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AN ENZYME FROM LUPIN SEEDS FORMING ALANINE DERIVATIVES OF CYTOKININS

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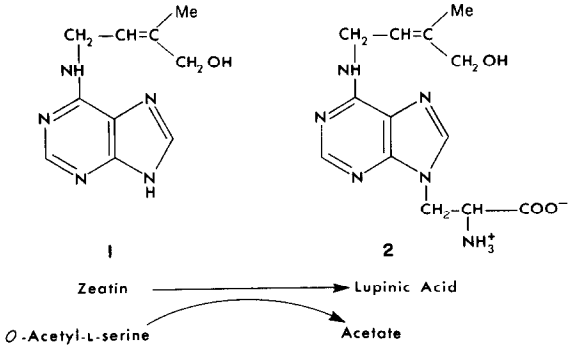
Key Word Index—*Lupinus luteus*; Leguminosae; cytokinin metabolism; enzyme forming alanyl conjugates; purification.

Abstract—A new enzyme, which catalyses the conversion of the cytokinin zeatin to the alanine conjugate lupinic acid, has been partly purified from developing lupin seed (*Lupinus luteus*). Paired-ion, reverse phase HPLC was adapted to analyse the enzyme reaction quantitatively. The enzyme used *O*-acetyl-L-serine as the source of the amino acid residue, and it interacted with substrates in a ping pong bi bi mechanism. A number of adenine derivatives served as substrates, but preference was shown for compounds with high cytokinin activity. The possible role of the enzyme, tentatively called β-(9-cytokinin)alanine synthase or lupinic acid synthase, in the regulation of hormone activity is discussed.

INTRODUCTION

When the natural cytokinin zeatin [**1**, 6-(4-hydroxy-3-methylbut-*trans*-2-enylamino)purine] or synthetic analogues are supplied to plants, a great diversity of metabolites are formed [1]. One metabolite formed from zeatin by lupins and apple seeds is an amino-acid conjugate termed lupinic acid (**2**) which has been identified as L-3-[6-(4-hydroxy-3-methylbut-*trans*-2-enylamino)-purin-9-yl]alanine [1–3]. With isotope dilution techniques, this compound has been found as a normal component of lupin pod walls [4].



Lupinic acid is metabolically stable and is very weakly active in cytokinin bioassays relative to zeatin [2]. Similar observations have been made for 7- and 9-glucosides of cytokinins [5]. To help define the role of glucosides in hormone metabolism, the enzyme reaction which formed the compounds was elucidated by Entsch *et al.* [6]. A similar approach was desirable for the alanine derivatives. The formation of traces of lupinic acid in cell-free extracts of plant tissues has been observed [7]. The reaction required the unusual substrate, *O*-acetylserine. This paper reports the partial purification and properties of a unique new enzyme for the synthesis of lupinic acid from developing lupin seeds. The acquisition of data with very small amounts of enzyme activity depended upon sensitive assays. This was achieved by using the paired-ion technique in HPLC. A part of the results presented were reported at the 10th International Plant Growth Substances Conference in Madison [1].

RESULTS

Enzyme assay

Analysis of products of the enzyme reactions by paired-ion HPLC (see Experimental) was the most successful approach to a quantitative assay. It was flexible, fast, accurate and sensitive, and products were detected without chemical modification. Alternative processes such as electrophoresis, ion exchange and use of radioactive tracer were not nearly as effective. With HPLC and UV detection, as little as 50 pmol of product could be measured directly in the reaction mixtures at the same time as a 1000-fold greater amount of starting substrates. This was achieved by chromatography on a C_{18}-substituted Si column. The mobile phase was a methanol–water mixture, containing small amounts of acetic acid and sodium heptane sulphonate. The acetic acid partially suppressed the ionization of the carboxyl group in the product formed by the enzyme, and protonated the purine ring of the aromatic substrate and product in the reaction. The heptane sulphonate paired and exchanged with the α-amino group in the product and the purine ring of substrate and product. The result was reproducible chromatograms with the product always eluted before the substrate in a reaction. Quantitative recovery of purine substrate and product was established over the range of concentrations studied.

Purification of enzyme

A summary of the preparation used for the enzyme is shown in Table 1. A low concentration of phosphate buffer was used to extract seed, since this minimized the solution of storage protein. However, sufficient storage protein was extracted to interfere with assays and fractionation. No alternative was found to a heat treatment as suggested by Murakoshi *et al.* [7] to obtain a clear, stable protein solution. It was not until the subsequent fractionation with ammonium sulphate that activity could be detected by the present method (Table 1), although a radioactive tracer method did confirm the presence of activity in heated extracts. The ammonium sulphate precipitate was then fractionated on DEAE-cellulose; all the activity applied to the column was retained and a single peak of activity was eluted by a gradient of sodium chloride (Fig. 1). After pooling the

Table 1. Purification of enzyme from developing lupin seeds

Fraction	Protein (g)	Total activity (units)	Specific activity (units/mg protein)
Crude extract	2.30	nd*	—
55° supernatant	1.60	nd	—
40–70% ammonium sulphate	0.27	24.5	0.09
DEAE-cellulose eluate	0.03	16.2	0.54

Developing lupin seeds (105 g) were extracted as described and the enzyme activity was assayed by the standard method. Protein was determined by the method of Lowry *et al.* [20].

*Not detected.

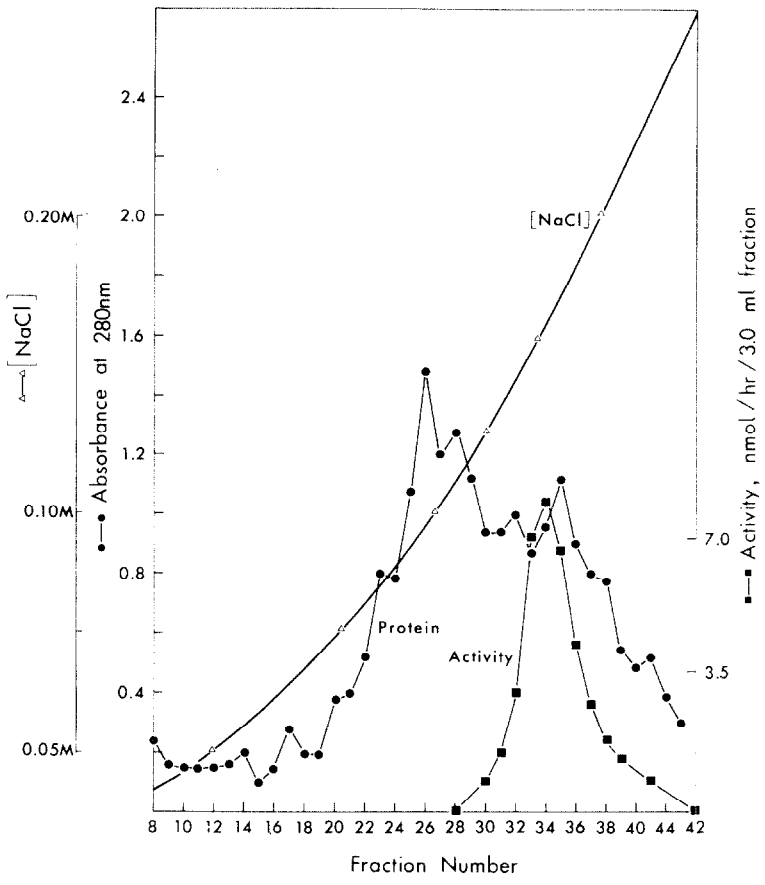


Fig. 1. Distribution of enzyme activity during gradient elution from DEAE-cellulose. A total of 250 mg protein was pumped onto the column before chromatography (see Experimental). The total activity eluted as a single peak in the salt gradient. Protein is represented by *A* at 280 nm.

central fractions of the peak, a substantial purification was achieved. The enzyme activity was stable in the final ammonium sulphate precipitate, and the preparation was suitable for kinetic studies, since no interfering reactions could be detected. After removal of salt just before use, the enzyme solution behaved normally, with a linear relationship between rate of product formation and amount of enzyme preparation added. The yield and specific activity of enzyme varied between preparations, and this probably reflected the difficulty in selecting uniform seed. The final specific activity of the preparation in Table 1 (*ca* 0.54 μmol lupinic acid formed/hr/mg protein) indicated that only a small proportion of the protein in the final preparation was the enzyme required, and the level of the enzyme in the seed was very low as a proportion of total protein. However, the activity measured would easily account for the amount of lupinic acid formed on a weight basis when labelled zeatin was supplied to lupin seeds [1].

Molecular weight

Size analysis of the enzyme was carried out by molecular sieve chromatography on a standardized Sephadex G-100 column, as described by Andrews [8]. The enzyme was detected by activity measurements, and the peak of activity was found to elute just prior to bovine serum albumin. The estimated MW was 64 500 ± 3000.

Stoichiometry

After incubation of the full enzyme assay mixture, one product was detected by HPLC, using UV absorbance. This compound accounted for all zeatin consumed in the reaction. Pooled incubations (see Experimental) were purified by chromatography on a cellulose phosphate column and paper. The purified compound had a mass spectrum identical to that of authentic lupinic acid [3]. The purification of lupinic acid by paired-ion, reverse phase HPLC was found to be unsuitable for mass spectrometry due to the difficulty in removing the paired ion reagent.

The alanyl residue of lupinic acid was derived from *O*-acetyl-L-serine, as suggested previously [7]; neither L-serine, *N*-acetylserine or *O*-phospho-L-serine would substitute for this compound. The inability of *O*-phospho-L-serine to function as a substrate was not due to phosphatase activity. The stoichiometry of the enzyme reaction could not be established, apart from demonstrating that zeatin is converted to lupinic acid. No more analysis was possible due to the instability of *O*-acetylserine in aqueous solution. The enzyme catalysed reaction must be as represented above.

O-Acetylserine as a substrate

O-Acetylserine is most stable in moderately acidic solutions [9], but undergoes an intramolecular rearrangement to *N*-acetylserine in neutral and moderately alkaline solutions [10], with a rate constant strongly dependent upon pH. In this work the formation of *N*-acetylserine from *O*-acetylserine was established by HPLC (see Experimental). It was found that the rearrangement was also dependent upon the type and concentration of buffer ions. No attempt was made to study the rearrangement in detail, though it was found that the buffer preferred for use with the enzyme, potassium phosphate, did not accelerate the rearrangement relative to other buffer solutions tested. In the presence of this unstable substrate, the thermodynamic equilibrium of the enzyme reaction could not be established.

There was a 10-fold increase in enzyme reaction rate as the pH increased from 7 to 8. With the use of short incubation times, it was found that the pH optimum for the reaction must be well above pH 8, where the rate of rearrangement of *O*-acetylserine made quantitation too difficult. A compromise set of conditions (25°, 25 mM phosphate as potassium salt, pH 7.65, with incubation times of 15–30 min) was thus established to make a study of the kinetics and specificity of this enzyme in the trace amounts available. Under these conditions, the non-enzymatic rearrangement of substrate occurred with a rate constant of *ca* 0.01 per min, and had a negligible effect on pH at 2.0 mM (or less) *O*-acetylserine.

Kinetic studies

The dependence of the initial velocity of the enzyme reaction upon substrate concentration was studied with zeatin and *O*-acetylserine. Concentrations of the substrates were varied systematically over ranges suitable to obtain kinetic constants, and corrections were made for small changes in substrate concentrations during incubation. To illustrate the results, double reciprocal plots of reaction rates against concentration of one substrate were combined at constant concentrations of the other substrate (Fig. 2). The complete matrix of concentrations shown was run on two separate occasions, and the same pattern of plots was obtained. The results fitted a pattern of linear, parallel plots. This pattern is unique to the ping pong bi bi mechanism for interaction of substrates with the enzyme, as described by Cleland [11]. Confirmation would require a series of product inhibition studies, and neither enzyme nor product were in adequate supply for this. The characteristic feature of the mechanism is the involvement of two different species of the enzyme. Each substrate interacts successfully with only one form of the enzyme, resulting in a reaction which produces a product and the alternative form of the enzyme. Thus, the likely sequence of events in catalysis is first, the reaction of the enzyme with *O*-acetylserine to form an alanyl-enzyme with release of acetate, followed by interaction of the alanyl-enzyme with zeatin to produce lupinic acid and the original form of the enzyme.

Secondary plots of intercepts on the rate axis (Fig. 2) against the reciprocal of the concentration of the alternate substrate gave the following values for the basic kinetic parameters [12]: K_m, zeatin 8.8×10^{-4} M; K_m, *O*-acetylserine 4.7×10^{-5} M; and V, 1.03 μmol/hr/mg protein. Without knowledge of enzyme concentration, V can only be given in terms of the specific activity of preparations of enzyme used. The low value of V probably indicates that there was a very small amount of the enzyme present in the preparation described, rather than a low molecular activity of the enzyme.

Purines and related compounds as substrates

A property of the enzyme preparation which is of great significance is its specificity towards cytokinins and related molecules. Substrates were found by incubation of potential compounds in reactions with and without enzyme preparation as described in Tables 2 and 4. The

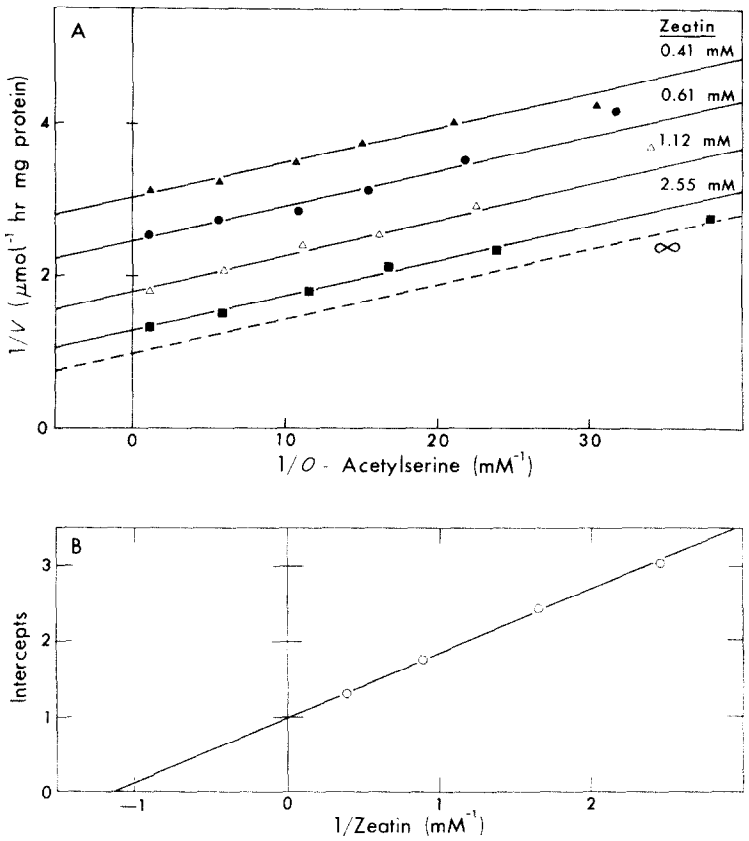


Fig. 2. Kinetic studies. (A) Double reciprocal plots of steady state initial velocity measurements of enzyme activity, varying the concentrations of both zeatin and *O*-acetylserine. Reactions were run under standard assay conditions with 0.15 unit of enzyme per incubation. The dashed line represents extrapolated infinite zeatin concentration. (B) Secondary plot from the data in (A). The intercepts on the velocity axis are re-plotted against reciprocal zeatin concentration.

Table 2. Relative rates of product formation from various N^6-substituted adenines with an aliphatic substituent

Compound	Relative rate
Adenine	7.5
Methylaminopurine	25.0
6-(2-Hydroxyethylamino)purine	20.0
6-Propylaminopurine	57.0
6-(Δ^2-Isopentenyl)aminopurine	290.0
6-Isopentylaminopurine	99.0
Zeatin	100.0
Dihydrozeatin	30.0
cis-Zeatin	91.0*
6-(6-Hydroxyhexylamino)purine	64.0
O-β-D-Glucopyranosylzeatin	34.0

Standard enzyme assay conditions were used. The reaction mixtures contained 1.5 mM of each compound listed, 0.10 units of enzyme, and 1.0 mM *O*-acetylserine (*ca* $\times 20 K_m$). The formation of products was established by HPLC (see Experimental) and each reaction was run at least twice.

*A much lower relative activity with *cis*-zeatin was suggested previously [1], but the result was not reproducible with a new preparation of *cis*-zeatin and enzyme. We suspect the presence of an enzyme inhibitor in the early experiments.

products formed were detected by UV absorption in HPLC as described in the Experimental. Of the compounds tested, only those with adenine as part of the molecule were found to be substrates of the enzyme. Based on the characteristic shift, caused by substitution at position 9, in absorbance of the protonated adenine chromophore observed in acetic acid solutions, the products formed were probably purin-9-yl alanines in analogy to lupinic acid.

The influence of an aliphatic side chain structure in N^6-substituted adenines on their ability to accept an alanine residue is surveyed in Table 2. All reactions were run under identical conditions, with a saturating concentration of *O*-acetylserine. The concentration of adenine derivatives (1.5 mM) was governed by the low solubility of some of the compounds under assay conditions. The relative rates of product formation should contain elements of both K_m and V. The results in Table 2 show that the enzyme accepted all the listed compounds as substrates. However, the enzyme showed a strong preference for compounds with an N^6-substituent of *ca* five carbon atoms with an olefinic linkage, structural features which enhance cytokinin activity [5, 13]. Dihydrozeatin was much less effective as a substrate than zeatin. However, the naturally occurring isomer of dihydrozeatin, which has an *S*-configuration [14], must be a substrate *in vivo* since

dihydrolupinic acid has been found to occur naturally in lupins [4]. Isopentenyladenine was the most reactive substrate. This may have some regulatory significance, since this compound is a biosynthetic precursor of zeatin [5]. The influence of the size of the side chain at position N^6 was investigated in greater detail with more kinetic measurements of three compounds under identical conditions (Table 3). The enzyme had a clear preference for zeatin, which was principally expressed in the binding interactions (K_m) of enzyme and substrate. Adenine, without a side chain, was much more weakly bound. The enlarged side chain of *O*-glucosylzeatin resulted in a less effective substrate. With the same stereochemistry involved at the site of reaction, it was not surprising to find little change in V (Table 3).

Table 3. Kinetic analysis of three selected substrates

Acceptor substrate	K_m (mM)	V (units/mg protein)
Adenine	26.0	1.47
Zeatin	0.88	1.03
O-Glucosylzeatin	2.50	0.55

Standard enzyme assay conditions were used. One substrate (*O*-acetylserine) was kept constant and saturating (1.5 mM). One preparation of enzyme with constant specific activity was used. The dependence of reaction rate on substrate concentration was analysed over an optimum range of concentration for each substrate (*ca* K_m). Double reciprocal plots were used to obtain V and K_m. Values for V were normalized to the uniform specific activity of enzyme used in this paper (Table 1).

Synthetic adenine derivatives with an aromatic substituent at position N^6 of the purine ring also serve as substrates (Table 4). These compounds were tested relative to zeatin at 0.25 mM due to their extreme insolubility in water. It was clear (Table 4) that kinetin and 6-benzylaminopurine, both potent cytokinins, were among the best substrates for the enzyme. However, addition of methoxy groups to the benzyl side chain markedly lowered substrate activity, presumably due to less effective binding to the enzyme. As indicated in Table 4, this addition also nearly abolishes cytokinin activity. Hypoxanthine was not a substrate under the conditions studied and hence only a small change in the purine ring abolished substrate activity. Indole auxins have a similar ring structure to purines (Table 4), but IAA was not a substrate for the enzyme. The pyrimidine ring of uracil is substituted with an alanyl residue by an enzyme from a range of species [15]. However, the enzyme under study did not catalyse this reaction.

DISCUSSION

This paper describes practical methods for the preparation and assay of a new enzyme which is clearly involved in the metabolism of cytokinins. The catalytic properties presented show that the enzyme probably belongs in a recently discovered group of enzymes in plants which have been neglected. These enzymes catalyse the synthesis of a group of unusual amino acids by using the substrates *O*-acetylserine [15] or *O*-acetylhomoserine [16] as the source of the zwitterion in the product. The reaction has similarities to those which result in formation of tryptophan and cysteine, and which involve serine as a substrate. The latter enzymes have been studied in detail [17, 18], and have been classified into sections EC 4.2.1 and EC 4.2.99 of the enzyme listing. The final grouping of the enzyme described here should depend on more extensive knowledge of the mechanism of the reaction. We propose the trivial name of β-(9-cytokinin)alanine synthase, or β-(6-alkylaminopurin-9-yl)alanine synthase, or lupinic acid synthase. With the emphasis on product formation, such an enzyme may be better classified as a *C–N*-ligase (EC 6.3.2).

The first investigation of the reaction forming lupinic

Table 4. Relative rates of product formation from cytokinins with an aromatic substituent at N^6 and from compounds with a modified ring nucleus

Compound	Concentration (mM)	Relative rate	Cytokinin activity
Zeatin	0.25	57.0	High
6-Benzylaminopurine	0.25	100.0	High
6-Furfurylaminopurine (kinetin)	0.25	138.0	High
6-(3,4-Dimethoxylbenzylamino) purine	0.25	7.5	Very low
6-Oxypurine (hypoxanthine)	1.5	No product detected	Nil
3-Indoleacetic acid	2.0	No product detected	Nil
2,4-Dioxypyrimidine (uracil)	1.5	No product detected	Nil
6-Benzoylaminopurine	0.85	135*	Moderate
Zeatin	0.85	100*	High

Standard enzyme assay conditions were used, with *O*-acetylserine constant and saturating (1.5 mM). Incubation times were increased to confirm negative results for non-substrates. The substrates tested at 0.25 mM were restricted by low solubility in aqueous solutions. Zeatin was included to compare with the data in Table 2.

*This value was obtained in a separate experiment and is expressed as a percentage of the rate found for zeatin.

acid was carried out with crude cell extracts and an assay which was slow and qualitative [7]. However, results did suggest that developing lupin seeds were a rich source of activity. At this stage, the variation in activity with seed development is unknown. As the amount of enzyme detectable was very small, a minimum of 100 g seed was needed each preparation. In addition, it was necessary to avoid the later stages of development, when large amounts of storage protein were laid down. Fractionation on DEAE-cellulose indicated that the activity eluted as a single peak from the column (Fig. 1). The final, partially purified enzyme solution did not catalyse side reactions with substrates. With very small amounts of enzyme, further purification was not justified.

The key to successful analysis of this enzyme reaction was the development of an analytical separation of the amino acid products from the substrates. The paired-ion technique of HPLC enabled both the polar product (an amino acid) and the purine substrate to be rapidly chromatographed (15 min) and simultaneously quantitated using a reverse-phase column. Since even a few nanograms of product could be measured, many assays were possible with small amounts of enzyme. The use of HPLC also has the potential advantage of rapid separation of product for further analysis or use in other reactions or physiological studies. Consequently, the enzyme becomes a useful tool in the synthesis of new purin-9-yl alanine derivatives.

The enzyme requires *O*-acetylserine as a substrate, which is extremely unstable under physiological conditions (see the Results). Only one enzyme using *O*-acetylserine has been studied in detail, and this resulted in the classic description of cysteine synthase [18], which requires a bifunctional protein complex. One enzyme produces *O*-acetylserine from acetyl-CoA, and the second enzyme uses it without release to the medium. We could not detect lupinic acid formation with acetyl-CoA plus serine, so any proposed complex in lupin seed may have been separated during isolation. In addition, we did not observe sensitivity of the enzyme activity to inhibitors of pyridoxal phosphate, which is required for the formation of cysteine in the cysteine synthase enzyme complex. The reaction mechanism remains a matter of speculation, and it would require much more enzyme to obtain useful information.

The specificity of the enzyme preparation was central to the question of its physiological role. The results in Tables 2 and 3 showed that the enzyme had a clear preference for binding and catalysing the reaction of adenine substituted at position N^6, the basic requirement for cytokinin activity [5]. The activity of the enzyme with a selected group of N^6-substituted adenines with aromatic substituents, and its inactivity towards the non-cytokinins hypoxanthine, uracil and IAA (Table 4), also merits note. The above specificity in enzyme activity, together with the natural occurrence of lupinic acid and dihydrolupinic acid [4], provide strong support for the hypothesis that the enzyme plays a role in metabolism of endogenous cytokinin.

A peculiarity of the reaction kinetics is the relatively high K_m for zeatin (0.88 mM). Since cytokinins are biologically active at tissue concentrations orders of magnitude lower than this, a reasonable, but negative, deduction could be that the enzyme has been modified in isolation, as suggested before. However, if the K_m is an accurate reflection of tissue action, then this enzyme has the ability to control cytokinin activity levels over a wide concentration range which extends above the upper limit of endogenous levels. This is because the product (lupinic acid) is only weakly active in cytokinin bioassays [2]. At least some information about biological function could be obtained by following quantitative changes in activity with stages of development (e.g. following anthesis in lupins). However, this would require development of reliable and reproducible crude protein extracts at all stages.

EXPERIMENTAL

Plant material. Lupin plants (*Lupinus luteus* L., cv Weiko III) were grown from seed (source: Westralian Farmers Co-op., Perth, Australia) in a greenhouse with natural light at 15–25° or in a field plot (late spring–autumn). Developing seed pods were harvested and seed (7–8 mm average diam.), which was soft and light green (containing little storage material), was separated from the pods, frozen in liquid N_2 and stored at −78° until required.

Chemicals. The principal enzyme substrates, *trans*-zeatin (< 1% *cis*-isomer) and *O*-acetyl-L-serine were purchased from Calbiochem. *Cis*-zeatin was prepared by sequential TLC and HPLC separation from a commercial preparation of mixed isomers of zeatin (Sigma). Analytical HPLC and GC/MS failed to detect any impurity in this preparation of *cis*-zeatin. Lupinic acid and *O*-β-D-glucopyranoside of zeatin were prepared in this laboratory [3]. Several of the N^6-substituted forms of adenine were synthesized by lit. procedures, and the remainder were purchased.

Enzyme assay. The enzyme was assayed in a standard incubation mixture of 0.5 ml. This contained 1.4 mM zeatin, 2.0 mM *O*-acetylserine, enzyme preparation and 25 mM Pi (made to pH 7.65 with KOH). The *O*-acetylserine was stored for a few days as a stock soln of 0.2 M in H_2O at −20°. Reaction was commenced by the addition of *O*-acetylserine, and incubated at 25° for time intervals between 15 and 60 min. Reaction was stopped by the rapid addition of an equal vol. of MeOH. After 15 min, any protein ppt was removed by centrifugation. An aliquot (20–50 μl) from the soln was chromatographed isocratically on a column of ODS-silica (0.39 × 30 cm) using the liquid chromatograph (see HPLC section). The mobile phase was MeOH–H_2O(3:7) containing 5 mM sodium heptane sulphonate and HOAc (reagent PIC-B7, Waters Associates). The effluent was monitored by UV *A* at 254 and 280 nm. Lupinic acid was eluted in *ca* 5 min and zeatin in *ca* 11 min (at 1.5 ml/min). Other soln components were not detected. The reaction was specific—no side reactions were detected, apart from the rearrangement of *O*-acetylserine (see Results). The amounts of zeatin and product formed from it were determined from the area of the peak for each compound detected at 280 nm, and a conversion factor obtained from plots of areas against amount of standard compound chromatographed. The enzyme activity was then calculated from the mol fraction of product formed.

With small amounts of enzyme to study, a unit of enzyme activity was defined as the formation of 1 μmol product/hr at 25°, with zeatin as substrate.

Enzyme preparation. All steps were carried out at 4° unless stated otherwise. Immature lupin seeds (100 g) were removed from liquid N_2 storage and blended for 2 min in the extraction buffer (2 ml/g; buffer 0.01 M KH_2PO_4, 1 mM EDTA, 2 mM dithiothreitol, adjusted to pH 7.4 with KOH). The brei was filtered through three layers of washed Miracloth before centrifugation at 25 000 *g* for 30 min. The resulting supernatant (275 ml; pH readjusted to 7.4 with KOH) was called the crude extract.

The crude extract was brought to 55° in a water bath and after

1 min at this temp. was removed to an ice bucket. After chilling, the heat treated crude extract was centrifuged at 10000 *g* for 20 min and the supernatant (pH adjusted to 7.4) retained.

The straw coloured supernatant was brought to 40% satn with $(NH_4)_2SO_4$ centrifuged at 10000 *g* for 20 min and the inactive pellet discarded. The supernatant, which contained the enzyme activity, was brought to 70% satn with $(NH_4)_2SO_4$, stirred for 30 min and the pptated protein removed by centrifugation at 10000 *g* for 20 min. The ppt was dissolved in 8 ml 0.01 M KH_2PO_4 (adjusted to pH 7.0); following the removal of insoluble material by centrifugation, the protein soln was desalted by passage through a small column of Sephadex G-25.

The protein soln (10–11 ml) was loaded immediately onto a column of microgranular DEAE-cellulose (1.6 × 25 cm) which had been equilibrated with 0.01 M KH_2PO_4, pH 7.0. After washing with one bed vol. of buffer, the column was eluted with a linear gradient of NaCl in the same buffer (0–0.5 M NaCl in 180 ml). The enzyme activity eluted as a single peak in the range of 0.18–0.24 M NaCl. The central fractions of the peak (*ca* 80% of the enzyme activity) were pooled and the protein (*ca* 40 mg) pptated by 70% satn with $(NH_4)_2SO_4$. The ppt was divided into four aliquots, centrifuged and stored under buffer (0.01 M KH_2PO_4, 80% $(NH_4)_2SO_4$, 2 mM EDTA, pH 6.9). The enzyme activity was stable for 2–3 months at 4°.

The pptated enzyme was prepared for assays by dissolving in 2–3 ml buffer (25 mM KH_2PO_4, pH 7.65) and desalting on a small Sephadex G-25 column. All the results presented were obtained with this enzyme preparation.

High Pressure Liquid Chromatography. The molecular changes in enzyme incubations were followed by HPLC. The equipment was supplied by Waters Associates, Milford, MA, U.S.A. Solvent delivery was controlled by two model M-6000 A pumps connected to a model 660 solvent programmer. After passage through the column, effluent was monitored by a model 440 *A* detector or a model 401 RI detector. The signals from the detectors were recorded with a two-pen recorder (Houston Instruments) with integration capability, or a Waters model 730 data module. Two types of columns were used: ODS-silica (Si substituted on the surface with *n*-octadecyl groups; μ Bondapak C_{18} column from Waters) and CX-silica (Si substituted on the surface with phenyl sulphonate groups; Bondapak CX/Corasil from Waters).

The enzymatic formation of products from aromatic substrates (mostly derivatives of adenine) was established by chromatography on a column of ODS-silica with MeOH–H_2O mixtures containing PIC-B7 (Waters) as the mobile phase. The principal resolving power of such columns is on the basis of polarity, with highest polarity being eluted first. Additional resolution results from variation in the hydrophobic portions of the molecule [19]. Any alanyl derivative was more polar than the starting material and would be eluted first. In addition, significant retention of such products depended upon the presence of PIC-B7. A chromatogram was run for each unknown reaction mixture until the substrate was eluted. Compounds eluted earlier were detected by UV absorption at 254 and 280 nm. Shifts in the *A* of adenine due to alkylation of a ring nitrogen (equivalent to formation of an alanyl derivative) were detected by shifts in the ratio of areas of peaks at 254 and 280 nm.

Changes in the substrate (*O*-acetylserine) and products without UV absorbance were detected by RI changes after separation on a column of CX-silica. The mobile phase was aq. citric acid (1.5 mM, pH 3.15). Isocratic conditions gave a separation with *N*-acetylserine at the front, followed by retention and resolution of *O*-acetylserine and serine, respectively.

Purification of lupinic acid. Enzyme incubation mixtures of active fractions off DEAE-cellulose columns (three enzyme preparations) were stored at −10°. The incubations were pooled, evaporated *in vacuo* (< 40°) and redissolved in H_2O (pH 3.0). The soln was percolated through a column of cellulose-phosphate (NH_4^+ form, pH 3.0) and eluted with 0.3 N NH_4OH. The eluate was extracted (× 3) with an equal vol. of H_2O satd *n*-BuOH and the aq. phase was evaporated. The residue was dissolved in 0.5 ml H_2O–MeOH (9:1) and chromatographed on exhaustively washed Schleicher & Schull 2040b paper: the paper chromatogram was developed in H_2O-satd *n*-BuOH in an atmosphere of NH_3. The UV absorbing zone at the R_f of authentic lupinic acid was cut out and exhaustively eluted with MeOH–H_2O (1:3) to yield a purified product (*ca* 25 μg) for characterization by mass spectrometry.

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