AN ABSTRACT OF THE THESIS OF

Susan C. Dixon for the degree of Doctor of Philosophy in Horticulture presented on May 1, 1990

Title: Characterization of Cytokinin Metabolic Enzymes from Phaseolus

Abstract approved: _____

Dr. David W. S. Mok

An enzyme catalyzing the formation of O-glucosylzeatin in immature embryos of P. lunatus was purified 2500 fold using ammonium sulfate precipitation followed by affinity and anion exchange chromatography. The enzyme uses trans-zeatin as substrate (Km 28 uM) but not cis-zeatin, ribosylzeatin, or dihydrozeatin. Both UDP-glucose and UDP-xylose can serve as glycosyl donors, with Kms of 0.2 and 2.7 mM respectively, for the formation of O-glucosylzeatin and O-xylosylzeatin. In comparison, the UDPxylose:zeatin O-xylosyltransferase (Turner et al., 1987) isolated by the same procedures from P. vulgaris embryos uses only UDP-xylose as donor substrate and the Kms for both zeatin and UDP-xylose are much lower (2 and 3 micromolar, respectively). The chromatographic behavior on affinity columns and the molecular weights (approximate Mr 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.

Characterization of Cytokinin Metabolic Enzymes from *Phaseolus*

by

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ABBREVIATIONS

Ade	adenine
Ado	adenosine
ADPG	adenosine diphosphate glucose
AMP	adenosine-5'-monophosphate
ATP	adenosine-5'-triphosphate
BAP	benzylaminopurine
BAP7G	benzylaminopurine-7-glucoside
BAP9G	benzylaminopurine-9-glucoside
DHZ	dihydrozeatin
DHZMP	dihydrozeatin-5'-monophosphate
DHZOG	dihydrozeatin-O-glucoside
DHZR	dihydrozeatin riboside
DHZROG	dihydrozeatin-O-glucoside riboside
GN	Phaseolus vulgaris cv. Great Northern
i^6Ade	N^6-(Δ^2-isopentenyl)adenine
i^6Ado	N^6-(Δ^2-isopentenyl)adenosine
i^6AMP	N^6-(Δ^2-isopentenyl)adenosine-5'-monophosphate
ipn^6Ade	N^6-isopentyladenine
ipn^6Ado	N^6-isopentyladenosine
K	Phaseolus lunatus cv. Kingston
UDP-galactose	uridine diphosphate galactose
UDPG	uridine diphosphate glucose
UDP-glucose	uridine diphosphate glucose
UDPX	uridine diphosphate xylose

UDP-xylose	uridine diphosphate xylose
Z	trans-zeatin
c-Z	cis-zeatin
[^{14}C]Z	trans-[8-^{14}C]zeatin
[^{3}H]Z	trans-[^{3}H]zeatin
ZAla	lupinic acid
Z7G	trans-zeatin-7-glucoside
Z9G	trans-zeatin-9-glucoside
ZMP	zeatin riboside-5'-monophosphate
ZOG	O-glucosylzeatin
ZOX	O-xylosylzeatin
ZROG	O-glucosylzeatin riboside
ZROX	O-xylosylzeatin riboside
ZR	zeatin riboside

CHARACTERIZATION OF CYTOKININ METABOLIC ENZYMES FROM PHASEOLUS

INTRODUCTION

Plant hormones regulate all aspects of plant growth and development. In order for development to follow a specifically programmed pathway, an appropriate balance of hormones is essential. This rather precise balance varies from species to species and from stage to stage in the developmental process. Abnormal or arrested development may occur if this balance is disrupted.

The five general classes of plant hormones are auxins, cytokinins, gibberellins, ethylene, and abscisic acid. Each class has specific functions during different stages of development. Because of interactions between hormones, separation of the actions of specific hormones is sometimes difficult. In order for us to understand hormonal control of coordinated plant development, examination of each group of hormones individually is necessary, as well as consideration of interactions between groups.

Despite the importance of plant hormones, very little is known about the mechanisms controlling hormonal levels and action. Mutants would provide an ideal system to study the effects of abnormal hormone production and

metabolism; however, very few cytokinin mutants have been found. The first observed effect of altered cytokinin levels on plant development was the crown gall tumor produced by Agrobacterium tumefaciens (Braun, 1958). Later, interspecific hybrids of Nicotiana, which produced tumors, were shown to have altered cytokinin levels (Smith, 1972). A mutant which produces abnormally high levels of cytokinins (ove) has been found in moss (Ashton et al., 1979).

Other approaches have been used to try to identify genetic mechanisms regulating the metabolism and biosynthesis of cytokinins. Callus cultures of Phaseolus were screened in order to identify variations of genetic interest. Large differences in requirements for, and responses to, cytokinins were found (Mok et al., 1978; Mok et al, 1980; Mok et al., 1982). Cytokinin autonomous genotypes were found in P. vulgaris, but not in P. lunatus. Genetic analysis demonstrated that the cytokinin requirement of P. vulgaris callus tissues may be regulated by one set of alleles (Mok et al., 1980). In callus cultures of P. vulgaris cv. Great Northern (GN), cytokinins with saturated sidechains (ipn^{6}Ade, ipn^{6}Ado, DHZ, and DHZR) were always more active than the unsaturated analogs (i^{6}Ade, i^{6}Ado, Z and ZR), while in P. lunatus cv. Kingston (K) cytokinins with unsaturated sidechains were either more active or equally as active as the saturated forms (Mok et al., 1982b).

Zeatin metabolism in immature embryos of P. vulgaris cv. GN and P. lunatus cv. K was compared to determine if genetic differences in cytokinin metabolism also occurred in organized tissues (Lee et al., 1985). In P. vulgaris cv. GN embryos two novel zeatin metabolites, designated at the time as Met I and Met II, were discovered. In P. lunatus cv. K these novel metabolites did not occur; rather, the majority of the zeatin was converted to metabolite B. The two novel zeatin metabolites from P. vulgaris cv. GN (Met I and Met II) were identified by chemical tests, GC-MS analysis, and chemical synthesis as O-xylosylzeatin and its riboside (Lee et al., 1985; Turner et al., 1987). Metabolite B from P. lunatus cv. K was identified as O-glucosylzeatin. Thus, interspecific qualitative differences in the O-glycosylation of zeatin were established.

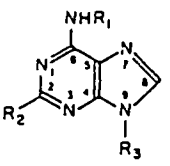
The differences found between P. vulgaris cv. GN and P. lunatus cv. K, in the glycosylation of zeatin, suggested that different cytokinin specific metabolic enzymes might be present. Subsequently, the O-xylosyltransferase enzyme was isolated from P. vulgaris cv. GN embryos and partially purified using gel filtration and anion exchange chromatography (Turner et al., 1987). The enzyme was highly specific, transferring the xylose from UDPxylose to zeatin. However, this enzyme did not catalyze the formation of O-glucosylzeatin from zeatin and UDPglucose.

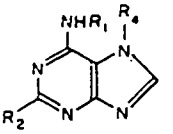
These results suggest that the presence of different O-glycosyl derivatives of zeatin in embryos of the two species is most likely the result of the presence of distinct metabolic enzymes, rather than substrate availability. This study was undertaken to confirm this hypothesis by isolating and characterizing the O-glucosylation enzyme from P. lunatus cv. K embryos and comparing its properties with the O-xylosyltransferase extracted from P. vulgaris cv. GN embryos.

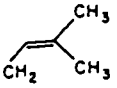
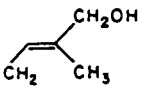
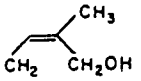
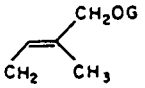
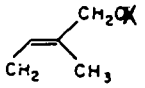
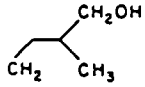
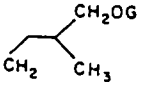
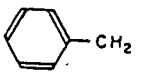
LITERATURE REVIEW

Cytokinins are a group of hormones that play a critical role in plant cell division and differentiation. Cytokinins are produced primarily in the roots and are transported through the xylem to actively growing regions (Englebrecht, 1972; Van Staden and Davey, 1979; Maab and Klambt, 1981). The naturally occurring cytokinins are generally adenine derivatives with N^6 substituents (Table I); however, a few derivatives of BAP have been reported (Horgan, 1975; Ernst et al., 1983). Compounds with a planar five carbon side chain have been shown to have optimal activity (Hecht et al., 1970). Some phenyl ureas, such as diphenylurea and thidiazuron and their derivatives, also exhibit cytokinin-like activity in bioassay systems (Shantz and Steward, 1955; Bruce et al., 1965; Mok et al., 1982a; Mok et al., 1987). Zeatin, the first naturally occurring cytokinin to be identified, was extracted and purified from Zea mays (Letham, 1963). In extracts of flowering plants zeatin and its derivatives are the dominant cytokinins (Letham and Palni, 1983). A number of investigators have attempted to elucidate the mechanism of action of cytokinins, but no precise mechanism has been determined (Burrows et al., 1971; Armstrong et al., 1976; Keim et al., 1981; Kulaeva, 1981).

Table 1. Cytokinin structures, nomenclature, and abbreviations.





R1	R2	R3	Common name	Abbreviation
	H	-	N^6 (Δ^2 isopentenyl)adenine	i^6Ade
	ribosyl	-	N^6 (Δ^2 isopentenyl)adenosine	i^6Ado
	ribotide	-	N^6 (Δ^2 isopentenyl)adenosine-5'-monophosphate	i^6AMP
	H	-	trans-zeatin	Z
	ribosyl	-	t-zeatin riboside	ZR
	glucosyl	-	t-zeatin-9-glucoside	Z9G
	-	glucosyl	t-zeatin-7-glucoside	Z7G
	alanyl	-	lupinic acid	ZAla
	ribotide	-	t-zeatin riboside-5'-monophosphate	ZMP
	H	-	cis-zeatin	c-Z
	H	-	O-glucosylzeatin	ZOG
	ribosyl	-	O-glucosylzeatin riboside	ZROG
	H	-	O-xylosylzeatin	ZOX
	ribosyl	-	O-xylosylzeatin riboside	ZROX
	H	-	dihydrozeatin	DHZ
	ribosyl	-	dihydrozeatin riboside	DHZR
	ribotide	-	dihydrozeatin riboside-5'-monophosphate	DHZMP
	H	-	dihydrozeatin-O-glucoside	DHZOG
	ribosyl	-	dihydrozeatin-O-glucoside riboside	DHZROG
	H	-	N^6 benzyladenine	BAP
	glucosyl	-	N^6 benzyladenine-9-glucoside	BAP9G
	-	glucosyl	N^6 benzyladenine-7-glucoside	BAP7G

Mechanism of Action of Cytokinins

Cytokinins occur as free bases, nucleosides, and nucleotides in plant cells (Letham and Palni, 1983). Modified bases are also found as components of specific tRNAs (Chen and Hall, 1969; Burrows et al., 1971; Letham and Wettenhall, 1977). However, biological activity is generally associated with the free forms of cytokinins. Although it was speculated that the modified bases found in tRNA may be related to cytokinin action, the evidence was not conclusive (Burrows et al., 1971; Armstrong et al., 1976). Protein synthesis is stimulated by the addition of exogenous cytokinins, but the exact mechanism is not known (Kulaeva, 1981; McGaw, 1987). At the transcriptional level cytokinins may activate a chromatin-bound RNA polymerase and thus enhance RNA synthesis (Kulaeva, 1981). At the post-transcriptional level, cytokinins may enhance the recruitment of untranslated mRNAs by activating polysomes or stimulating polysome formation (McGaw, 1987). The precise target of cytokinins is not known.

Cytokinin binding proteins (CBPs) may take part in cytokinin action <u>in vivo</u>, or they may be involved in stabilizing cytokinins. The best documented case involved the wheat germ CBPs, which have a high affinity for BAP, i^6Ade and i^6Ado, but a very low affinity for Z (Keim et al., 1981). These CBPs were found in high

concentrations (Polya and Davis, 1978), and the ligands were structurally diverse (Polya and Bowman, 1979), which suggests that these CBPs are probably not involved in the mechanism of action of cytokinins; rather these CBPs may bind cytokinins in order to stabilize them (Letham and Palni, 1983). If synthesis did not keep up with metabolism, then the cytokinins would be released from the CBPs and available to the cell for metabolism.

A protein kinase which phosphorylases CBPs has been isolated and characterized (Polya and Davies, 1983). The consequences of phosphorylation on the CBPs are unknown, but perhaps phosphorylation triggers cytoplasmic/nuclear cytokinin receptors. CBPs which were isolated from barley leaves (Romanov et al., 1988) have a number of properties of receptor proteins (high affinity for zeatin, low concentration of binding sites, and no crossreaction with other plant hormones). Some CBPs may be free in the cytosol, while other CPBs appear to be loosely bound to ribosomes (Fox and Erion, 1977), which suggests that these binding proteins may have a receptor function in the translation process. A direct biological function of a cytokinin-binding protein complex has not been demonstrated; however, the evidence suggests that these CBPs are probably involved in cytokinin action <u>in vivo</u>, though they could also play a role in regulating free levels of cytokinins in the cell.

Biosynthesis of Free Cytokinins

Endogenous concentrations of cytokinins are extremely low, and the most likely precursors for their biosynthesis are adenine and its nucleotides or nucleosides. Since these precursors are also central to many other metabolic pathways in the cell, it is difficult to study cytokinin biosynthesis. The two pathways that have been suggested for the biosynthesis of free cytokinins are via tRNA degradation (Chen and Hall, 1969) and/or de novo synthesis (Miura and Miller, 1969). Studies with the primary roots of Zea mays (Klemen and Klambt, 1974) and Lactobacillus acidophilus (Leineweber and Klambt, 1974) have shown that tRNA half-life seems to be correlated with cellular metabolic activities. These tRNA half-life data have been used to calculate daily cytokinin production and to support the hypothesis that free cytokinin production is due to the turnover of tRNAs containing cytokinins.

The turnover of plant tRNAs may contribute to the free cytokinin pool, but there is strong evidence that the free pool is generally synthesized via another pathway (Letham and Palni, 1983): 1) Many of the cytokinins that occur as free bases in higher plants are not found in the tRNA of the same species (Burrows, 1978). 2) The dominant cytokinin in plant tRNA is cis-zeatin, while trans-zeatin is a common free compound

(Letham, 1978). 3) In many plant tissues the tRNA turnover rate is too low to make a significant contribution to the free cytokinin pool (Hall, 1973; Trewavas, 1970).

An alternate mechanism is direct biosynthesis wherebyΔ^2-isopentenyl pyrophosphate is transferred to AMP to yield i^6AMP, which is then transhydroxylated to yield trans-zeatin. A number of enzymes that have been investigated support this pathway. Cell-free extracts from the slime mold Dictyostelium discoideum catalyzed the synthesis of i^6AMP from isopentenylpyrophosphate and AMP (Taya et al., 1978), and a similiar transferase was partially purified from cytokinin-autonomous tobacco callus tissue (Chen and Melitz, 1979). Isopentenyl transferase activity has also been exhibited by a number of crown gall tumor cell lines (Morris, 1986). Gene 4 from the T-DNA of Agrobacaterium tumifaciens has been shown to code for an isopentenyl transferase (Barry et al., 1984) and has been compared to a similiar gene in Pseudomonas savastanoi (Morris, 1986). Overall amino acid homology is at least 50% and is confined to the coding regions, and conservation of the first and second bases occurs.

There is evidence that hydroxylation occurs rapidly following transferase activity. When Vinca rosea crown gall tissue was incubated with [^{14}C]Ade, labelled zeatin derivatives were recovered (Stuchbury et al., 1979; Palni

et al., 1983). In this crown gall tissue hydroxylation appeared to occur at the nucleotide level. Following incubation with [^{14}C]Ade, AMP was recovered first, followed by ZMP. In addition, more ZMP than ZR or Z was always recovered (Stuchbury et al., 1979). It has been reported that [^{14}C]i^{6}Ade is stereospecifically trans hydroxylated in several tissues (Palni and Horgan, 1983). An enzyme extracted from cauliflower was able to trans-hydroxylate i^{6}Ade to Z and i^{6}Ado to ZR (Chen and Leisner, 1984). It is still not clear whether hydroxylation always occurs at the free base, nucleotide, or the nucleoside level. One study suggested that there may be variations in different species in the biosynthesis of zeatin. In several woody species examined, only certain species were able to hydroxylate i^{6}Ade to Z (Einset, 1986).

Metabolism of Cytokinins

The zeatin derivatives that result from hydroxylation undergo many metabolic reactions to produce about twenty other derivatives. Feeding studies with radioactively labelled cytokinins have been used to identify a variety of metabolites of the basic purine cytokinins. Examination of these products has been useful in elucidating the pathways involved in cytokinin metabolism, though exogenous compounds are probably

subject to much more intense metabolism than are the endogenous compounds (Letham and Palni, 1983). Plant tissues convert cytokinin bases into a great diversity of metabolites, which include products of ring substitution and products of isoprenoid sidechain reduction, cleavage, and substitution.

Interconversion between free base, ribonucleoside and ribonucleotide forms

The most abundant naturally occurring cytokinins are the ribosides and their phosphate forms (McGaw et al., 1984). In plant tissues the free base, nucleoside, and nucleotide forms of cytokinins can all be interconverted. In tissues incubated with [^{14}C]Z, ZR and ZMP were recovered. When time course studies were performed, nucleotides were formed first and then hydrolyzed to yield other, more stable, forms (McGaw et al., 1984).

Various enzymes are probably responsible for the interconversions of the cytokinin bases, ribosides and ribotides. The following enzymes have been purified from wheat germ by Chen and coworkers: adenosine phosphorylase (Chen and Petschow, 1978), adenosine nucleosidase (Chen, 1981), adenosine phosphoribosyltransferase and adenine kinase (Chen and Eckert, 1977), and 5'-ribonucleotide phosphohydrolase (Chen and Kristopeit, 1981).

Time course labelling studies have shown that nucleotides are the dominant metabolites in the early

stages; thus, nucleotide formation may be associated with cytokinin uptake and transport across membranes (Letham and Palni, 1983). Labelling studies also suggest that ribosides are important cytokinin forms for translocation in the xylem (Goodwin et al., 1978; Letham, 1979), and that free bases may be the biologically active forms (Letham and Palni, 1983).

Side chain cleavage

The N^6-isopentenyl side chain is often cleaved from Z, ZR, i^6Ade and i^6Ado to yield Ade and its derivatives (Letham and Palni, 1983). Specifically, in tobacco cell cultures incubated with labelled i^6Ado and i^6Ade, the side chain was removed to yield adenosine, adenine, and adenylic nucleotides (Laloue et al., 1977), and in Phaseolus vulgaris cv. Great Northern (GN) i^6Ado was rapidly degraded to Ado (Mok et al., 1982b).

The side chain is cleaved by the enzyme cytokinin oxidase, which was first purified from kernels of Zea mays (Whitty and Hall, 1974). When enzyme extracts were incubated with i^6Ado or Z, the side chain was cleaved to yield Ado or Ade. Oxidases have also been purified from tobacco tissue (Paces et al., 1971), Vinca rosea tumor tissue (Scott et al., 1982), and callus tissues of Phaseolus vulgaris L. cv. GN (Chatfield and Armstrong, 1986).

Cytokinin oxidase was shown to require oxygen and is

specific for unsaturated side chains. If the double bond was moved from the Δ^2 to the Δ^3 position, the side chain was saturated, or other functional groups were substituted, the enzyme was not active (Whitty and Hall, 1974; McGaw and Horgan, 1983). This enzymic cleavage serves to permanently inactivate cytokinins.

Amino acid conjugation

A less common metabolic pathway is the addition of alanine to zeatin at the 9-position to form lupinic acid (ZAla). Traces of ZAla were first found in cell-free extracts of plant tissues (Murakoshi et al., 1977), and when labelled zeatin was fed exogenously to nine-day old lupine seedlings, ZAla was recovered (Parker et al., 1978).

The enzyme that mediates this reaction (lupinic acid synthetase) has been partially purified from developing lupine seed (Entsch et al., 1983). O-acetyl-L-serine donated the amino acid residue, and a number of adenine derivatives served as substrates; however, preference was shown for compounds with high cytokinin activity.

Lupinic acid was much more stable than Z in lupine leaves (Parker et al., 1978) and more stable than ZR in soybean callus tissue (Palni et al., 1983). In these tissues both Z and ZR were rapidly degraded by sidechain cleavage. This alanine conjugate was only weakly active in bioassays (Letham et al., 1983), again suggesting a

mechanism for lowering cytokinin activity levels.

Side chain reduction

The unsaturated N^6 side chain of zeatin may be reduced to yield DHZ. DHZ derivatives are common in plant tissues and have frequently been found as metabolites of applied Z (Letham and Palni, 1983). DHZ and DHZR were products obtained after feeding derooted lupine seedlings [^{3}H]Z (Parker et al., 1978), and when detached leaves of P. vulgaris L. were incubated with [^{14}C]Z, the side chain was reduced (Palmer et al., 1981a.)

Recently two isozymes of a reductase catalyzing the conversion of zeatin to DHZ were partially purified from immature embryos of P. vulgaris (Martin et al., 1989). This enzyme was specific for zeatin and did not recognize closely related compounds, nor did it convert c-Z or DHZ to Z, thus suggesting that side chain reduction occured at the free base level.

Side chain reduction seems to be related to biological activity. In P. vulgaris callus bioassays saturated cytokinins were more active than their unsaturated counterparts (Mok et al., 1978, Mok et al., 1982b). In tobacco callus bioassays, DHZ was ten times more active than zeatin, while in other bioassays DHZ and its conjugates were equally as active as their Z analogues (Letham et al., 1983). Reductases may be important in regulating cytokinin levels in plant tissues

(Palni et al., 1988), since saturated cytokinins are resistant to attack by cytokinin oxidase (Chatfield and Armstrong, 1988). Thus, in plants where cytokinin oxidase is present, the presence of a reductase would serve to protect cytokinins by converting them to the oxidase resistant saturated form.

Glycosylation

Glycosylation involves the addition of a sugar moiety to the 7- or 9- position of the purine ring producing N-glycosides (Cowley et al., 1978; Fox et al., 1973), or to the oxygen in the isoprenoid side chain of zeatin producing O-glycosides (Letham et al., 1977; Morris, 1977; Duke et al., 1979; Scott et al., 1982b). In addition to zeatin, BAP has also been converted into 3-, 7-, and 9- glycosides (Entsch and Letham, 1979).

The major cytokinins in tobacco (Scott and Horgan, 1984) and Vincea rosea (Palni et al., 1983) crown gall tissues are Z7G and Z9G respectively. In radish tissues, Z7G is the predominant free cytokinin (McGaw et al., 1985), while Z9G is most abundant in maize roots (Parker et al., 1973). O-glucosides appear to be widely distributed endogenous cytokinins. The glucoside cytokinins, ZOG, ZROG, DHZOG and DHZROG have been identified in a number of plant tissues. O-Glucosides appear to account for most of the extractable cytokinin activity in soybean callus (Horgan, 1975), in mature or

senescing leaves (Wang et al., 1977; Duke et al., 1979; Palmer et al., 1981a; Van Staden and Davey, 1981), and in lupine pod walls and seeds (Davey and Van Staden, 1978; Summons et al., 1979; Summons et al., 1981).

Labelling studies have confirmed that glycosides can be synthesized from exogenously supplied cytokinin. N-glucosylation was the predominant fate of exogenously applied cytokinin in radish tissues (McGaw et al., 1985). In lupine seedlings, exogenous application of labelled zeatin led to the formation of large amounts of O-glucosyl derivatives which remained unmetabolized over long periods (Parker et al., 1978). When derooted radish seedlings were supplied with low levels of [^{14}C]Z, the principal metabolite was Z7G; however, when high levels of labelled zeatin were applied, both ZR and ZOG were recovered (McGaw et al., 1985). Studies of zeatin metabolism in Phaseolus embryos, seeds, roots, axes, and leaves also yielded ZOG (Sondheimer and Tzou, 1971; Wang et al., 1977; Palmer et al., 1981b; Lee et al., 1985). In some cases xylose, rather than glucose was conjugated as demonstrated with embryos of Phaseolus species where O-xylosyl derivatives of zeatin were recovered (Mok and Mok, 1987).

Three enzymes that are responsible for the formation of glycosyl conjugates have been studied (Entch et al., 1979; Turner et al., 1987). Two N-glucosyltransferases that catalyze the formation of 7- and 9-glucosyl

derivatives of several cytokinins as well as other purines have been isolated (Entsch et al., 1979). These enzymes were extracted from radish cotyledons and converted BAP into 7- and 9-glucosides using UDPG as the glucose donor. Glucosylation ratios for the two enzymes were compared and the ratio of BAP7G/BAP9G was 1.5 for the major enzyme and 10.5 for the minor enzyme (Entsch and Letham, 1979). The molecular weight was determined to be 46,500, 76 ug of the major enzyme was purified from one kg of cotyledons, and specificity and kinetics were examined (Entsch et al., 1979). A third glycosylation enzyme was isolated and purified from P. vulgaris embryos. This enzyme catalyzed the formation of O-xylosylzeatin, recognized only UDP-xylose as a substrate, and had a molecular weight of about 50,000 (Turner et al., 1987).

N-glucosides seem to serve a different function than O-glucosides. The 7- and 9- glucosides were only weakly active in bioassays (Letham et al., 1983). The 7-glucosides were very stable in tobacco tissue culture cells (Gawer et al., 1977), soybean callus cultures (Fox et al., 1973), and radish cotyledons (Parker and Letham, 1973). N-glucosides were resistant to glycolysis by B-glucosidase (Duke et al., 1979; Duke et al., 1978); thus, N-glycosylation may be a mechanism for lowering levels of physiologically active cytokinins in tissues.

In contrast, the O-glucosides were highly active in

tissue culture and leaf senescence bioassays (Letham et al., 1983); however, this activity was probably due to the action of B-glucosidase enzymes, which hydrolyzed the glucosides to the aglycones (McGaw et al., 1985). In other cases, the O-glucosides remained unmetabolized (Parker et al., 1978). One hypothesis for the role of O-glycosides is that they may serve as cytokinin storage forms which are metabolized to yield biologically active forms when needed. The following evidence supports this hypothesis: 1) Several metabolic studies with labelled ZOG indicated that it was readily hydrolyzed to its aglycone (Palmer et al., 1981a; Van Staden and Davey, 1981; McGaw et al., 1984; McGaw et al., 1985). 2) When plant tissues accumulate active cytokinins, much of the increase in activity appears to be due to metabolites of O-glucosides (Palmer et al., 1981b; Van Staden and Davey, 1981). 3) During some phases of plant development, the levels of O-glucosides appear to change rapidly. In potato tubers, as dormancy was broken and apical growth began, a decline in the level of a glucoside (possibly RZOG) was accompanied by a rise in the level of a cytokinin which cochromatographed with ZR (Van Staden and Dimalla, 1978). Similiar changes were seen in germinating maize seeds (Smith and Van Staden, 1978) and developing bean seedlings (Palmer et al., 1981a). Decapitation of bean plants was followed by a rapid rise in DHZOG in the leaves. However, as lateral buds

developed, the DHZOG rapidly decreased.

An alternate hypothesis is that the O-glycoside derivatives themselves are biologically active compounds. To determine the biological activity of O-xylosylzeatin, callus bioassays of P. vulgaris cv. GN and P. lunatus cv. Kingston (K) were examined (Mok et al., 1978). O-Xylosylzeatin was equally active in both systems, but was 100 times more active than zeatin in P. vulgaris cv. GN and ten times less active than zeatin in P. lunatus cv. K tissues. These data suggest that in this system O-xylosylzeatin itself was biologically active. If O-xylosylation serves merely as a storage role, conversion of O-xylosylzeatin back to zeatin should be accompanied by a reduction in activity of O-xylosylzeatin in P. vulgaris cv. GN as compared to P. lunatus cv. K. Such a reduction in activity was not found, and in short term incubation studies very little conversion to zeatin was detected.

Both hypotheses for the function of O-glycosides may be correct. Perhaps O-glucosides are storage forms, while O-xylosides are active forms. When four species of Phaseolus were examined (P. vulgaris, P. lunatus, P. acutifolius, and P.coccineus), all converted [^{14}C]Z to ZROG (Mok and Mok, 1987). However, major differences were detected between these species. In P. lunatus, the major metabolite recovered was ZOG, while ZOX and ZROX were found in the other three species. Thus, in three of

the species examined both O-glucosides and O-xylosides were found.

In summary, the various forms of cytokinins may serve various functions. The ribosides are important as translocation forms in the xylem, while the nucleotides may be involved in cytokinin uptake as well as transport. The free bases may be the active forms, while the O-glycosides may be active forms as well as storage forms. Side chain cleavage, alanine conjugation, and 7- or 9- glucosylation may all be mechanisms for lowering the physiological levels of active cytokinins in tissues.

Cytokinin metabolism in Phaseolus

In attempts to understand how cytokinins regulate development, zeatin metabolism has been examined in both unorganized callus tissues and organized plant organs in a variety of species of Phaseolus. Differential cytokinin structure-activity relationships were examined in P. vulgaris cv. GN and P. lunatus cv. K callus tissues (Mok et al., 1978). Bioassays were used to determine genotypic responses to eight cytokinins. Major species differences in cytokinin responses to cytokinins bearing unsaturated isoprenoid side chains (io^6Ade and i^6Ade) were established. P. vulgaris callus tissues were relatively unresponsive to cytokinins with unsaturated side chains. Further studies with callus tissues of P.

vulgaris cv. GN and P. lunatus cv. K and the interspecific hybrid GN X K confirmed that in P. vulgaris GN callus tissues, cytokinins bearing saturated side chains were always more active than unsaturated analogs (Mok et al., 1982b). In addition, differences in the metabolism of exogenously applied [^{14}C]i^{6}Ado were also established in these callus tissues. In GN tissues i^{6}Ado was rapidly degraded to Ado, while in K tissues i^{6}Ado was rapidly phosphorylated to the nucleotide form. Hybrid tissues were intermediate between the two parental types in both growth responses and metabolic products.

The proper balance of cytokinins is important at all stages of development. Quantitative differences in endogenous cytokinin compounds were found during seed and embryo development in P. coccineus (Lorenzi et al., 1978). These data suggest that the embryo-suspensor system contained high levels of Z and very few other cytokinins in early stages of development, while at intermediate and late stages Z decreased, and ZR and ZG became dominant. Both heart-shaped and middle cotyledonary stages of embryos were then cultured in vitro on media containing various concentrations of Z or ZR (Bennici and Cionini, 1979). Growth of heart-shaped stage embryos was inhibited by high concentrations of ZR and stimulated by equal concentrations of Z. In contrast, low concentrations of ZR enhanced growth at the same stage, while corresponding levels of Z had no effect. At

more advanced stages the P. coccineus embryos seemed to become less dependent upon cytokinins, as indicated by the lesser effect of both Z and ZR on in vitro growth of middle cotyledonary embryos. These studies suggest that Z is an important factor for the in vitro growth of early P. coccineus embryos.

In fruits of P. vulgaris L. var. Pinto, the major cytokinin identified was DHZ, along with minor amounts of Z, ZR, and DHZR (Krasnuk et al., 1971). These compounds and their nucleotides were later identified in immature seeds of P. coccineus (Sodi and Lorenzi, 1982). The metabolism of exogenously applied ^{14}C-zeatin has been examined in embryos of several species of Phaseolus. ZR, ZMP, DHZR, and DHZMP were recovered when P. vulgaris embryo axes were incubated with [^{14}C]Z, (Sondheimer and Tzou, 1971). A comparative study of zeatin metabolism included P. acutifolius, P. coccineus, P. vulgaris and P. lunatus (Lee et al., 1985; Mok and Mok, 1987). All species metabolized [^{14}C]Z to ZR, ZMP, and ZROG. In addition, there were species specific differences. In P. lunatus, the major metabolite recovered was ZOG, while in all other species, ZOX and ZROX were recovered. These studies confirm that embryos of different species differ in [^{14}C]Z metabolism. Two cytokinin specific metabolic enzymes which have been isolated from Phaseolus embryos are a xylosyltransferase which mediates the transfer of xylose from UDPX to zeatin, and a reductase which

converts zeatin to DHZ. The embryo appears to autonomously regulate cytokinin metabolism, and the cytokinins found in embryos are produced endogenously, rather than being transported from maternal tissues (Singh et al., 1988).

The major organs of intact Phaseolus plants have also been examined with respect to cytokinin production and metabolism. When roots formed on the petioles of detached bean leaves, high levels of cytokinins accumulated in the leaves which suggests that cytokinins are produced in the roots (Englebrecht, 1972). The major cytokinin extracted from roots was ZOG, along with minor quantities of Z, ZR, and ZROG (Scott and Horgan, 1984). When labelled zeatin was fed to the roots of bean plants, DHZ, DHZOG, DHZROG, and ZROG were recovered in the stems and petioles (Waring et al., 1977). High levels of ZR, DHZR, and their nucleotides were extracted from stems of decapitated, disbudded P. vulgaris (Palmer et al., 1981a). Minor cytokinins found in stems included DHZOG and ZROG. Thus, ribosides and nucleotides may be the forms in which cytokinins are transported from the roots to the leaves.

The major cytokinin that accumulated in the leaves of decapitated, disbudded P. vulgaris was DHZOG (Wang et al., 1977), along with minor amounts of DHRZ (Wang and Horgan, 1978). In order to determine the stability of exogenous cytokinins in bean leaves, detached bean leaves

were incubated with ^{14}C analogues of Z, ZOG, DHZ, and DHZOG (Palmer et al., 1981b). ZOG was significant in all experiments, for it was not readily metabolized and it was the major product recovered after incubation with both Z and DHZ. Zeatin was rapidly metabolized in these tissues, while DHZOG and ZOG were quite stable. Thus, zeatin metabolism in leaves is different than in stems in P. vulgaris. The glucosides were considerably more stable in the leaves than in the stems. Palmer et al. (1981b) have proposed that cytokinin concentration gradients exist within the plant from the roots to the leaves, with high levels of zeatin derivatives in the roots and high levels of DHZ derivatives in the leaves. These concentration gradients are maintained by endogenous cytokinin metabolic enzymes and are important in establishing the proper levels of various active cytokinins required at specific stages in plant development.

Summary

In the area of cytokinin research there are still many unsolved questions. Cytokinins play a crucial role in plant development; however, the mechanism of action is not known. The plant can synthesize zeatin from AMP and and Δ^2-isopentenyl pyrophosphate, and then zeatin can be converted to about twenty other cytokinin derivatives.

In some cases precise identification of metabolic derivatives is questionable because two derivatives with similiar properties would not be separated. In addition, metabolites are often present in very small quantities which also makes specific identification difficult or impossible.

Specific functions have been suggested for the various metabolites, based on a variety of experiments. The specific functions of the O-glycosides are still questionable. A variety of evidence supports the role of O-glucosides as storage forms, but the O-xylosides may be directly active. More work needs to be done with O-xylosides, as only one experiment has looked at the function of these compounds.

In spite of many metabolic studies which have been done, the regulation of cytokinin metabolism is still not understood. A number of cytokinin metabolic enzymes have been purified, and have been used to confirm specific metabolic pathways. In Phaseolus, cytokinin metabolism varies from species to species and from tissue to tissue. Interspecific variations in cytokinin metabolism suggest that there is selective regulation of specific cytokinin metabolic enzymes. The goal of this research was to confirm interspecific differences in glycosylation enzymes in Phaseolus. This research is part of a larger project to identify genetic mechanisms regulating cytokinin metabolism and biosynthesis in Phaseolus.

MATERIALS AND METHODS

Plant Materials

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

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 (Kadir et al., 1984). [^{14}C]-Labeled *trans*-ribosylzeatin and dihydrozeatin were obtained by incubating [^{14}C]zeatin with crude extracts of PRPP-ribosyltransferase (Chen et al., 1982) and zeatin reductase (Mok et al., 1990) respectively.

Enzyme Isolation

For the isolation of O-glucosyltransferase, immature embryos of P. lunatus were homogenized in 1 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 2 bed volumes of extraction buffer. The enzyme was eluted with 4 bed volumes of buffer containing

UDPG (2.5 mM). The eluate was concentrated to 150 ul 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 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 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 ul), UDPG (3 mM), ATP (0.5 mM), $MgCl_2$ (0.05 M) and 0.025 uCi of [^{14}C]zeatin (0.001 umol) in 200 ul at pH 8 (buffered with 1 mM Tris) and 27 ° C. One ml of cold ethanol was added after 1 hr 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 ul <u>in vacuo</u> (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 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 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 B-glucosidase, following the procedures reported earlier (Lee et al., 1985), and

rechromatographed by HPLC at pH 4.8. In addition, standards of O-glucosylzeatin (synthesized in the laboratory of Dr. G. Shaw), O-xylosylzeatin and [^{14}C]-O-xylosylzeatin (synthesized earlier {Shaw et al., 1987}), 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 6.0-7.5 and pH 7.5-10, respectively. 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 Sephadex G-100 gel filtration using a 0.9 x 90 cm column and a flow rate of 0.25 ml/min. Mol wt standards used were bovine serum albumin (Mr 66,000), carbonic anhydrase (Mr 29,000), Cyt c (Mr 12,400) and aprotinin (Mr 6,500).

Substrate Specificity and Km 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 Km 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 uM, with 0.4 uM increments, at 2.6 mM UDPG. Kms for UDPG and UDPX were determined using concentration ranges from 0.05 to 4 mM with 0.15 mM increments, at 5 uM [^{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 co-eluted with authentic samples of O-glucosylzeatin (Fig. 1B), and after treatment with B-glucosidase, the radioactivity shifted to the position of zeatin (Fig. 1 C). 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 II), with a two fold purification over the fraction

Figure 1. Separation of cytokinin standards and analyses of products of enzymic reactions by HPLC. (A). Cytokinin standards. (B). Product of enzymatic reaction after incubation with labeled zeatin (Z), UDP-glucose and purified enzyme (extracted from 100 mg of embryos). (C). Products (approximately 11,000 cpm) obtained from (B) treated with B-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.

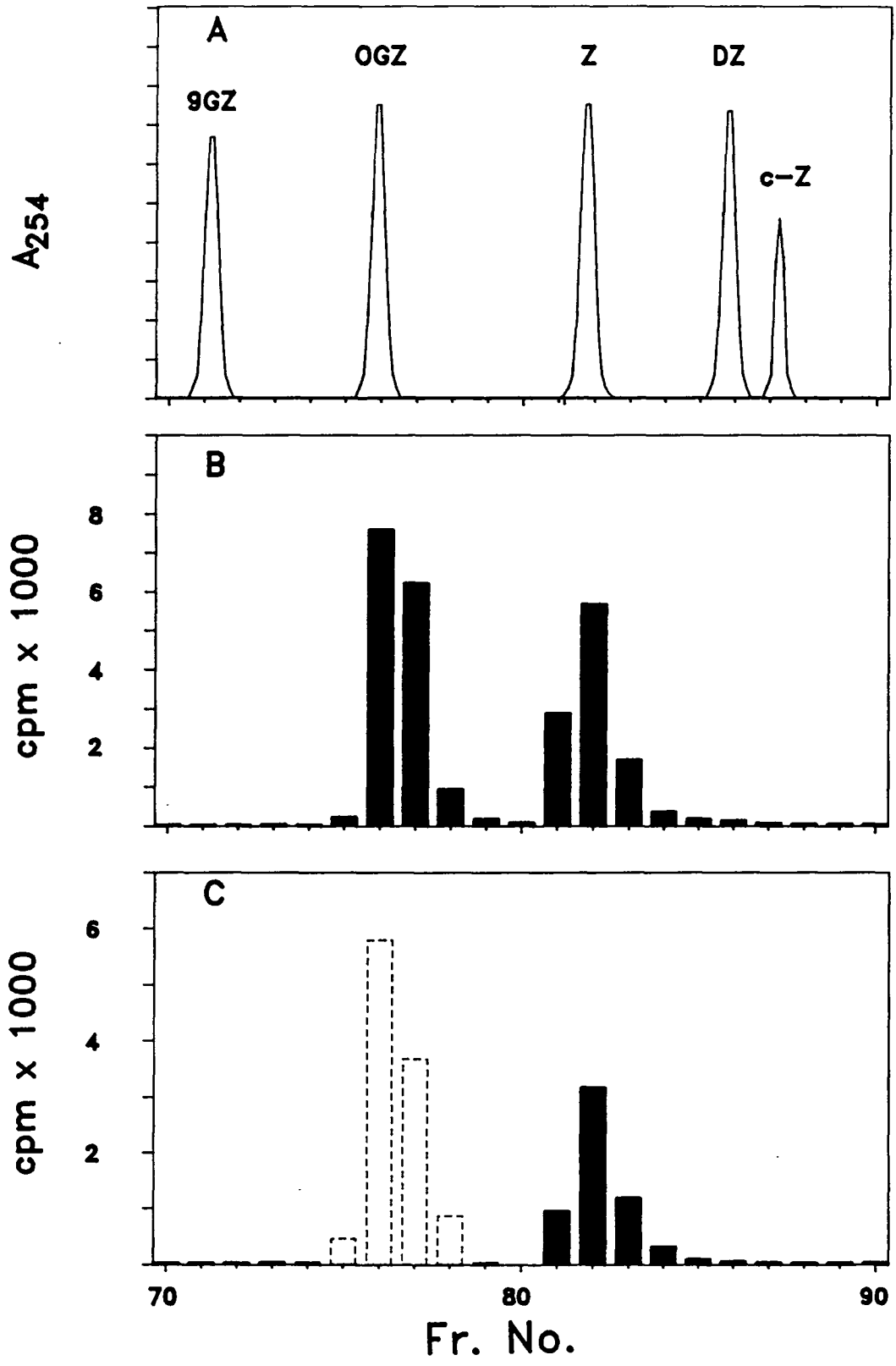

A
9GZ
OGZ
Z
DZ
c-Z
A254
B
cpm x 1000
8
6
4
2
C
cpm x 1000
6
4
2
70
80
90
Fr. No.

Figure 1

Table 2. Purification of O-glucosyltransferase from embryos of *P. lunatus* cv Kingston.

Sample[a]	Protein (ug/assay)	Activity (cpm of product)	Specific activity (cpm/ug protein)	Enrichment (fold)	Recovery (%)
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.

obtained after the ammonium sulfate precipitation. Blue Sepharose, which has high affinity for a wide range of enzymes using dinucleotides as substrates or co-factors, retained the O-glucosyltransferase whereas cytokinin oxidases were removed in the initial wash. The enzyme was selectively eluted from the Blue Sepharose column by 4 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 II). The enzyme was further purified by anion exchange HPLC. Enzyme activity was detected in fractions 24-28 (Fig. 2). The fraction with the highest activity represented a further 12-fold purification. The specific activity of the purified enzyme was 34 pmol/ug 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.

Characterization of the Enzyme

The conversion of zeatin to O-glucosylzeatin was linear up to 50 min. (Fig. 3). The pH optimum of the reaction was approximately 8 (Fig. 4). From the elution postions of the enzymes and the protein standards in relation to that of blue dextran (Vo) after gel filtration, the molecular weight of the enzyme was 44,000

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.

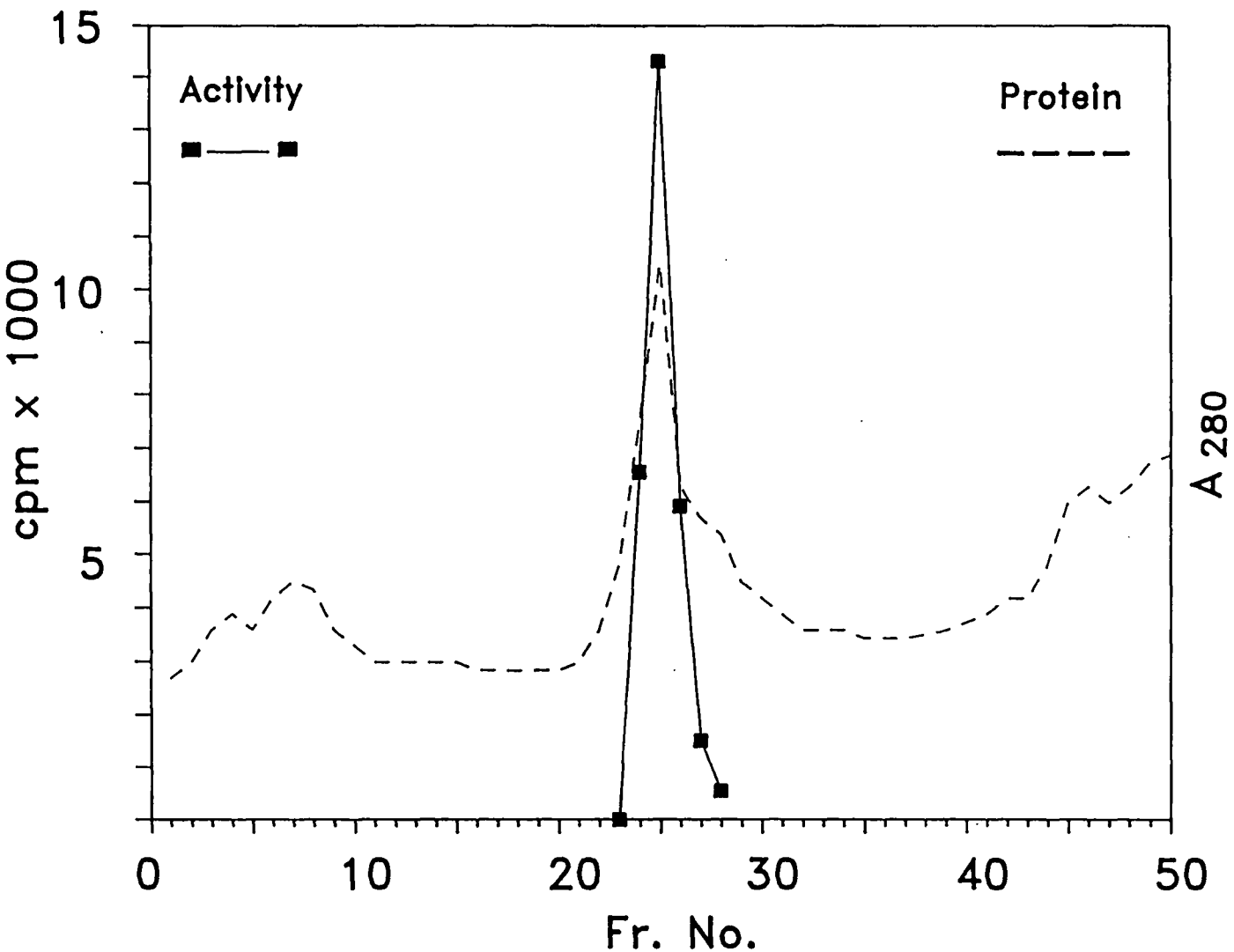


Figure 2

Figure 3. Time course of conversion of zeatin to O-glucosylzeatin.

Percent O-glucosylzeatin formed as a product of enzymatic reaction after incubation of labelled zeatin with UDP-glucose and purified enzyme for various lengths of time. Each assay contained the equivalent of enzyme extracted from 100 mg of embryos. Extracts were purified by ammonium sulfate precipitation prior to anion exchange HPLC.

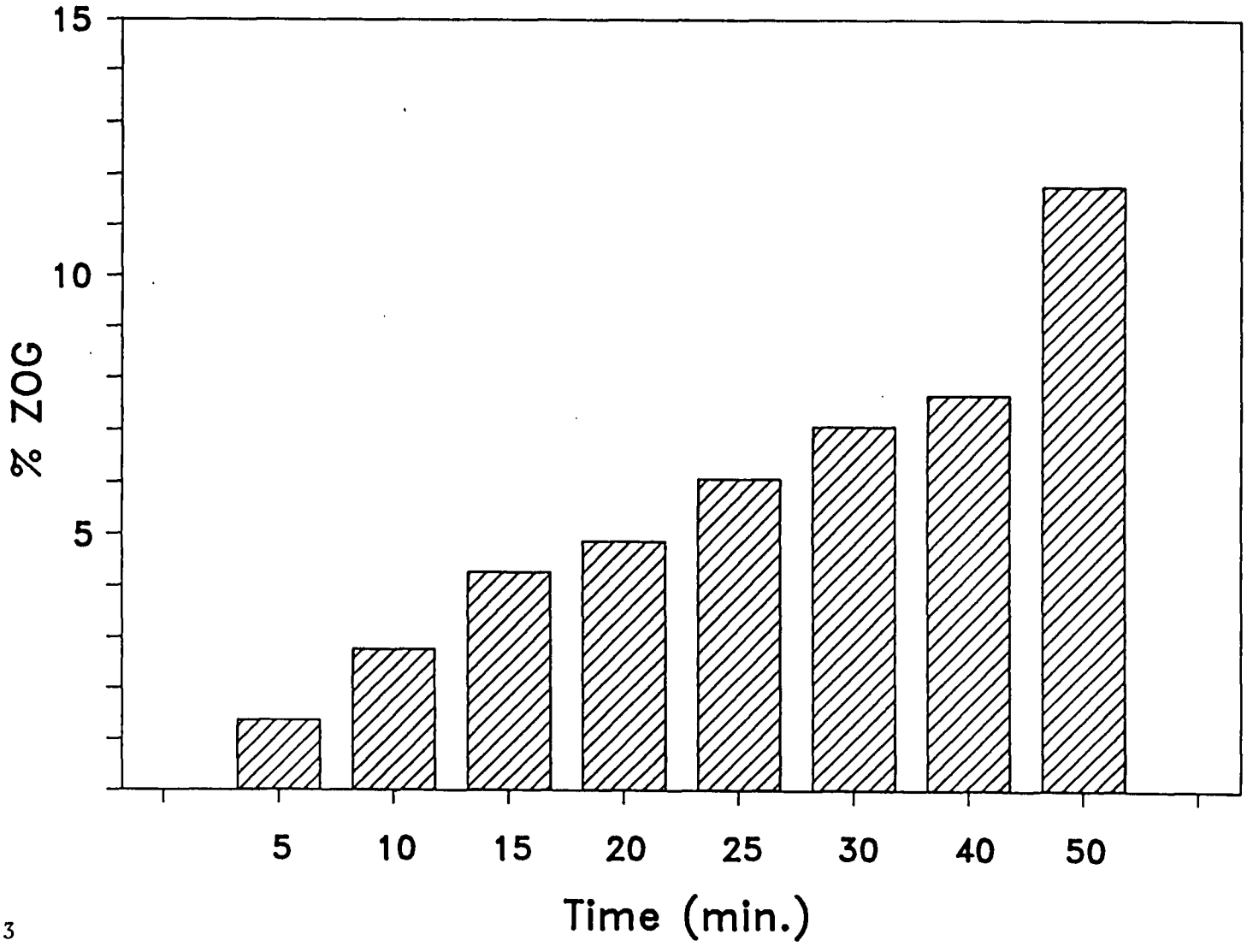


Figure 3

Figure 4. Effect of pH on the conversion of zeatin to O-glucosylzeatin.

Percent O-glucosylzeatin formed as a product of enzymatic reaction after incubation of labelled zeatin with UDP-glucose and purified enzyme. Phosphate and Tris-HCl buffers were used for pH 6.0-7.5 and pH 7.5-10, respectively. Each assay contained the equivalent of enzyme extracted from 150 mg of embryos. Extracts were purified by ammonium sulfate precipitation prior to anion exchange HPLC.

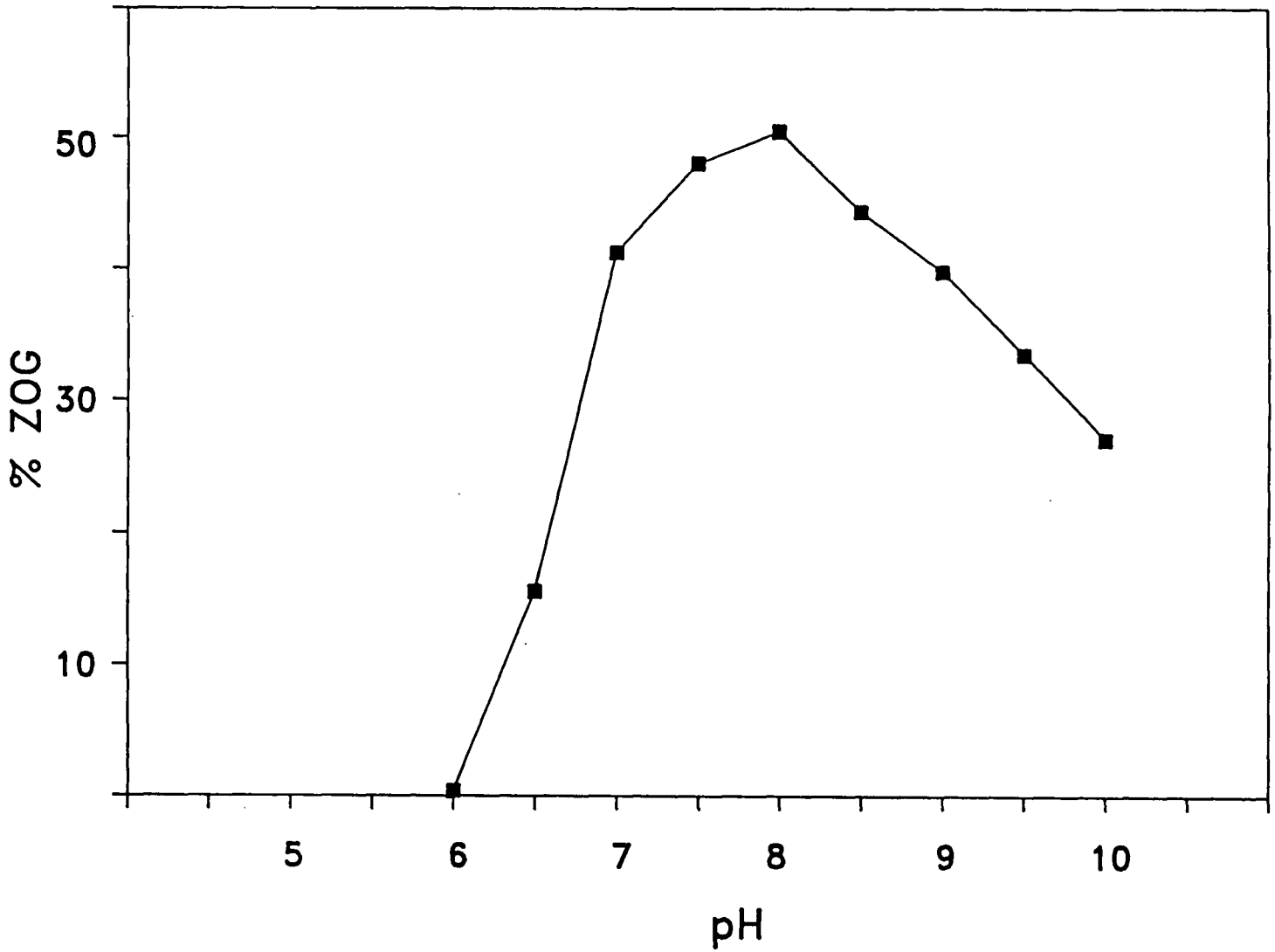


Figure 4

D ±4,000 (Fig. 5). Of the four cytokinins tested, zeatin was the only substrate for the enzyme (Table III). The Km of zeatin was 28 uM (Table III, Fig. 6). A number of compounds were tested as glycosyl donors in the enzymatic reaction (Table III). UDPG served efficiently as the sugar donor with a Km of 216 uM. In addition, UDPX was found to be a substrate, but the Km (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 (Turner et al., 1987). These observations prompted a series of experiments to compare the O-glucosylation enzyme from P. lunatus with the O-xylosyltransferase of P. vulgaris.

O-xylosyltransferase from P. vulgaris was purified using the same procedure as used for the purification of the O-glucosyltransferase from P. lunatus. The recovery of enzyme based on the HPLC fraction with the highest activity was 13.5 %.

Figure 5. 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.)

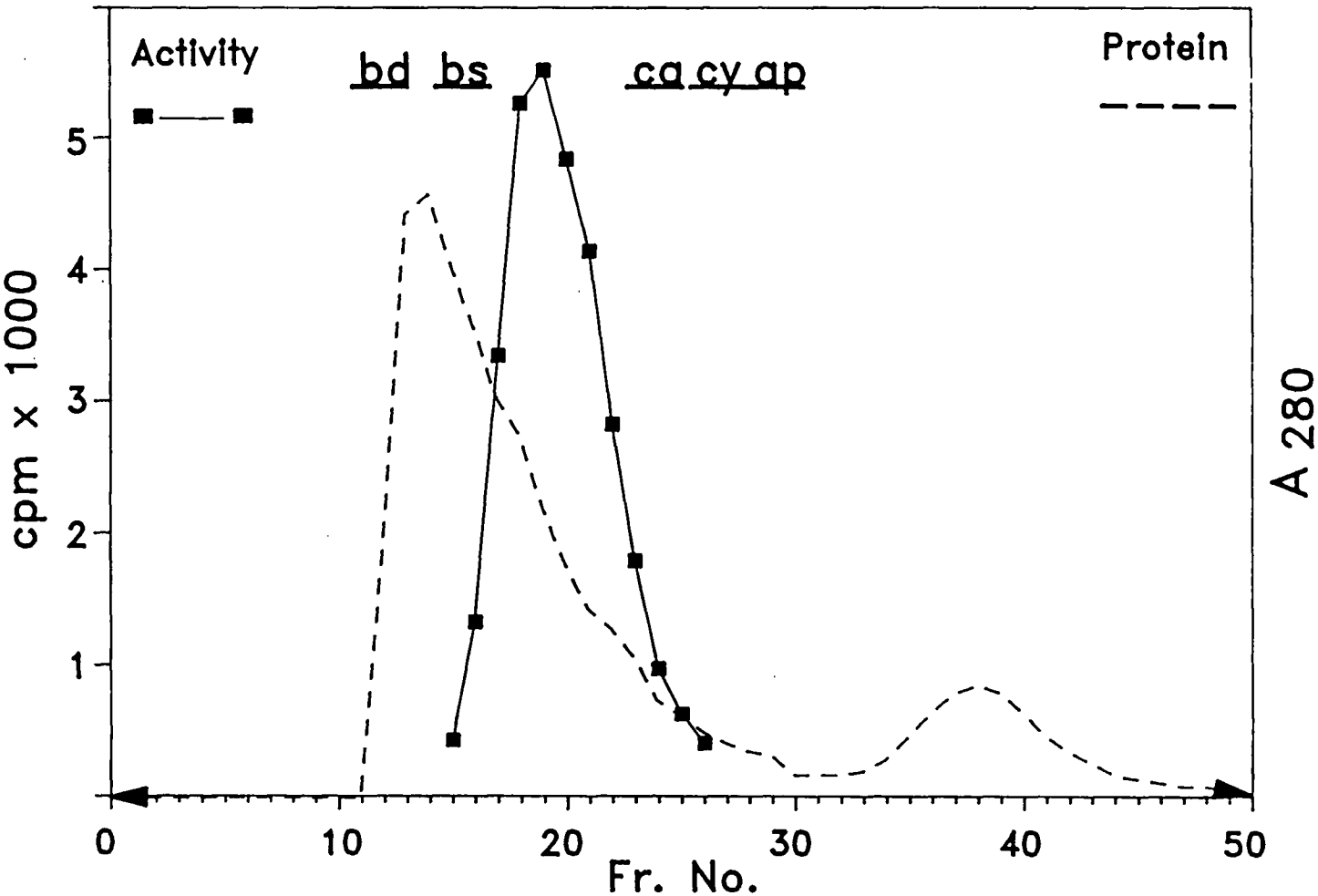

Activity
bd bs
ca cy ap
Protein
cpm x 1000
A 280
Fr. No.
0
10
20
30
40
50
1
2
3
4
5

Figure 5

Table 3. Substrate specificity and Kms of O-glucosyltransferase isolated from embryos of P. lunatus cv Kingston.

Substrate	Reaction	Km (uM)
trans-zeatin	+	28
ribosylzeatin	-	
dihydrozeatin	-	
cis-zeatin	-	
UDP-glucose	+	216
UDP-xylose	+	2,700
ADP-glucose	-	
UDP-galactose	+	ND

Figure 6. Lineweaver-Burk plot for conversion of zeatin to O-glucosylzeatin.

Each assay contained the equivalent of enzyme extracted from 800 mg of embryos. Extracts were purified by ammonium sulfate precipitation and affinity chromatography prior to anion exchange HPLC. Concentrations of [^{14}C]zeatin ranged from 0.8 to 7.2 uM, with 0.4 uM increments, at 2.6 mM UDPG.

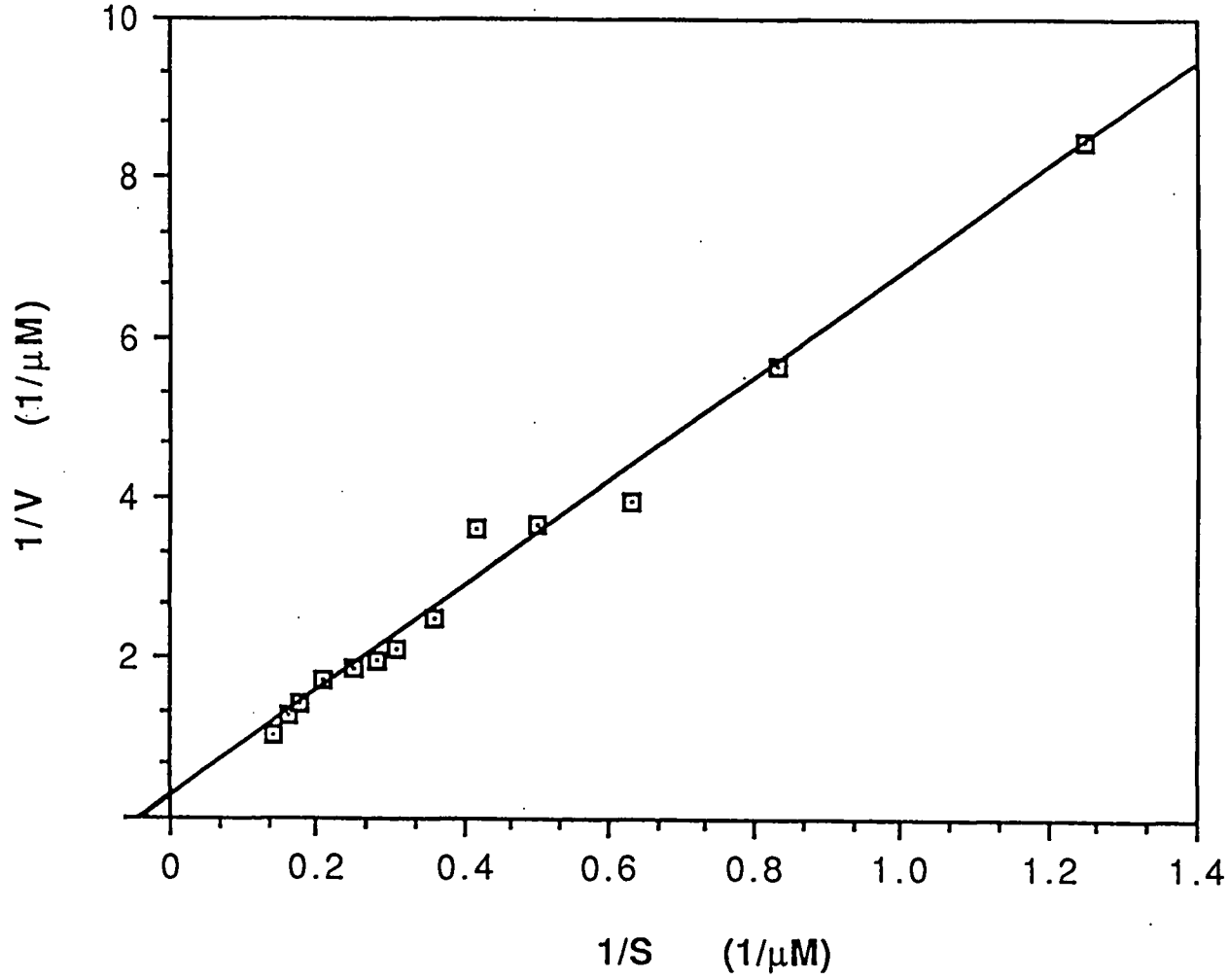


Figure 6

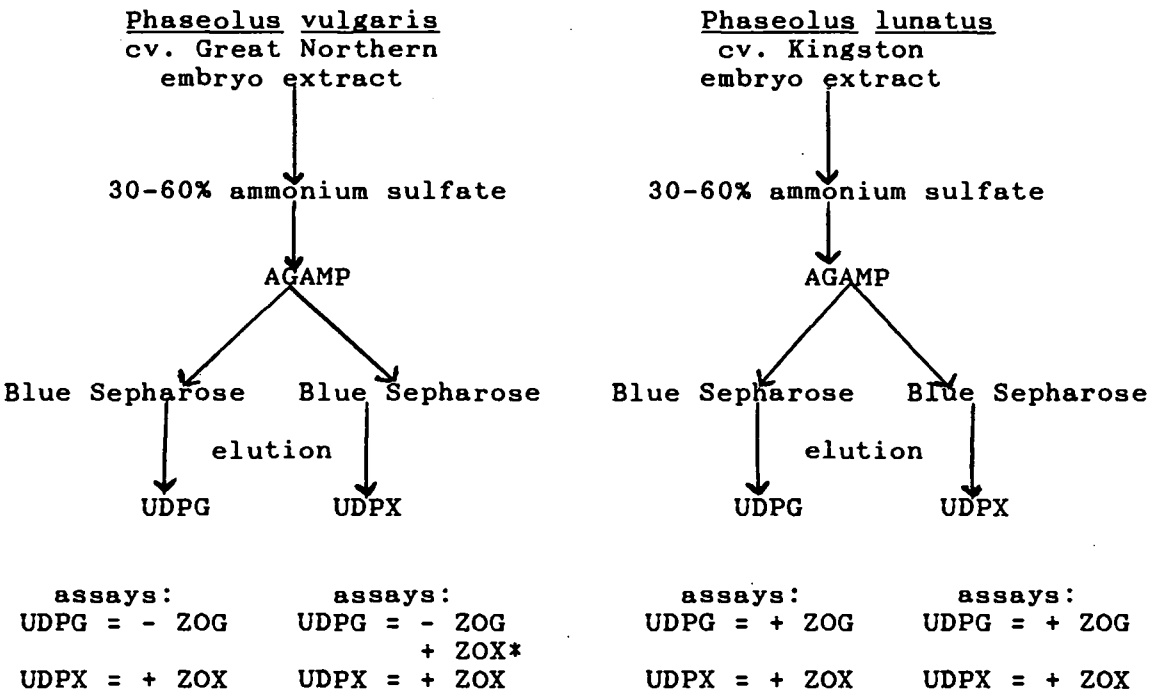
To examine the possibility that additional glycosylation enzymes were lost from embryos during Blue Sepharose affinity chromatography, UDPX and UDPG were applied separately to affinity columns to elute enzyme preparations of P. vulgaris or P. lunatus. Interestingly, enzymes of both species could be eluted from the affinity column using either sugar dinucleotide (Fig. 7). 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. 8A). The enzyme preparation isolated from P. lunatus recognized both donor substrates and in the competition experiment O-glucosylzeatin was formed almost exclusively (Fig. 8B). These results suggest that these 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.

Figure 7. Differential elution of glycosylation enzymes extracted from embryos of P. vulgaris and P. lunatus and applied to Blue Sepharose CL-6B affinity columns.

Each enzyme was eluted from the Blue Sepharose column with either UDPG or UDPX. Each Blue Sepharose eluate was assayed with both UDPG and UDPX. Each assay contained the equivalent of enzyme extracted from 425 mg of embryos.



* This enzyme sample was washed three times with buffer after elution. No UDPX was added to the assay; however, ZOX was still recovered. This conversion to UDPX is due to UDPX used in elution buffer which remained tightly bound to the enzyme.

Figure 7

Figure 8. 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, respectively.

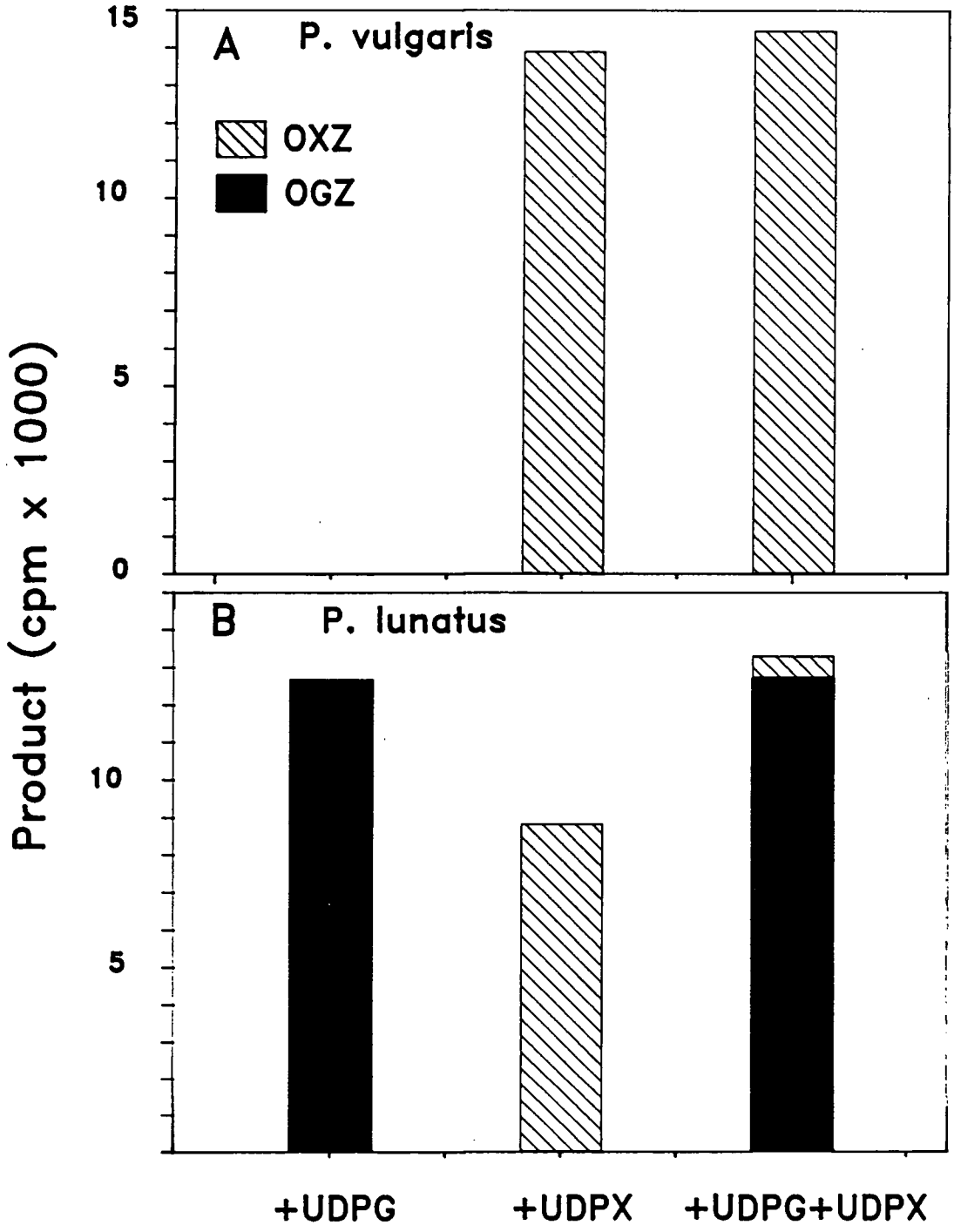

A
P. vulgaris
OXZ
OGZ
B
P. lunatus
Product (cpm x 1000)
15
10
5
0
10
5
+UDPG
+UDPX
+UDPG+UDPX

Figure 8

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. 9A) as well as O-xylosylzeatin (Fig. 9B), characteristic of the glucosyltransferase. Enzyme contained in fractions 27 to 31 catalyzed only the formation of O-xylosylzeatin (Fig. 9B), 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. 9C), 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.

Figure 9. Distribution of enzyme activity, of combined samples, eluted from AX300 anion exchange column.

(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 CL-6B affinity columns. The preparations were then combined and purified on an AX-300 anion exchange column. Fractions of 1 ml were collected and assayed for enzyme activity by incubating with the glycosyl donors.

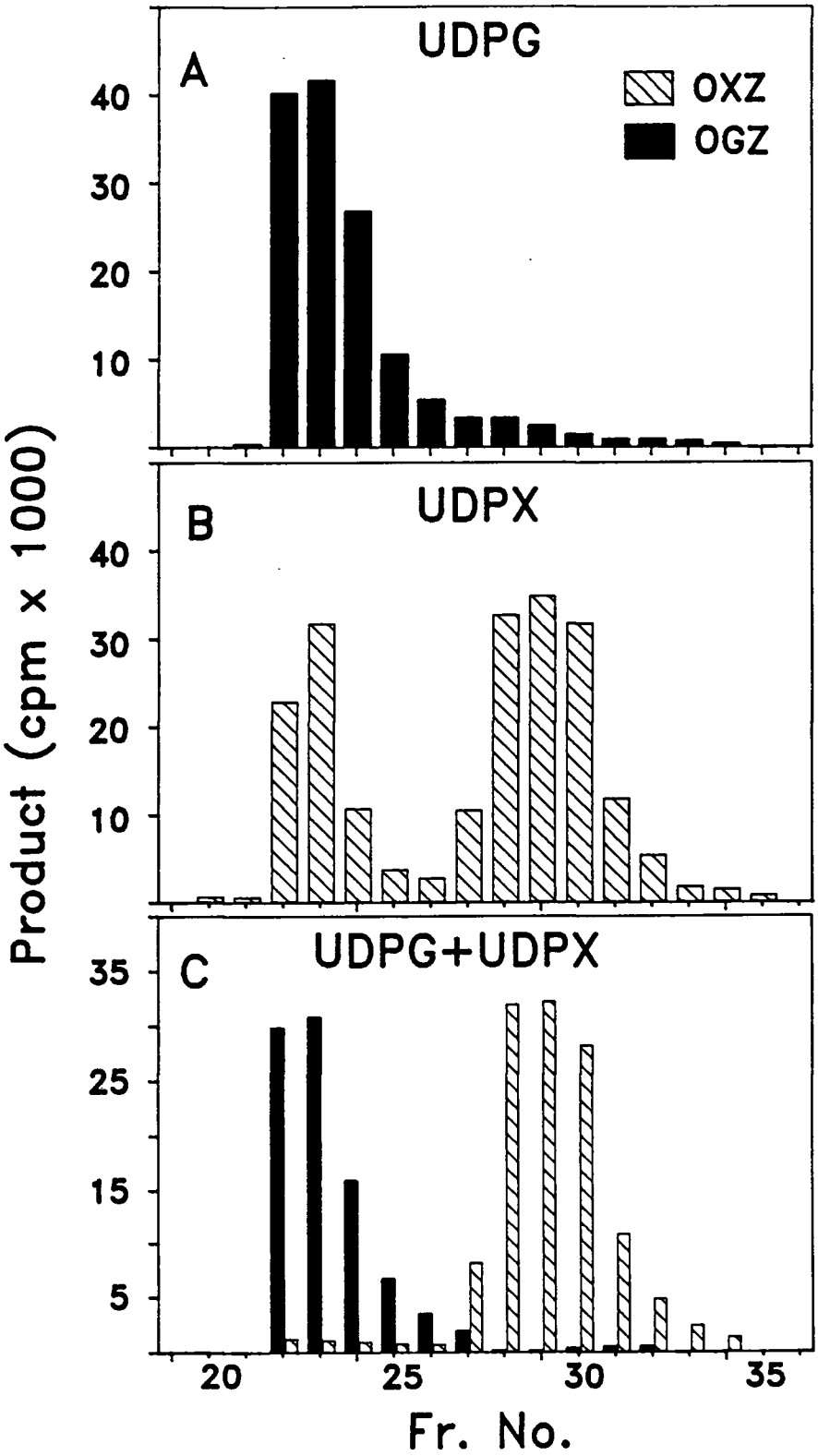

UDPG
A
OXZ
OGZ
40
30
20
10
UDPX
B
40
30
20
10
UDPG+UDPX
C
35
25
15
5
Product (cpm x 1000)
20
25
30
35
Fr. No.

Figure 9

DISCUSSION

The results presented indicate the occurrence of an 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 (Mok et al., 1990).

O-Glucosylzeatin has been identified in many other plant species (Duke et al., 1979; Morris, 1977; Scott et al., 1982a), 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 (Entsch et al., 1979). As expected, the O-glucosyltransferase described here does not mediate formation of N-glucosides. N-glucosyl derivatives were not detected in either in vivo or in vitro metabolism studies using Phaseolus embryos (Lee et al., 1985; Turner et al., 1987). It appears that N-glucosylation of zeatin (Fox et al., 1971; Fox et al., 1973; Parker et al., 1978) may not be pronounced in this genus.

O-glycosylation markedly enhances cytokinin stability. As leaves mature, glycosides accumulate to high levels (Palmer et al., 1981a). Mature leaves undergo senescence despite high levels of O-glucosides, which suggests that the glucosides may be compartmentalized so that they are not exposed to degradative enzymes (Letham and Palni, 1983). When [^{3}H]DHZ was applied to suspension cultures of Chenopodium

rubrum the predominant metabolites recovered were DHZOG and DHZROG (Fusseder and Ziegler, 1988). Both of these compounds were shown to be compartmentalized within the vacuole, while DHZ and DHZR were found outside the vacuole. After 36 hr of incubation, no export of O-glucosides could be detected; however, substantial export of other cytokinin metabolites had occurred. The plasma membrane is impermeable to cytokinin glucosides, while ribosides and free bases are permeable (Laloue et al., 1981). If cell membranes restrict the passage of glucosides, then the accumulation of O-glucosides in the vacuole must result from either of two possibilities. Glycosylation could take place within the vacuole, or glycosylation could take place outside the vacuole, and glucosides could be transported into the vacuole via specific tonoplast carriers (Fusseder and Ziegler, 1988). In order to differentiate between these two possibilities the location of glycosylation enzymes must be known. Antibody probes to glycosylation enzymes I have purified could be produced and used to determine the cellular locatization of these enzymes.

A number of genotypic differences in cytokinin metabolism in Phaseolus embryos as well as in callus tissues have been identified (Lee et al., 1985, Mok et al., 1978; Mok et al., 1980). Many of the differences are related to differential side chain removal or conjugation of naturally occurring cytokinins. Recently,

genetic differences in side chain reduction of zeatin were also identified (Mok and Mok, 1987; Mok et al., 1990), and the enzyme, zeatin reductase, has been partially purified (Martin et al., 1989). 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. Once antibodies are obtained, they will be used as probes for gene cloning experiments to further characterize the interspecific differences in zeatin metabolism.

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