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Partial Purification of a *cis-trans*-Isomerase of Zeatin from Immature Seed of *Phaseolus vulgaris* L.[1]

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Investigation of the conversion of exogenous *cis*-zeatin to *trans*-zeatin in immature seeds of *Phaseolus vulgaris* L. led to the isolation of a *cis-trans*-isomerase from the endosperm. The enzyme was purified more than 2000-fold by chromatography on a series of fast protein liquid chromatography (anion exchange, gel filtration, and hydrophobic interaction) and concanavalin A columns. The enzymic reaction favors conversion from the *cis* to the *trans* form and requires flavin, light, and dithiothreitol. *cis*-Zeatin riboside is also a substrate for the enzyme. Retention on the concanavalin A column indicated that the enzyme is a glycoprotein. The enzyme was stable for at least 8 weeks when stored at −80°C. The occurrence of *cis-trans*-isomerization suggests that *cis*-zeatin and *cis*-zeatin riboside formed by tRNA degradation could be precursors of biologically active cytokinins.

Cytokinins play an important role in the regulation of cell division, growth, and differentiation of plants. The most prevalent cytokinins in plant tissues are *trans*-zeatin and its derivatives. Two pathways have been suggested for the biosynthesis of *trans*-zeatin, de novo synthesis and indirect synthesis. De novo synthesis consists of formation of N^6-(Δ^2-isopentenyl)adenosine phosphate from AMP and Δ^2-isopentenylpyrophosphate mediated by an ipt, followed by hydroxylation of the side chain. The transferase has been isolated from *Dictyostelium discoideum* (Taya et al., 1978) and tobacco callus tissues (Chen and Melitz, 1979). Similar enzyme activity was found to be associated with the Ti plasmid of *Agrobacterium tumefaciens*, and the bacterial gene (*ipt*) encoding the enzyme has been cloned (Akiyoshi et al., 1984; Barry et al., 1984; Buchmann et al., 1985; Morris et al., 1985). However, no sequence homology to the *ipt* gene of *A. tumefaciens* has been found in plant tissues.

The second pathway involves breakdown of nucleic acids, in particular tRNAs that contain cytokinins adjacent to the anticodon (Skoog and Armstrong, 1970; Letham and Palni, 1983). These nucleosides include N^6-isopentenyladenosine, *cis*-zeatin riboside, *trans*-zeatin riboside, and the methylthio derivatives of these compounds (Skoog and Armstrong, 1970). A major challenge to this pathway is that the predominant cytokinin in plant tRNA is *cis*-zeatin riboside (Vreman et al., 1972, 1978; Edwards et al., 1981), which is at least 100-fold less active than *trans*-zeatin in the tobacco callus bioassay (Schmitz et al., 1972). Critical support for this pathway would require identification of mechanisms by which the weakly active *cis*-isomers can be converted to their active *trans* counterparts.

Recently, we observed that extracts of immature bean seeds convert *cis*-zeatin to its *trans*-isomer in the light (Mok et al., 1992b). In this study, we confirmed the existence of an isomerase, and we report here the steps for its partial purification and key features of the reaction. The occurrence of a *cis-trans*-isomerization indicates that *cis*-isomers of zeatin and zeatin riboside released by tRNA degradation can serve as a source of biologically active cytokinins.

MATERIALS AND METHODS

Plant Material

Immature seeds (3–10 mm long) of *Phaseolus vulgaris* L. cv Great Northern were used for enzyme isolation.

Chemicals

trans-Zeatin, *cis*-zeatin, *cis*-zeatin riboside, FAD, flavin adenine mononucleotide, and α-D-mannopyranoside were obtained from Sigma. *cis*-[8-^{14}C]Zeatin was synthesized from 6-Cl-[8-^{14}C]purine (24 mCi mmol^{-1}) by the procedures reported previously (Kadir et al., 1984). BSTFA was obtained from Pierce.

Enzyme Isolation

Preliminary experiments in which the enzyme activities in extracts of various components of the seeds were compared indicated that endosperm was a rich source of the enzyme. Liquid endosperm removed from seeds with a microsyringe was mixed with an equal volume of buffer A (55 mM Tris-HCl [pH 7.5] and 4 mM DTT). Proteins precipitating between 30 and 60% ammonium sulfate saturation were collected after centrifugation at 12,000*g* for 30 min, desalted on an Econo-Pac 10DG column (Bio-Rad), and concentrated by centrifugation at 4000*g* in Centricon 30 ultrafiltration tubes (Amicon).

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Abbreviations: BSTFA, *N,O*,-bis(trimethylsilyl)trifluoroacetamide; FAD, flavin adenine dinucleotide; FPLC, fast protein liquid chromatography; ipt, isopentenyl transferase.

Enzyme Purification

Anion-Exchange Chromatography

The enzyme extract was applied to a Mono-Q HR 5/5 anion-exchange FPLC column equilibrated with buffer A and fractionated with a linear gradient of 0 to 500 mM KCl in buffer A over 20 min at a flow rate of 1 mL min^{-1}. Fractions of 0.5 mL were collected. Individual fractions were assayed for enzyme activity, and the positive fractions were pooled and concentrated by Centricon 30 ultrafiltration.

Gel Filtration FPLC

The active fractions obtained after Mono-Q FPLC were loaded on a Superose 12 FPLC column equilibrated with buffer A plus 150 mM NaCl. Proteins were eluted with this buffer at a flow rate of 0.5 mL min^{-1}. Fractions of 0.25 mL were collected and assayed for enzyme activity. The M_r of the enzyme was estimated by its elution relative to BSA (M_r 66,000), carbonic anhydrase (M_r 29,000), Cyt *c* (M_r 12,400), and aprotinin (M_r 6,500).

Phenyl Superose FPLC

The positive fractions from gel filtration FPLC were brought to 30% ammonium sulfate saturation and applied to a Phenyl Superose HR 5/5 FPLC column (Pharmacia) equilibrated with buffer B (55 mM Tris-HCl [pH 7.5], 2 mM DTT, and 30% ammonium sulfate). Enzyme was eluted with a linear gradient of 30 to 0% ammonium sulfate over 30 min. The flow rate was 0.5 mL min^{-1}, and the fraction size was 0.5 mL.

PAGE

Proteins purified by the FPLC steps were concentrated and separated by PAGE on 1.5-mm-thick 10% acrylamide gels. The running buffer consisted of 5 mM Tris-HCl and 3.8 mM Gly. Electrophoresis was performed overnight at constant voltage (90 V) and 4°C. Gel sections (5 mm) were transferred to dialysis tubes (mol wt cut-off of 14,000) containing 1.5 mL of buffer (55 mM Tris-HCl [pH 7.5] and 1 mM DTT), and proteins were eluted in an electrophoresis chamber (200 V for 1 h).

Con A-Sepharose 4B Chromatography

Con A-Sepharose 4B was washed with 50 bed volumes of 50 mM bis-Tris-HCl (pH 6.5) containing 0.25 M ammonium sulfate, 0.1 M methylmannose, 1 mM $CaCl_2$, and 1 mM $MnCl_2$, followed by 50 bed volumes of 50 mM bis-Tris-HCl (pH 6.5), with 0.25 M ammonium sulfate. Column material was packed in a Poly-Prep column (Bio-Rad) and equilibrated with 50 mM bis-Tris-HCl buffer containing 0.25 M ammonium sulfate and 2 mM DTT. The Phenyl Superose-purified enzyme preparation was loaded onto the column, which was then washed with 10 bed volumes of equilibration buffer. Enzyme was eluted with 5 bed volumes of the same buffer containing 0.1 M methyl-α-D-mannopyranoside.

Enzyme Assay

The assay mixture consisted of 70 μL of Tris-HCl buffer (55 mM [pH 7.5]) with or without enzyme extract, 10 μL of *cis*-[8-^{14}C]zeatin (0.01 μCi; 0.4 nmol), 10 μL of FAD (1 mM), and 10 μL of $MgCl_2$ (0.4 M). DTT concentrations ranged from 2 to 0.15 mM, depending on the purity of the enzyme (see "Results"). The assay mixture was incubated at 35°C under cool-white fluorescent light (110 μmol m^{-2} s^{-1}) for 1 h. Ice-cold methanol (100 μL) was added to terminate the reaction. The mixture was centrifuged at 27,000*g* for 10 min, and the supernatant was stored at −80°C until analyzed.

Cytokinin Analysis

To detect and quantify cytokinins resulting from the reaction, a Beckman model 110A dual-pump HPLC system with a reversed-phase C_{18} column (Ultrasphere ODS, 5-μm particle size, 4.6 × 250 mm; Altex) was used. The aqueous buffer consisted of 0.2 M acetic acid adjusted to pH 3.5 by triethylamine. Samples were eluted with a linear gradient of methanol (5–50% over 90 min). The flow rate was 1 mL min^{-1}, and 0.5-mL fractions were collected. Radioactivity was determined in Ready-Gel scintillation fluid with a Beckman LS 7000 scintillation counter. The enzyme activity was calculated after the background conversion, which was determined in control samples without the enzyme, was subtracted.

Protein Determination

The amount of protein in each fraction was determined with a Bio-Rad protein assay kit and procedures recom-

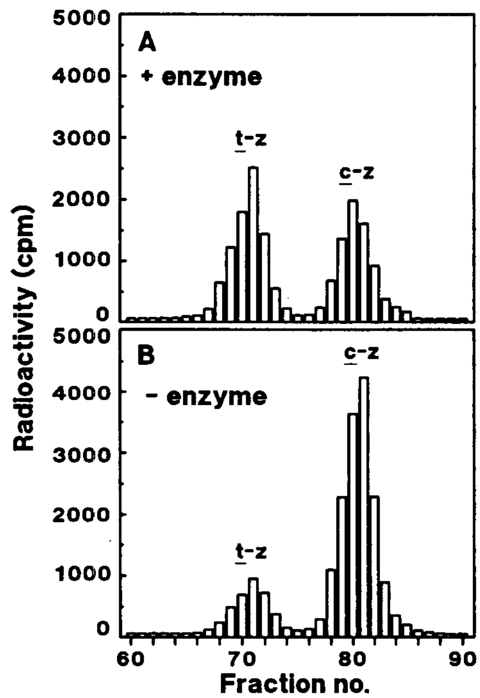

Figure 1. Separation by HPLC on reversed-phase C_{18} of the radiolabeled product (*trans*-zeatin; t-z) and substrate (*cis*-zeatin; c-z) after incubation with (A) or without (B) isomerase.

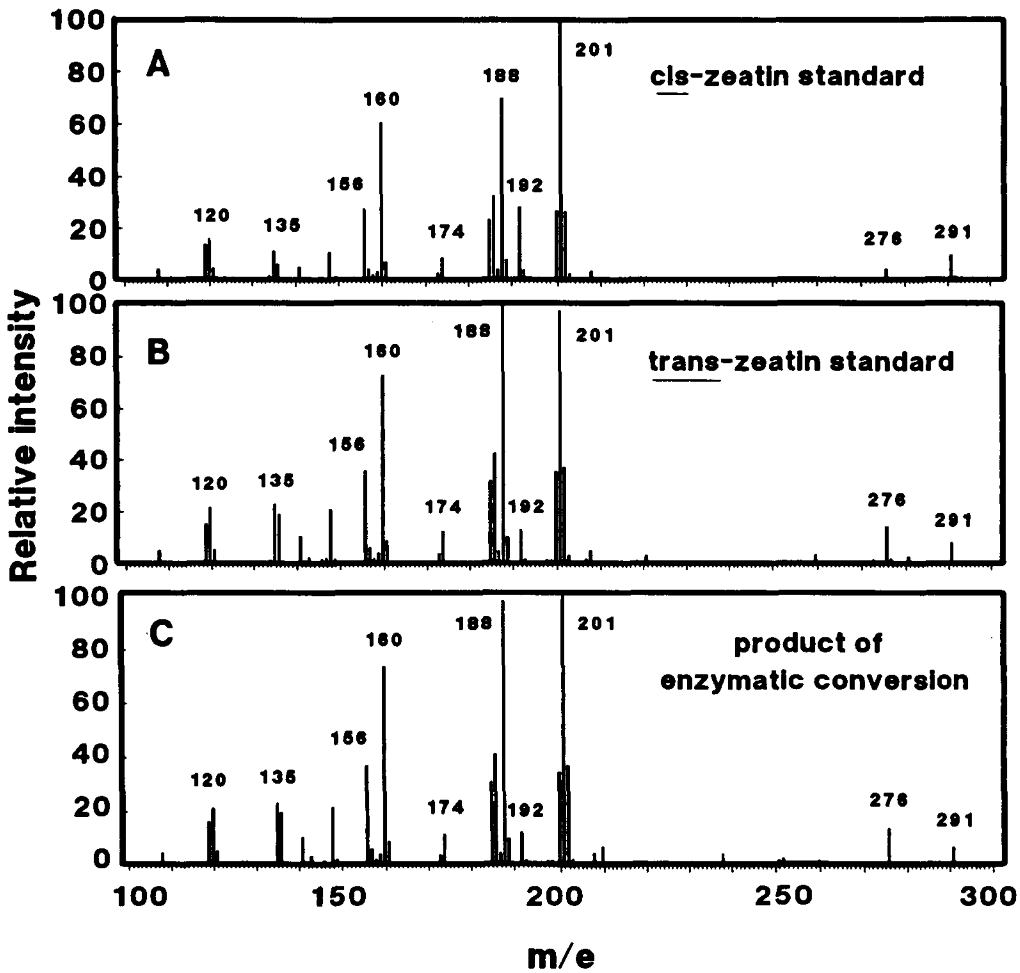

Figure 2. Mass spectra of *cis*-zeatin (A), *trans*-zeatin (B), and the product of enzymic conversion of *cis*-zeatin (C) after derivatization with BSTFA.

mended by the manufacturer, with BSA as the standard. The protein composition of various fractions was determined by SDS-PAGE (Laemmli, 1970); for details see Martin et al. (1990).

MS

The product of the enzymic conversion was separated from the original *cis*-zeatin substrate by HPLC, dried, and derivatized in 5 μL of pyridine and 5 μL of BSTFA for 15 min at 60°C. GC/MS analyses of the trimethylsilylated product and standards (*cis*- and *trans*-zeatin) were performed with a Finnigan model 4023 instrument in the electron impact mode. Samples were injected in a 30-m × 0.25-mm SE-54 column and eluted at a 25 cm s^{-1} linear velocity of the carrier gas (purified helium). The temperature was increased from 100 to 300°C over 10 min.

RESULTS

Substrates and Products

Incubation of radiolabeled *cis*-zeatin under the standard assay conditions (1 h under cool-white fluorescent light, 110 μmol m^{-2} s^{-1}), with or without enzyme, resulted in formation of a product that cochromatographed with *trans*-zeatin (Fig. 1). The nonenzymic conversion was 13% in 1 h, whereas enzymic conversion could be as high as 70%. When the enzyme extract was boiled for 10 min, conversion was the same as without enzyme. Incubation with radiolabeled *trans*-zeatin under the same conditions indicated that the reverse reaction could also take place. However, conversion in this direction was only 2% in the absence of enzyme and up to 25% in the presence of enzyme. Thus, the conversion from *cis*- to *trans*-zeatin is favored.

The occurrence of isomerization was confirmed by characterization of products by GC/MS. Derivatization with BSTFA led to formation of two products, the mono- and ditrimethylsilyl derivatives, which were separated by GC. The spectra of the mono derivatives are shown in Figure 2. The product resulting from incubation of *cis*-zeatin with enzyme (Fig. 2C) had the characteristic MS pattern of *trans*-zeatin (Fig. 2B). Specifically, the relative intensity of the m/e 188 ion was higher for the product and *trans*-zeatin than for *cis*-zeatin, whereas that of the m/e 192 ion was lower.

To determine whether ribosides could serve as substrates

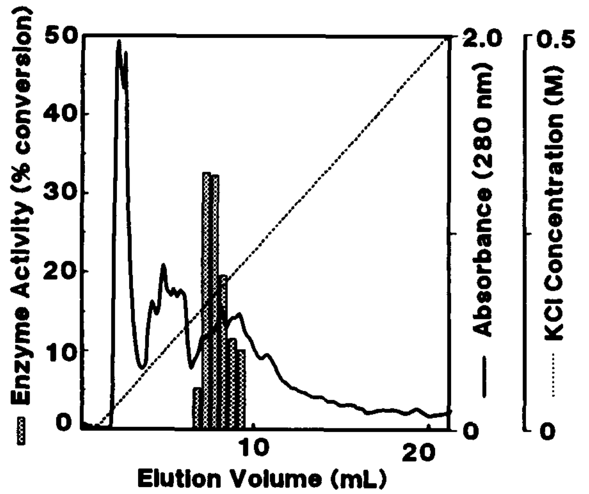

Figure 3. Elution profile of zeatin *cis-trans*-isomerase after Mono-Q HR 5/5 FPLC. Protein from 5 mL of endosperm was applied to the column and eluted with a linear gradient of 0 to 500 mM KCl in 55 mM Tris-HCl buffer (pH 7.5) with 4 mM DTT for 20 min at a flow rate of 1 mL min^{-1}. Enzyme activity was determined with 10 μL from each 0.5-mL fraction.

for the enzyme, the *cis*-^{14}C-zeatin in the reaction mixture was replaced with 40 nmol of *cis*-ribosylzeatin. Under standard enzyme assay conditions, more than 40% of the *cis*-ribosylzeatin was converted to *trans*-ribosylzeatin, and only a small *trans*-ribosylzeatin peak appeared after nonenzymic conversion. Thus, the enzyme can also use the corresponding riboside as substrate.

Reaction Conditions and Cofactors

Light and flavin were necessary for *cis-trans*-zeatin isomerization. Both flavin adenine mononucleotide and FAD were effective cofactors. DTT was required for the reaction and to limit breakdown of zeatin (to adenine) under the assay conditions. However, the optimal DTT concentration for isomerization decreased as the purity of the enzyme increased. For instance, 2 mM was optimal after ammonium sulfate precipitation and after anion-exchange FPLC, 1 mM after gel filtration FPLC and Con A chromatography, and 0.15 mM after PAGE.

Enzyme Purification

Formation of *trans*-zeatin is not expected to be linear over time because the isomerase also mediates the reverse reaction under the same conditions. For the same reason, product formation may not be proportional to the amount of enzyme. However, we have established that conversion over 1 h was proportional to the amount of enzyme used if less than 40% of the *cis*-zeatin was converted to *trans*-zeatin. Therefore, to compare activity in different fractions, the assay time was kept at 1 h, and if conversion was higher than 40% in any assay, it was repeated with less enzyme.

It was difficult to determine enzyme activity in crude endosperm preparations because of the large volume. Proteins were concentrated by precipitation with ammonium sulfate (30–60% saturation), and this fraction was used as the initial extract from which further purification of the enzyme was calculated.

Anion-exchange FPLC on a Mono-Q column separated proteins into three major regions with the enzyme eluting with the third peak (Fig. 3); 6-fold purification of the enzyme was obtained (Table I). Gel filtration on a Superose 12 FPLC column (Fig. 4) increased the purity 3-fold. Based on the elution volume relative to standards, the molecular mass was estimated to be 68 ± 4 kD. However, this estimate may not reflect the true molecular size because the enzyme seems to be a glycoprotein (see below). Hydrophobic interaction FPLC was effective in separating the enzyme from other proteins (Fig. 5), resulting in about 9-fold purification over the active fraction from gel filtration. In addition to the major peak at 15 mL, two smaller peaks were present in the activity region. It is not clear whether this signifies the presence of isozymes; all other purification methods resolved only a single peak of activity. Either PAGE or Con A chromatography was the final purification step. Although PAGE resulted in increased specific activity, it also caused substantial loss of the enzyme (Table I). When substituted by a Con A column, loss of enzyme was minimal and purification was 15-fold. The enzyme can be stored at −80°C for at least 8 weeks without loss of activity.

The sequential purification steps resulted in more than 2000-fold purification of the enzyme (Table I). Because the calculation was based on the sample obtained after ammonium sulfate fractionation, the actual extent of purification was probably higher. Enzyme recovery was 44% after Con

Table I. *Purification of zeatin cis-trans-isomerase from P. vulgaris endosperm*

For details of purification, see "Materials and Methods." Total activity and protein are for 10 mL of endosperm equivalent.

Purification Step	Total Activity	Protein	Specific Activity	Purification	Recovery
	pmol h^{-1}	*μg*	*pmol μg^{-1} h^{-1}*	*fold*	%
30–60% $(NH_4)_2SO_4$	39,187	26,823	1.5	1	100
Mono-Q	25,769	2,763	9.3	6	66
Superose 12	33,089	1,313	25.2	17	84
Phenyl Superose	24,329	105	232.7	155	62
PAGE	3,668	3	1,079.0	719	9
or					
Con A	17,030	5	3,406.0	2,271	44

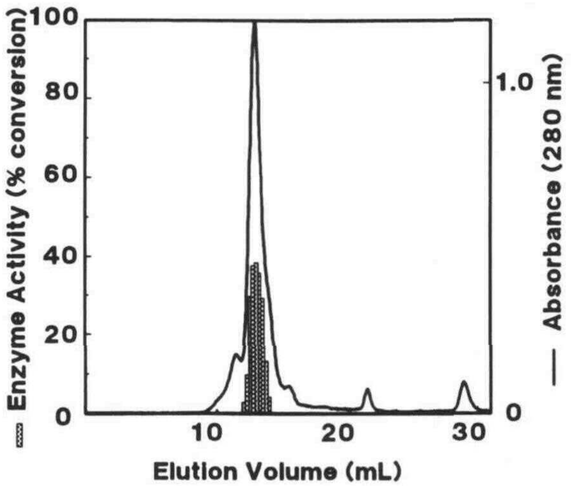

Figure 4. Elution profile of zeatin *cis-trans*-isomerase after gel filtration FPLC on Superose 12. The column was equilibrated and developed with 55 mM Tris-HCl buffer (pH 7.5) containing 4 mM DTT and 150 mM NaCl. Active fractions from Mono-Q FPLC (equivalent to 10 mL of endosperm) were applied to the column and eluted with the same buffer at a flow rate of 0.5 mL min^{-1} for 60 min. Enzyme activity was determined with 2.5 μL from each 0.25-mL fraction.

A chromatography. The protein composition after each purification step was analyzed by SDS-PAGE (Fig. 6). As expected, the complexity of the protein profiles decreased with each purification step. After PAGE, the gel slice with the highest activity contained a prominent protein band with estimated mass of about 64 kD.

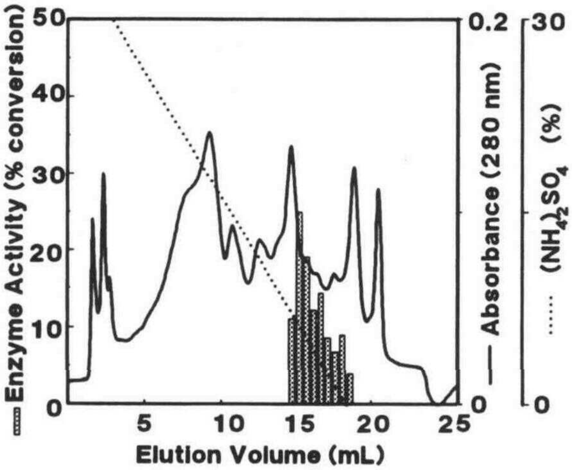

Figure 5. Elution profile of zeatin *cis-trans*-isomerase after Phenyl Superose HR 5/5 FPLC. Active fractions from Superose 12 FPLC (equivalent to 10 mL of endosperm) were applied to the column equilibrated with 55 mM Tris-HCl (pH 7.5) containing 2 mM DTT and 30% ammonium sulfate. Enzyme was eluted with a linear gradient of 30 to 0% ammonium sulfate at a flow rate of 0.5 mL min^{-1} for 30 min. Enzyme activity was determined with 5 μL from each 0.5-mL fraction.

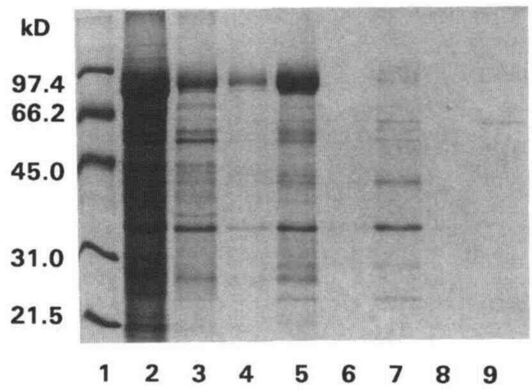

Figure 6. Coomassie brilliant blue-stained SDS-PAGE gel of pooled active fractions after each purification step. Protein in each lane is from 10 μL of endosperm or multiple, if indicated. Lanes: 1, Molecular markers; 2, 30 to 60% ammonium sulfate precipitation; 3, anion-exchange chromatography; 4, Superose 12 FPLC; 5, Superose 12 FPLC (10×); 6, Phenyl Superose FPLC; 7, Phenyl Superose FPLC (10×); 8, PAGE; 9, PAGE (50×).

DISCUSSION

The isolation and partial purification of the *cis-trans*-zeatin isomerase confirmed our earlier observation that interconversion of the two forms of zeatin occurs in immature seeds of *Phaseolus*. Although low nonenzymic conversion occurs when these factors are provided in vitro, presence of the enzyme enhances conversion significantly. The two essential requirements for isomerization are flavin and light. A very similar situation was reported for the isomerization of geraniol and geranyl phosphate to nerol and neryl phosphate (Shine and Loomis, 1974).

The possibility that cytokinins may be derived from degradation of tRNA, in addition to being synthesized de novo, has been debated for many years. According to the calculations of Barnes et al. (1980) and Maass and Klämbt (1981), cytokinins in tRNA could contribute 40 to 50% of the total cytokinin pool. Others have disputed the importance of the indirect pathway (see Letham and Palni, 1983, for a summary). A major problem with this pathway is the predominance of *cis*-zeatin riboside in tRNA, whereas the free cytokinins are the *trans*-isomers. Thus, our discovery of a *cis-trans*-isomerase forges a critical link between biologically inactive and active pools of cytokinins. However, we cannot predict at this point the extent of the contribution of isomerization to the overall biosynthesis of cytokinins.

The occurrence of *cis*-zeatin and its riboside has been reported for a number of plant species, including potato (Mauk and Langille, 1978), sweet potato (Hashizumi et al., 1982), tobacco (Tay et al., 1986), rice (Izumi et al., 1988), wheat (Parker et al., 1989), and oat (Parker et al., 1989). The *cis*-derivatives were prominent in underground parts, roots of rice and tubers of potato and sweet potato, and in xylem sap of wheat and oat collected just above ground level. It is tempting to speculate that the absence of light may have precluded isomerization to the *trans* forms. Significant quantities of *cis*-zeatin were also found in cones of hops (Watanabe et al., 1981) and in *Mercurialis* (Durand et al., 1992). In the latter, formation of male-fertile and male-sterile flowers was correlated with the occurrence of *trans*- and *cis*-zeatin derivatives, respectively (Durand et al., 1992). Thus, interconversion between *cis*- and *trans*-zeatin may play a role in developmental events such as shoot differentiation and male gamete production.

As part of a project to characterize the regulation of cytokinin biosynthesis and metabolism, we have isolated several zeatin-specific enzymes, including an *O*-xylosyltransferase, an *O*-glucosyltransferase, and a reductase (Turner et al., 1985; Dixon et al., 1989; Martin et al., 1989). Antibodies specific to two of these enzymes have been obtained (Martin et al., 1990) and used to isolate genes involved in cytokinin metabolism (Mok et al., 1992a) and to study their spatial expression (Martin et al., 1993). We intend to use similar strategies for further analyses of the isomerase.

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