45 min) dissolved in 0.02 M phosphate buffer at pH 7.2. Fractions of 1 ml were collected; 500 ul of each fraction was concentrated to 50 ul and assayed for enzyme activity.

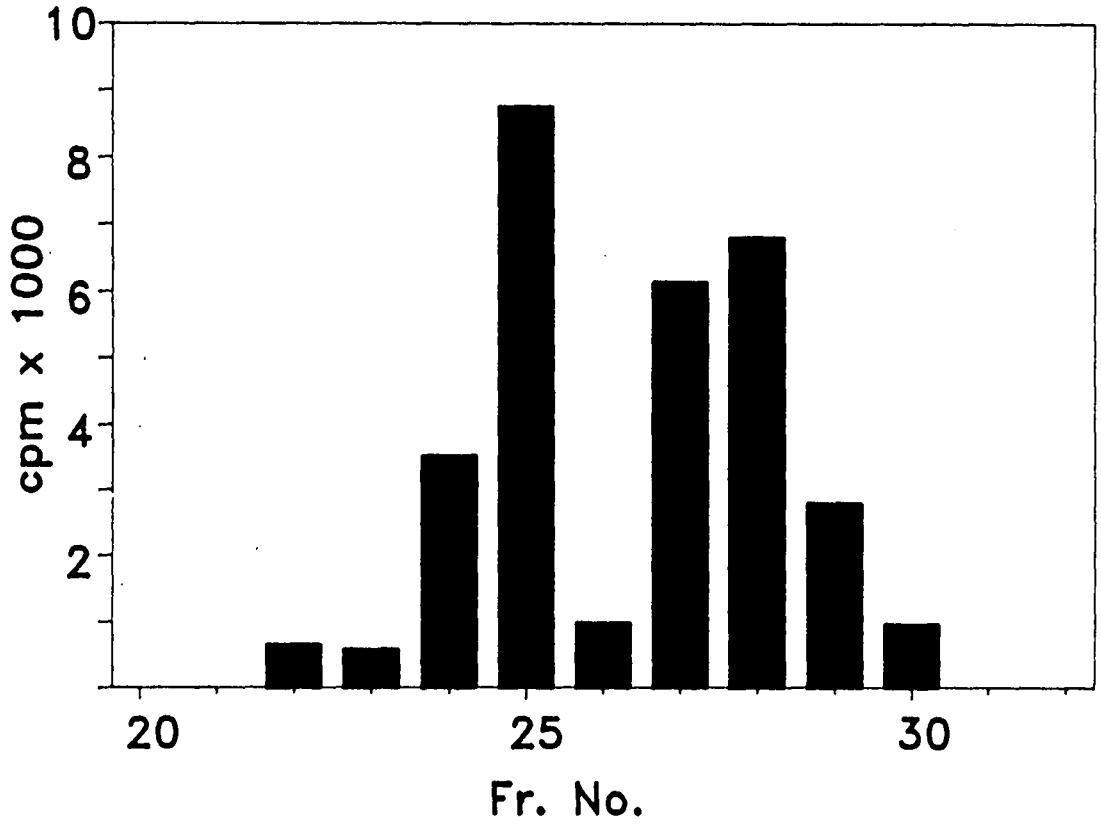

Figure 5

Figure 5. Distribution of HMW and LMW isozymes initially 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 molecular weight 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.

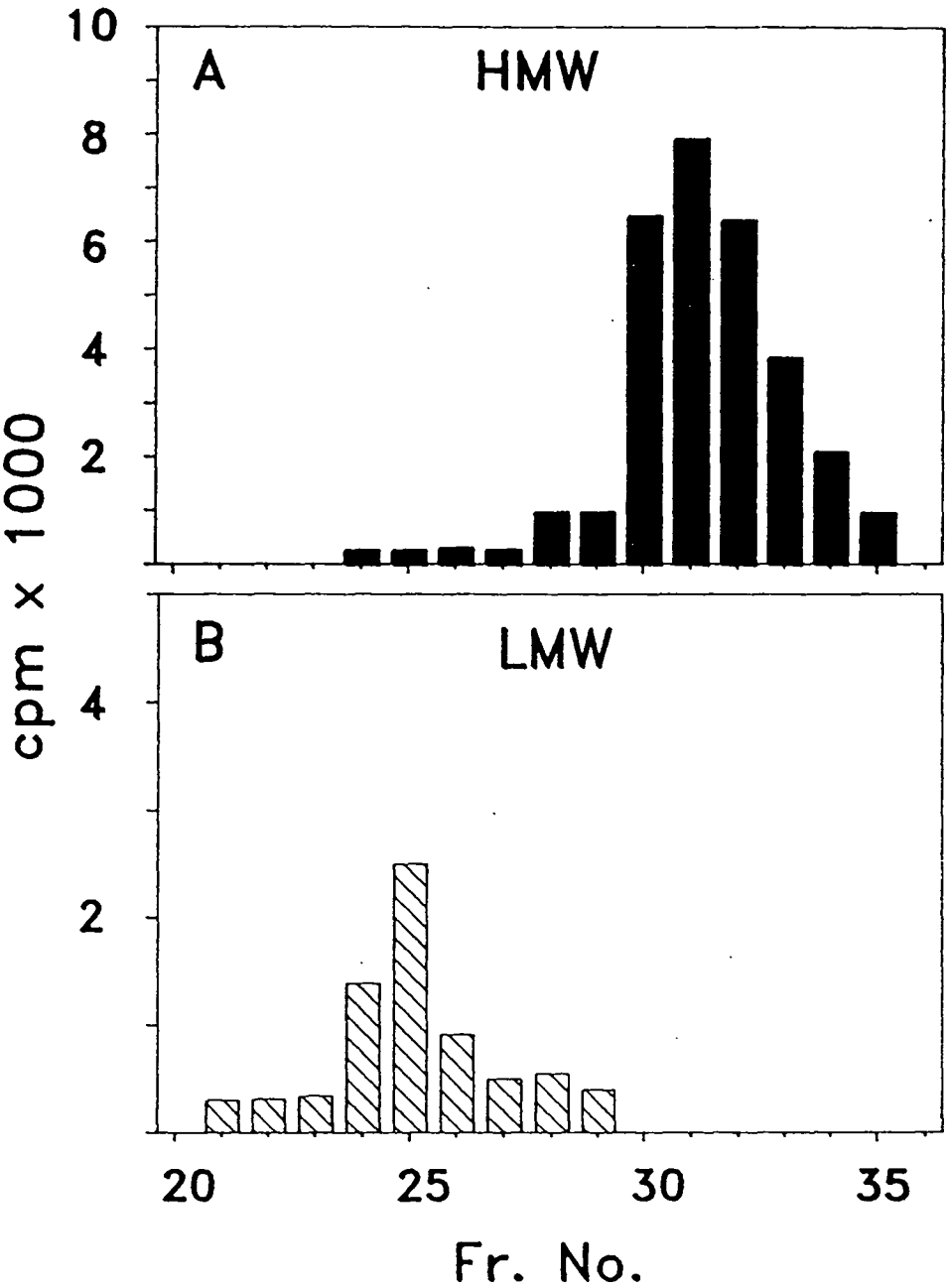

Figure 5

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 3) 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 (Gee et al., 1988; Ishikura et al., 1988; Schlieper and Barz, 1987). 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 3). 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 enzyme after anion exchange HPLC as compared with gel filtration (Table 3) further supports this interpretation. Addition of glycerol (20%) to concentrated preparations prevented the loss of activity during short term storage (Table 4), however, glycerol in the anion exchange buffers was less effective in preventing the loss of enzyme activity (Table 3).

Table 3. Purification of zeatin reductase from embryos of Phaseolus.[a]

Sample	Activity (cpm of DHZ)	Specific Activity pmol/ug protein/h	Enrichment (fold)	Protein Recovery (ug/g fresh weight)	Protein Recovery (%)
Crude	1977	0.014	1	13,034	100
$(NH_4)_2SO_4$	2587	0.036	2.59	8,797	67
Affinity Columns	7936	0.156	11.33	1,156	8.8
Gel filtration (HMW)	6287	0.057	4.12	503	3.8
Gel filtration (LMW)	10407	0.200	14.54	236	1.8
AX-300 (HMW)	7847	0.438	31.74	20	0.14
AX-300 (LMW)	1495	0.168	12.17	10	0.07

[a]Enzymes were extracted from 30 g of embryos and purified by the steps listed. Fractions containing the high molecular weight (HMW) and low molecular weight (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 pmole = 55 cpm).

Table 4. Effect of glycerol on the stability of zeatin reductase stored at -20°C.[a]

Treatment	Enzyme activity (cpm of DHZ)	
	Day 1	Day 3
Phosphate buffer	6943	1652
Phosphate buffer with 20% glycerol	6803	6242

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

Characterization of Zeatin Reductase

The conversion of zeatin to dihydrozeatin was linear up to 1 h (Fig. 6) with a pH optimum between 7.5 and 8 (Fig. 7). 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^6Ade and i^6Ado did not result in the formation of ipn^6Ade or ipn^6Ado (Fig. 8). 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 and other cofactors. No formation of [^{14}C]zeatin was detected. The Km for trans-zeatin of the HMW isozyme was 70 +/- 10 uM. The estimated Km for the LMW isozyme varied greatly between experiments with values ranging from 100 to 230 uM. Instability of the isozyme after gel filtration may have contributed to the variation.

Quantitative Differences in Reductase Activity Between Species

Quantitative differences in reductase were detected between P. vulgaris, P. lunatus, and P. coccineus. P. coccineus had the highest level of reductase followed by P. vulgaris, with P. lunatus having low enzyme activity (Table 5).

Figure 6. Reaction velocity of zeatin reductase.

Aliquots were removed from a large volume assay (equivalent to 7 assays) at 10 minute intervals. Each aliquot contained enzyme extracted from 500 mg of GN embryos purified by affinity columns.

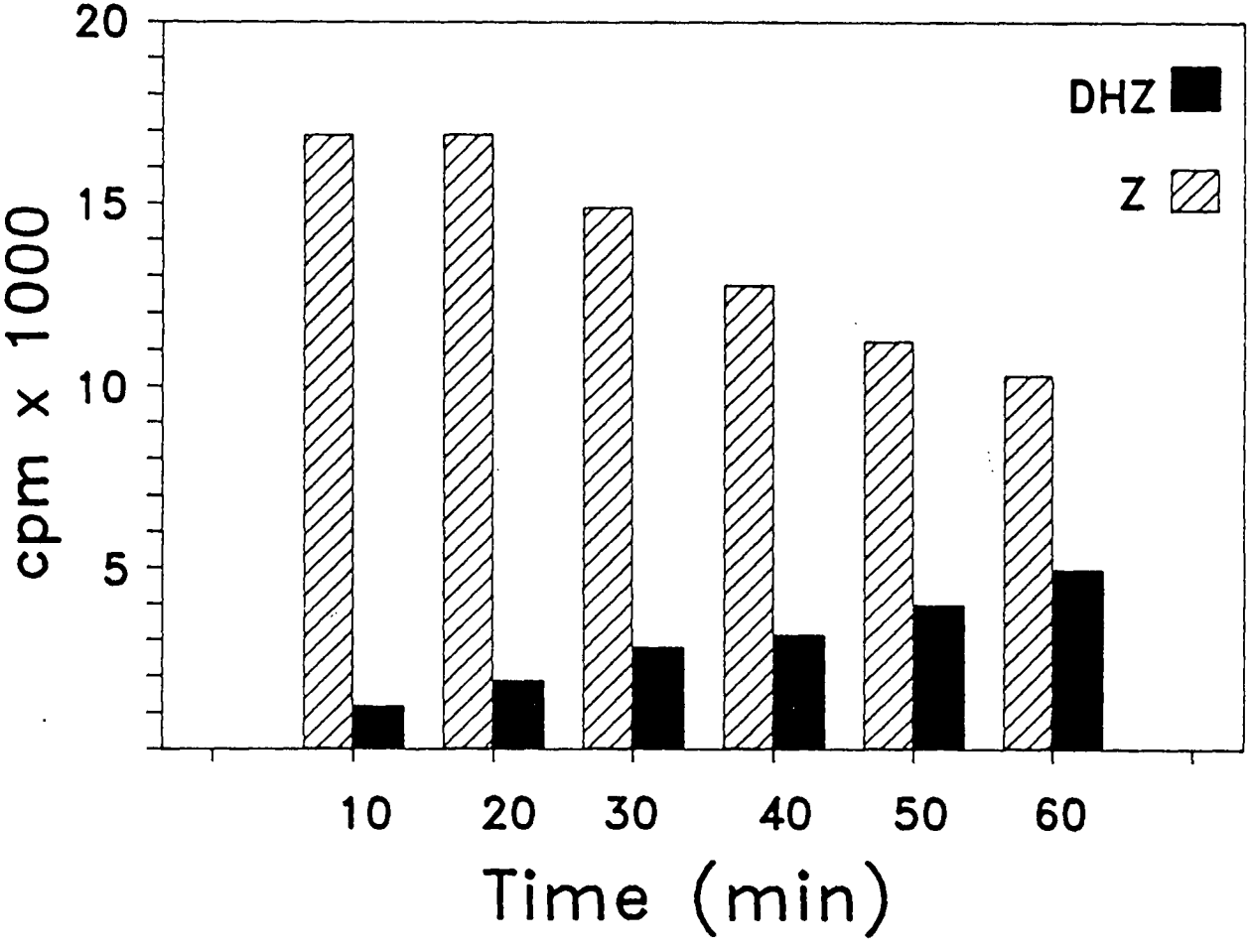

Figure 6

Figure 7. 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.

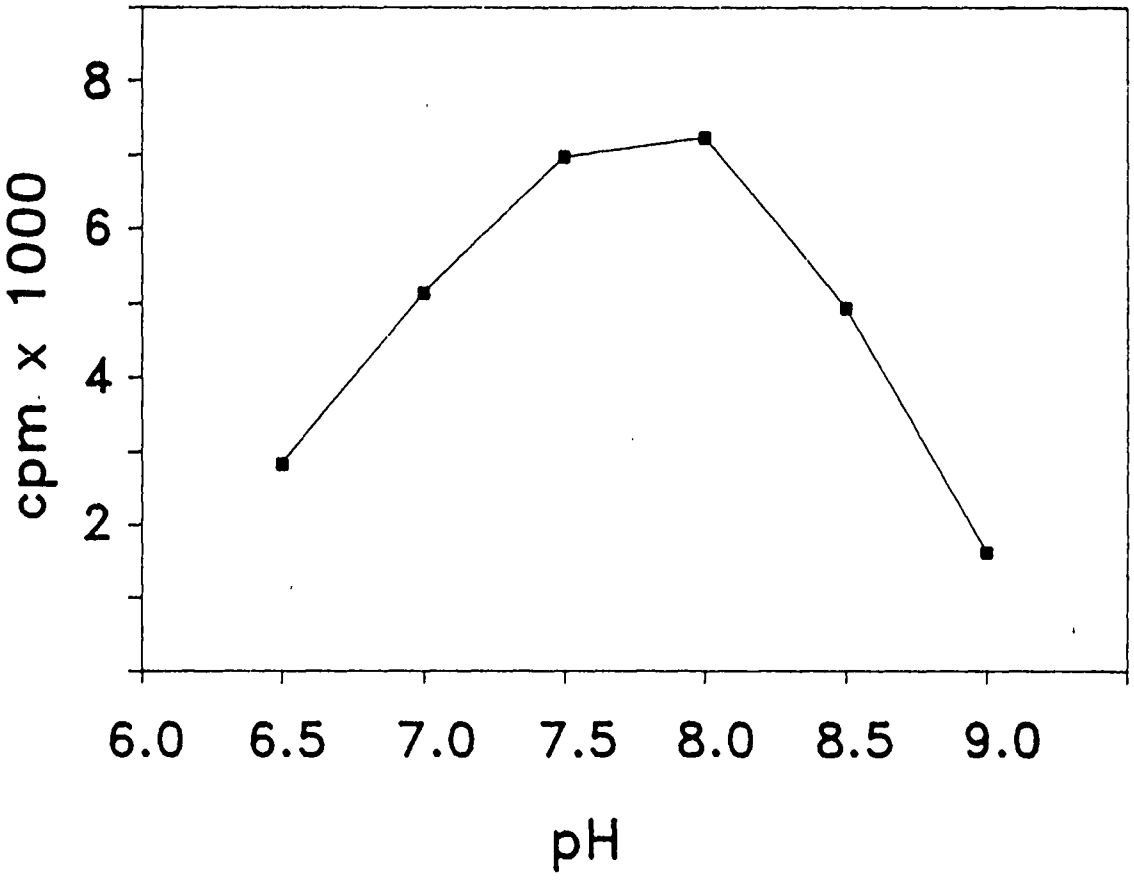

Figure 7

Figure 8. Incubation of N^6-(Δ^2-isopentenyl)adenine and N^6-(Δ^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 analysed by reversed phase C_{18} column at pH 3.5. Samples were eluted with increasing gradient of MeOH (15 to 100% over 85 min). Fractions of 0.5 ml were collected.

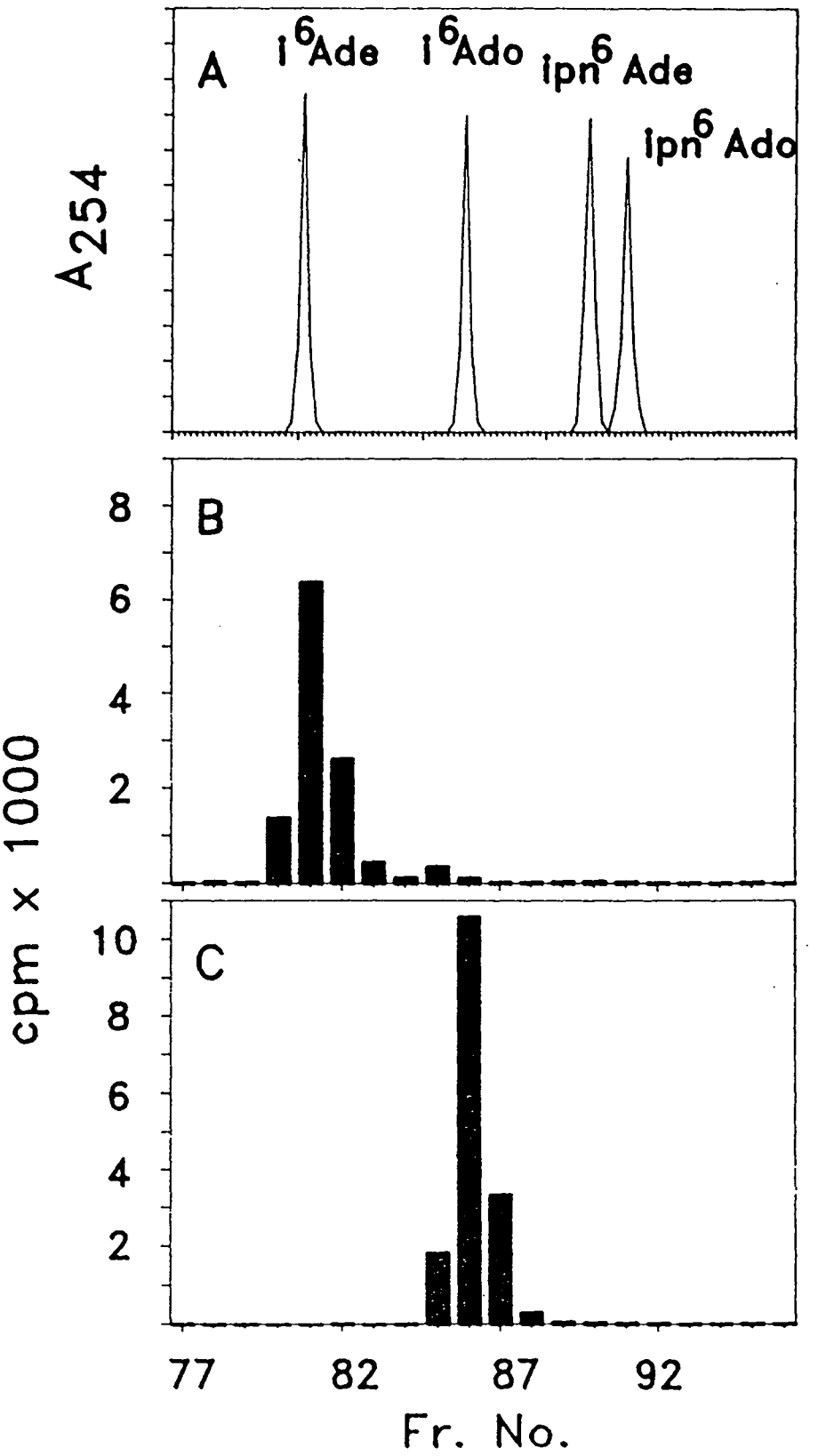
A
i6Ade
i6Ado
ipn6 Ade
ipn6 Ado
A254
B
C
cpm x 1000
8
6
4
2
10
8
6
4
2
77
82
87
92
Fr. No.

Figure 8

Table 5. Reductase activity in Phaseolus species.

	Specific activity[a]	
	pmol/mg protein	pmol/g fr. wt.
P. coccineus	210	362
P. vulgaris	67	174
P. lunatus	48	82

[a]Each assay contained enzyme extracted from 700 mg of embryos, purified by ammonium sulfate precipitation and affinity columns.

DISCUSSION

The results described above demonstrate that a NADPH-dependent zeatin reductase can be isolated from Phaseolus embryos. The two most interesting features of the enzyme are its high substrate specificity and the presence of isozymes with different molecular weights. The enzyme recognizes trans-zeatin, but not cis-zeatin. Neither ribosylzeatin nor cytokinins such as i^6Ade which lack 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 (Dixon et al., 1989; Mok et al., 1988; Turner et al., 1987) suggest that metabolism of zeatin is different than other cytokinins.

The two isozymes could be clearly distinguished by gel filtration and anion exchange chromatography. The molecular mass of the HMW isozyme was approximately two-fold 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. These steps, however, 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 similar circumstances have been reported (Gee et al., 1988; Ishikura et al., 1988; Schlieper and Barz, 1987).

The function of the enzyme may be related to the regulation of cytokinin levels in plant tissues (Palni et al., 1988). 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 (Chatfield and Armstrong, 1988; McGaw and Horgan, 1983; Whitty and Hall, 1974); 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 (Singh et al., 1988), 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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