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PREPARATION AND CHARACTERIZATION, USING HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY, OF AN ENZYME FORMING GLUCOSIDES OF CYTOKININS

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Summary

Cytokinins can occur naturally as glycosides with β-D-glucose as the sugar substituent. From radish (*Raphanus sativus*) cotyledons, an enzyme has been partly purified which synthesizes the 7-glucopyranoside of zeatin [6-(4-hydroxy-3-methylbut-*trans*-2-enylamino)purine], a compound known to occur in this species. High-performance reverse-phase liquid chromatography was uniquely useful as the analytical procedure for quantitative study of the minute amounts of enzyme available. The enzyme uses UDPglucose as the source of the sugar residue. A large number of derivatives of purine are glucosylated, but adenine derivatives with an alkyl side chain at least three carbon atoms in length at position N^6 are preferentially glucosylated. This corresponds to the structural features required for high cytokinin activity. The 7-glucoside of zeatin is known to be very weakly active in cytokinin bioassays. Hence, this enzyme, and others catalyzing the same reaction, have a role in the regulation of cytokinin activity.

Introduction

When supplied to many plant species, the phytohormones termed cytokinins (N^6-substituted adenines) are converted to 7-glucopyranosides (see Refs. 1, 2).

Abbreviations: HPLC, high-performance liquid chromatography; ODS-silica, silica with *n*-octadecyl groups bound to the surface; PIPES, piperazine-*N*,*N*′-bis[2-ethanesulphonic acid].

Recently compound I (see Fig. 3), the 7-glucopyranoside of zeatin, 6-(4-hydroxy-3-methyl-but-*trans*-2-enylamino)purine, has been shown to occur as an endogenous cytokinin in radish seed [3]. The 7-glucosides of cytokinins are metabolically stable [4—6], and very weakly active relative to the unsubstituted cytokinin [7,8]. In other cytokinin glucoside metabolites, the glucose substituent is attached to position 3 or 9 of the purine ring [2], and in the case of zeatin metabolites, to the oxygen of the isoprenoid sidechain [2,9,10].

The physiological significance of these glucosides remains uncertain. They may simply be waste products formed by glucose transferases which catalyse the formation of glucoside metabolites characteristic of particular species [11], or be part of the control exerted by plants on hormone activity. It has been suggested that 7-glucosides may function as storage forms of cytokinins [4,6]. The substitution of the purine ring at position 7 is rare in nature, being found only in a few compounds related to vitamin B-12, which have a 7-ribofuranosyladenine moiety [12], and in rare bases in RNA which carry a 7-methyl group. Physiological studies of cytokinin 7-glucosides would be enhanced by examining their enzymic synthesis.

With the exception of cytokinin oxidase [13], enzymes of cytokinin metabolism have not been studied. This paper reports the preparation and characterization of one of two enzymes from radish cotyledons which glucosylate cytokinins. The detection of these enzymes was reported previously by Entsch and Letham [14].

Materials

Radish (*Raphanus sativus* L., cv. Long Scarlet) seed was obtained from Arthur Yates and Co., Australia. Seed was sown thinly in trays of sterilized soil and germinated in a glasshouse receiving prevailing daylight. The seedlings were grown (9—11 days) until the cotyledons had expanded and the first leaves were just appearing at the shoot apex.

The following media for chromatography were used: microgranular DEAE-cellulose (DE-82 from Whatman), modified dextrans (Sephadexes G-25 and G-100 from Pharmacia), ODS-silica (silica with *n*-octadecyl groups bound to the surface; μBondapak C_{18}, 0.39 × 30 cm column from Waters), and amino-alkyl-silica (Carbohydrate Analysis, 0.39 × 30 cm column from Waters).

General laboratory chemicals used were all analytical reagent grade. Triton X-100, a neutral detergent, was from Rohm and Haas; enzyme grade ammonium sulphate from BDH Chemicals; and Miracloth was a product of Chicopee Mills, New York. The following nucleotides and sugar derivatives were purchased from Sigma: uridine, UMP, UDP, UDPglucose, and other compounds listed in Table II.

Zeatin was obtained from Calbiochem, and hypoxanthine, 6-benzylaminopurine and kinetin were from Sigma, while 6-benzylamino-2-(2-hydroxyethylamino)purine and its 7-glucoside were prepared in this laboratory [15]. The 7- and 9-β-D-glucopyranosides of 6-benzylaminopurine and zeatin were synthesized as described previously [1]. Adenine 7- and 9-glucopyranosides, 4-furfurylaminopyrazolo[3,4-*d*]pyrimidine (II) and the remaining compounds of Tables III and IV were synthesized by literature procedures. Compound III,

3-methyl-7-*n*-pentylaminopyrazolo[4,3-*d*]pyrimidine, was provided by Dr. S.M. Hecht, Massachusetts Institute of Technology.

HPLC was performed with equipment supplied by Waters Associates, Milford, MA. Solvent delivery was controlled by two model M-6000 A pulseless pumps connected to a model 660 solvent programmer. After passage through the column, effluent was monitored by a model 440 absorbance detector. The signal from the detector was recorded with a two-pen recorder, such that an analogue and integrated trace were obtained for one wavelength.

Methods

Enzyme preparation

Radish cotyledons (0.9—1.0 kg) were chilled to 0 to 4° C in a cold room and all manipulations were done in this temperature range. An equal volume for weight of extraction buffer was added (0.1 M KH_2PO_4, 1 mM EDTA, 0.4% (v/v) Triton X-100, and 3 mM dithiothreitol, adjusted to pH 7.1 with KOH). The mixture was blended for 1 to 1.5 min to give finely divided leaves, and squeezed through 3 layers of Miracloth. The extract was centrifuged at 12 000 × *g* for 30 min and the green supernatant was siphoned off. More dithiothreitol (1 mM) was added to ensure a reducing solution.

The yellow precipitate formed between 35 and 70% saturation with ammonium sulphate was collected and dissolved in buffer (15 mM Tris solution adjusted to pH 7.6 at 22° C with HCl, plus 1 mM dithiothreitol) to give a total volume of about 250 ml. The solution was clarified by centrifugation and was subjected to molecular sieve chromatography by passage through a column of Sephadex G-25 (7.5 × 35 cm, giving a bed volume of 1500 ml) equilibrated with 15 mM Tris made to pH 8.1 with HCl. From 350 to 400 ml of void volume was collected.

The resulting solution of protein and nucleic acid was loaded immediately on a column of microgranular DEAE-cellulose (2.6 × 26 cm) equilibrated with 15 mM Tris-Cl, pH 8.1. The column was washed with one bed volume of buffer. Elution was carried out with a linear gradient of NaCl in the same buffer (0 to 0.5 M NaCl in 380 ml). All the enzyme activity was eluted in two separate peaks; the first at 0.05 M NaCl and the second at 0.155 M NaCl (Entsch and Letham [14]). The central portion of the latter peak (85% of the activity in the peak) was collected for further processing.

After the addition of EDTA to a final concentration of 0.6 mM, the protein solution was fractionated with solid ammonium sulphate. The precipitate formed between 41 and 56% saturation with ammonium sulphate was dissolved in buffer (25 mM KH_2PO_4 with 0.4 mM EDTA, adjusted to pH 7.4 with KOH) and salt was removed by passage through a small column of Sephadex G-25 equilibrated with this buffer. The protein solution (4 to 5 ml) was chromatographed by molecular size on a column of Sephadex G-100 (2.6 × 35 cm) equilibrated with the phosphate buffer. A single peak of enzyme activity was found. The fractions corresponding to the central portion of the peak (80% of the activity in the peak) were combined and used as the enzyme preparation. This solution contained about 35 mg of protein, and the enzyme present was stable for months if stored at —75° C. The activity of fractions is summarized in the purification table (Table I).

Enzyme assays

The following procedure was used routinely during enzyme preparations. Incubations were commenced in a total volume of 0.5 ml containing these components — 1.5 mM UDPglucose, 0.15 mM 6-benzylaminopurine, extract containing enzyme, 22 mM potassium phosphate buffer and 0.3 mM EDTA, final pH 7.35. The reaction was started by the addition of 6-benzylaminopurine in 20 µl methanol, and run at 35° C for 2.0 h. At 30-min intervals, the incubations were supplemented with 0.75 mM UDPglucose to maintain intact substrate in the presence of phosphatase and pyrophosphatase activity [14]. The reaction was stopped by the rapid addition of an equal volume of absolute methanol. After 15 min, protein precipitate was removed by centrifugation, leaving a clear solution.

An aliquot (20 to 50 µl) from the reaction was chromatographed on a column of ODS-silica using the liquid chromatograph (see Materials). A typical chromatogram is shown in Fig. 1. The amounts of 6-benzylaminopurine and glucosides 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 mole fraction of products formed.

Benzylaminopurine is cheap and available pure, but is very insoluble and not natural. For experiments with purified enzyme, the natural hormone zeatin was used in assays. The standard incubation contained the following in 0.50 ml — 1.5 mM UDPglucose, 1.4 mM zeatin, enzyme, potassium phosphate buffer (22 mM in phosphate), and 0.3 mM EDTA, final pH 7.35. The enzyme preparation was in the same buffer, and was added to start the reaction. With the purified enzyme preparation, substrates and products were stable in the incubation. The reaction was halted with methanol as above, and chromatography was similar to that above, and is illustrated in Fig. 1.

Because of the extremely small amounts of enzyme studied (see Results), the unit of enzyme activity used was defined as the formation of 1 nmole of glucoside per h at 35° C, with 6-benzylaminopurine as substrate.

Detection and analysis of products

The formation of a few nmol of unknown product glucosides from about a µmole of substrate was followed by chromatography on columns of ODS-silica with methanol and water mixtures as the mobile phases. 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 molecules [18]. The glucoside products were intermediate in polarity between the two substrates. Hence, a chromatogram was run for each unknown reaction mixture until the lower polarity substrate was eluted. Compounds running between the substrates were detected by ultraviolet absorption at 254 and 280 nm on high sensitivity.

Products formed from zeatin were compared with known glucosides by mass spectrometry. After six incubations of 5 h with enzyme, about 50 µg of major product and 5 µg of minor product from zeatin were purified from the reaction by chromatography as described in the legend to Fig. 1. The compounds were trimethylsilylated using a procedure found suitable for cytokinin glucosides

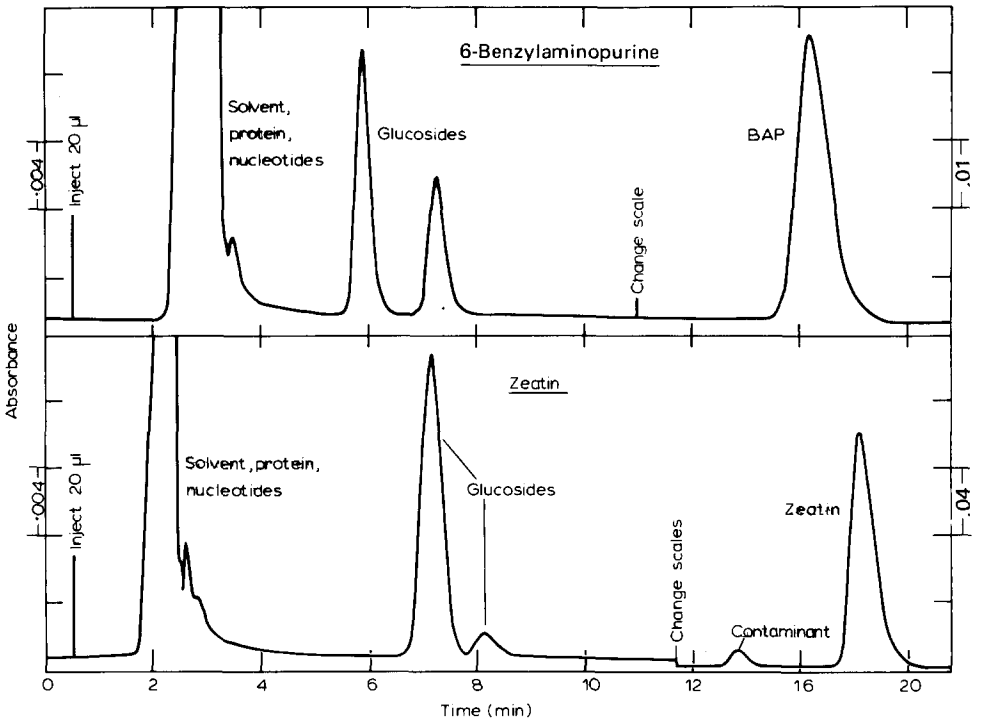


Fig. 1. Chromatograms obtained for quantitative analysis during enzyme assays. The traces were recorded at 280 nm, and resulted from injections of 20 µl aliquots of the respective incubation mixtures. The column was ODS-silica. For 6-benzylaminopurine, the mobile phase was 42% methanol and 58% H_2O, run at 1.0 ml · min^{-1}. For zeatin, the mobile phase was 20% methanol and 80% H_2O, run at 1.5 ml · min^{-1}. At the position marked 'change scales', the recorder speed was halved for zeatin only.

and the products were subjected to GC-MS, as detailed by MacLeod et al. [19].

In a number of cases, known glucosides of purine derivatives used in this work were available for comparison with products by chromatography. When known glucosides were not available, an indication of the position of substitution with glucose was obtained from absorption properties. Adenines substituted in the N^6 position undergo characteristic changes in ultraviolet spectrum depending on position of substitution in the purine ring [20]. From chromatographic records, the ratio of A_{280nm}/A_{254nm} was calculated from peak areas, and compared with that for similar known glucosides under the same chromatographic conditions.

Chromatography of nucleotides

Examination of the fate of UDPglucose in an enzyme incubation was followed by liquid chromatography on a column of aminoalkyl-silica. The column was equilibrated with 10 mM H_3PO_4, adjusted to pH 3.1 with ammonia. An aliquot from a reaction mixture (20 to 40 µl), after removal of protein by precipitation with methanol, was loaded onto the column. Elution was achieved by running a convex gradient in 15 min to 0.13 M H_3PO_4, adjusted to pH 5.1 with ammonia. With this arrangement, uridine was eluted at the front, followed in order by UMP, UDPglucose, and UDP. Quantitation of

TABLE I

PURIFICATION OF ENZYME FROM COTYLEDONS OF RADISH

The figures presented were obtained in a preparation using 940 g of freshly harvested cotyledons. Protein was determined by the methods of Lowry et al. [16] and Warburg and Christian [17]. The yield and purification of enzyme are based upon total activity, which is all that is measured initially. These factors would improve significantly if they were based upon the activity due to the purified enzyme only.

Fraction	Total activity (units)	Total protein (g)	Specific activity ($U \cdot mg^{-1}$)	Yield of activity (%)	Purification of activity
Crude extract	*	approx. 21 **	—	—	—
35 to 70% ammonium sulphate and G-25 column	approx. 4200 **	12.5	0.335	100	0
DEAE-cellulose (collected)					
low salt enzyme	1090	0.68	1.6	26	5
high salt enzyme (continued below)	2100	0.48	4.4	50	13
41 to 56% ammonium sulphate	1220	0.165	7.4	29	22
Molecular sieve (G-100 column) (collected)	910	0.032	28.4	22	85

* Enzyme activity was not detected at this stage.

** Several preparations demonstrated that these values could only be taken as a guide due to problems in measurement.

nucleotides was achieved by measuring areas of peaks on a chromatogram run at 254 nm.

Results

Enzyme assays

The use of fixed wavelength detection of purine derivatives with HPLC on ODS-silica columns permitted accurate quantitation of as little as 50 picomoles of product directly in an enzyme assay mixture, which also contained a thousand fold greater amount of substrate. With known glucosides, plots of area against amount injected gave linear relationships that passed through the origin over a wide range (at least 0.02 to 10 nmol). It was clear that complete recovery of the smallest amounts of compound occurred, together with reproducible retention times and peak shapes over an extended period of use. Reaction rates as low as 0.50 nmol of product formed per hour were measured. This sensitivity was adequate for quantitative studies of the enzyme isolated.

Assaying a plant enzyme at 35° C was an unusual step. At times this was used to reduce the incubation time (from 4—5 h to 2.0—2.5 h) and most importantly to conserve enzyme. An overriding consideration throughout this study was the critical shortage of enzyme. At 35°C, the reaction rate was linear up to about 4 h and the same products were formed as at 25° C.

Enzyme preparation

No significant enzyme activity was detectable in initial extracts (Table I). This was probably due to the very small amount of enzyme and interferences from enzymes which catalysed reactions with the substrates [14]. There was a substantial purification (Table I) of enzyme activity, though the final preparation contained less than 1% by weight of enzyme in the protein present. When calculated from the measured molecular weight and assumed low molecular activity of 10 min^{-1}, 1 kg of leaves gave 76 μg of enzyme under optimum conditions, with a total activity of approximately 1000 units. With the sensitive HPLC procedure, the preparation was sufficient for about 80 assays.

The preparation was halted after molecular sieve chromatography, since at this stage, neither substrates nor products were significantly susceptible to attack by other enzymes present. Further purification would have reduced total protein to a low level, resulting in losses of enzyme for no useful gain. The only major loss of activity during the preparation occurred upon ammonium sulphate fractionation before molecular sieve chromatography (Table I). This step was retained because it provided a compromise between recovery and removal of some of the interfering hydrolytic enzyme activity which resulted in destruction of UDPglucose. The presence of phosphatase and pyrophosphatase activity was detected by following the fate of UDPglucose in incubations (see Methods). Most of this activity was separated from the enzyme during chromatography on DEAE-cellulose and subsequent steps were necessary for complete removal.

Extractable enzyme activity depends strongly on the state of development of the plants. Complete uniformity of conditions of growth was not available, and the optimum stage for harvest is not clear; however it approximates to the

seedling development stage defined under Materials and this appears to be critical.

Analysis of products

The two products formed from zeatin by the enzyme were converted to trimethylsilyl (TMS) derivatives for GC-MS. The mass spectrum and retention time of the TMS derivative of the major product were identical with those of derivatized synthetic 7-β-D-glucopyranosylzeatin (I). This spectrum is characteristic of I and markedly different from those of closely related compounds [19]. Similar investigation of the minor product established that it was 9-β-D-glucopyranosylzeatin. *O*-Glucosylzeatin was not detected as a product.

Products formed from 6-benzylaminopurine by the enzyme were found to co-chromatograph with authentic 7- and 9-β-D-glucopyranosides on ODS-silica columns. In a similar manner, 7-glucosides were found to be enzyme products from adenine, 6-benzylamino-2-(2-hydroxyethylamino)purine and 2-benzylamino-6-hydroxypurine. It was concluded, by analogy, that the enzymic products formed from other substituted purines were either 7- or 9-glucosides. The position of substitution was then determined from ultraviolet absorption ratios. Although the 3-glucoside forms as a minor metabolite when 6-benzylaminopurine is supplied to radish cotyledons, it was not detected as a product in the enzyme reaction.

Stoichiometry

The reaction catalysed is similar to that for other transferase enzymes using sugar nucleotides in plants [21]. The glucose from UDPglucose is transferred to an acceptor, yielding UDP (demonstrated chromatographically) and glucosides. Quantitation of the enzyme reaction components established the following equation for zeatin:

$$1 \text{ zeatin} + 1 \text{ UDPglucose} \rightarrow x \text{ zeatin-7-glucoside} + (1 - x) \text{ zeatin-9-glucoside} + 1 \text{ UDP}$$

With 6-benzylaminopurine as substrate, almost equal amounts of the two glucosides were formed (see Table IV).

The evidence available supports the conclusion that a single enzyme is responsible for these reactions. The enzyme activity studied eluted off DEAE-cellulose as a single peak [14]. Assays of fractions across the peak gave a constant ratio of products from 6-benzylaminopurine. The same behaviour was recorded later during molecular sieve chromatography. Slow inactivation of the enzyme upon storage at 0°C over several days, did not change the ratio of products when the preparation was assayed. Variation in substrate (zeatin) concentration over a wide range gave a constant ratio of products. The same result was obtained when pH was varied over the limited range of 6.7 to 7.7 with constant phosphate concentration in the buffer. Changing the buffer ions did cause small changes in product ratios. This observation is still consistent with a single enzyme which is influenced by ion binding.

Molecular properties

Size analysis of the enzyme was carried out by molecule sieve chromatography on Sephadex G-100, as described by Andrews [22]. The enzyme was

detected by activity measurements, and its elution pattern was almost coincident with that of egg albumin. Estimated molecular weight was 46 500 ± 1500.

The influence of pH on the reaction was studied with zeatin in the range 6.2 to 8.6. In this range, there was negligible effect from ionization of the substrates in solution. Initial analysis showed little dependence of the overall reaction on pH. A broad optimum at pH 7.3 to 7.4 was observed, and a maximum change in activity of less than two fold. It was found later that some of the decrease in activity at lower and higher pH was due to inactivation.

More important influences on the reaction were the buffer ion used and ionic strength. These parameters may be indistinguishable from specific anion influences. Four buffer solutions were tried at pH 7.4 and similar concentrations: potassium phosphate, potassium PIPES, Tris-chloride, and Tris-sulphate. Activity and stability were highest in potassium phosphate and lowest in Tris-chloride (only 40% as active). Between 10 and 100 mM in phosphate, activity

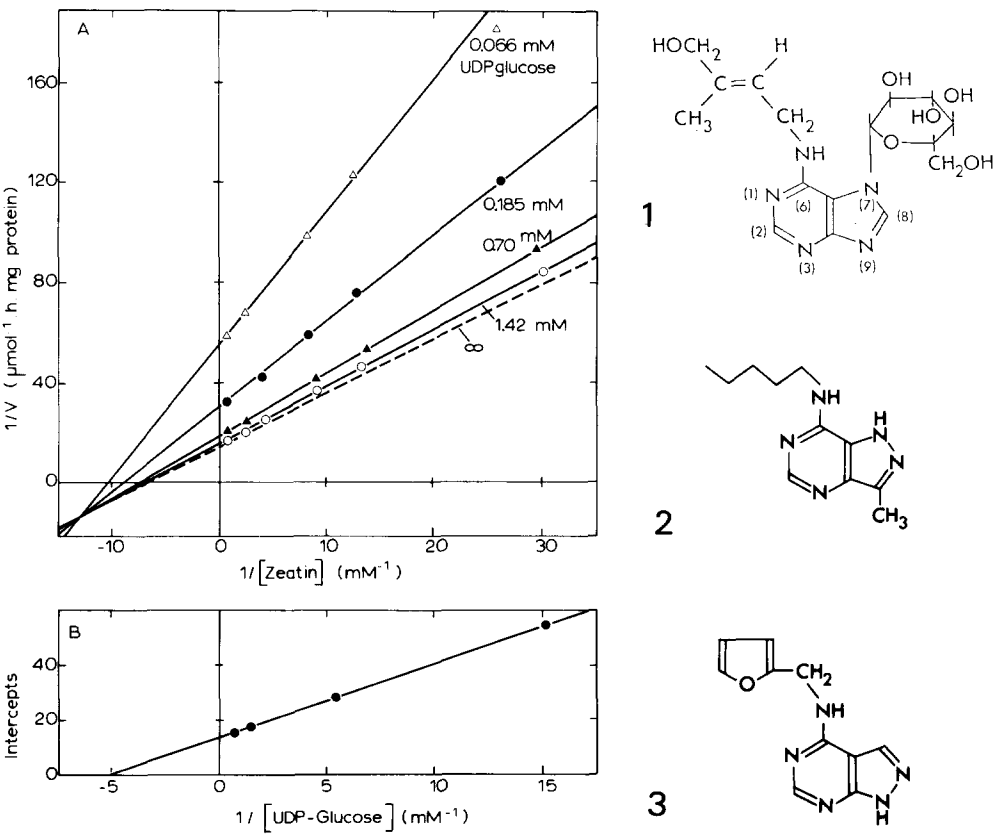


Fig. 2. A, double reciprocal plots of steady state initial velocity measurements of enzyme activity, varying the concentrations of zeatin and UDPglucose. Reactions were run at 35° with 12 units of enzyme. Rates were based upon combined 7- and 9-glucosides produced. The dashed line represents extrapolated infinite UDPglucose concentration. B, secondary plot from data in A. The intercepts on the velocity axis are plotted against reciprocals of UDPglucose concentration.

Fig. 3. Structural formulae for compounds I, II and III as mentioned in the text.

fell by a factor of two. Divalent metal ions (Mg^{2+} and Mn^{2+}) had negligible influence on the reaction in the range from 1 to 10 mM.

The enzyme was thus routinely studied at 22 mM phosphate with 0.3 mM EDTA adjusted to pH 7.35 with KOH, as a compromise between adequate buffering capacity and inhibition by phosphate.

Steady state analysis

The reaction of the enzyme was analysed with UDPglucose and zeatin as substrates. Concentrations of the substrates were varied systematically, and double reciprocal plots of reaction rates against concentration of one substrate were combined at constant concentrations of the other substrate (Fig. 2). Linear plots were obtained which intersected at one point. This behaviour is consistent with one group of mechanisms for an enzyme with two substrates [23]. Their common feature is the combination of both substrates with the enzyme to form a ternary complex, which then undergoes reaction, followed by the release of products. Detailed knowledge of the order of interaction and release of products required more enzyme than was available. Secondary plots of intercepts against reciprocal substrate concentration (Fig. 2) gave values for the basic kinetic parameters [24].

K_m, UDPglucose	$1.9 \cdot 10^{-4}$ M
K_m, zeatin	$1.5 \cdot 10^{-4}$ M
V	0.072 μmol $\cdot$ h^{-1} $\cdot$ mg^{-1} protein.

The observation that the reciprocal plots interact below the concentration axis (Fig. 2) indicates that the affinity of the enzyme for the substrate (expressed as $1/K_s$) is greater than that indicated by the K_m values [24]. Without knowledge of enzyme concentration, V cannot be given in absolute terms of molecular activity. The value given indicates the very small amounts of enzyme being studied.

TABLE II

SPECIFICITY TOWARDS DONOR OF SUGAR RESIDUE

Assays were run in 0.5 ml at 35°C for 3 h. Reactions contained donor substrate (below), 1.4 mM zeatin, 12 units of enzyme, potassium phosphate (20 mM in phosphate), 0.3 mM EDTA, final pH 7.35. The reaction was halted by the addition of 0.55 ml methanol and the products analysed. Total glucosides produced in the reaction were used to calculate rates. The minimum product detectable was approximately 0.5% of the amount formed with UDPglucose.

Potential substrate	Concn. used (mM)	Relative rate of reaction forming sugar conjugate
UDPglucose	1.5	100
TDPglucose	1.5	100
CDPglucose	1.5	<0.5
ADPglucose	1.7	<0.5
GDPglucose	1.7	2.3
UDP-*N*-acetylglucosamine	1.7	2.0
UDPgalactose	1.7	8.4
Glucose-1-phosphate	4.0	<0.5
Glucose-1-phosphate + UMP	2.0 each	<0.5

It was found that incubation of the enzyme with a near saturating concentration (2 mM) of UDPglucose alone under normal assay conditions gave no detectable UDP, by chromatography on a column of alkylamino-silica. Thus, the enzyme has control over the consumption of UDPglucose.

Specificity towards nucleotide sugar

The limited supply of enzyme forced a careful selection of possible substrates to study under fixed conditions. The findings are summarized in Table II. Zeatin was used as the common acceptor molecule at a concentration that was saturating to the enzyme, based on the data from Fig. 2. To optimize the detection of activity, the potential donor molecules were presented to the enzyme at a concentration many fold greater than the K_m for UDPglucose. The enzyme was specific for UDP- and TDPglucose, which differ by only one methyl group. The only other significant substrate, UDPgalactose, differs in the orientation of groups about one carbon atom. This activity was not due to epimerase formation of UDPglucose, as the products formed did not co-chromatograph with zeatin glucosides. The last entries in Table II show that the intact nucleotide was essential for activity.

TABLE III

SPECIFICITY TOWARDS THE ACCEPTOR OF GLUCOSE RESIDUE — PURINES WITH AN ALIPHATIC SUBSTITUENT

Reaction mixtures contained 1.5 mM UDPglucose, from 1.4 to 1.5 mM acceptor molecule (about 10 × K_m for zeatin), 14 units of enzyme, potassium phosphate (20 mM in phosphate) and 0.3 mM EDTA, final pH 7.35 at 35°C. After 3.5 h, methanol was added to stop the reaction, and the products analysed. Rates were based on total product produced. Apart from the first four compounds listed below, all other compounds are known to have hormone activity, with zeatin the most active.

Group substitution at position 6 of purine	Relative rate of glucoside formation	Ratio of glucoside products 7 position/9 position
—OH	no product detected	—
$-NH_2$	15	Trace of 9-glucoside
$-NH-CH_3$	20	Trace of 9-glucoside
$-NH-CH_2-CH_2-OH$	17.5	Trace of 9-glucoside
$-NH-CH_2-CH_2-CH_3$	113	27
$-NH-CH_2-CH_2-CH(CH_3)(CH_3)$	156	Trace of 9-glucoside *
$-NH-CH_2-CH=C(CH_3)(CH_2OH)$ (H) (zeatin)	100	10.5
cis isomer of zeatin	65	21
$-NH-CH_2-CH_2-CH(CH_3)(CH_2OH)$ (DL)	150	Trace of 9-glucoside *
$-NH-(CH_2)_6-OH$	113	Trace of 9-glucoside *
zeatin-*O*-glucoside	72	Trace of 9-glucoside *
zeatin-9-riboside	No product detected	—

* Less than 3% of total glucoside produced.

Specificity towards glucose acceptor

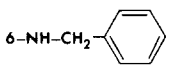
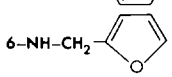
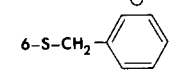
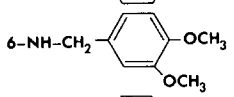
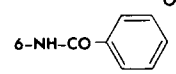
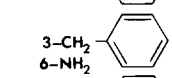
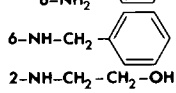
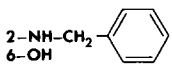
The influence of aliphatic side-chain structure in N^6-substituted adenines on enzyme activity is summarized in Table III. Activity was studied under conditions which were optimal for zeatin. All acceptor molecules were at the same concentration (1.4 to 1.5 mM), as was also UDPglucose. Some potential substrates may not have saturated the enzyme, but the conditions (Table III) should have given a good measure of relative effectiveness. In the case of N^6-isopentyladenine, the concentration chosen may have exceeded its solubility.

The results in Table III show that the enzyme accepted adenine and N^6 derivatives of adenine as substrates. It had a clear preference for a side chain with 3 to at least 6 carbon atoms. The products from every substrate were predominantly the 7-glucoside. It was clear that an unsubstituted position 9 of the purine ring was essential for activity. Zeatin-9-riboside was not a substrate, and neither was 6-benzylamino-9-(3-hydroxypropyl)purine. The *cis* isomer of zeatin was the least active of the four isopentyl substrates studied. It has been found

TABLE IV

SPECIFICITY TOWARDS THE ACCEPTOR OF GLUCOSE RESIDUE — PURINES WITH AN AROMATIC SUBSTITUENT

Reactions were carried out as described in Tables II and III. Acceptor molecules were present at 0.20 to 0.22 mM. The concentration used was close to the K_m for zeatin (0.15 mM). The column Cytokinin activity is a rough qualitative guide to the effect of the compounds listed in bioassays, as described in the literature [2,15]. The products were identified from compounds 1, 2, 4, 7 and 8 below. It was assumed that the products formed from compound 3 below were the 7- and 9-glucosides. Mass spectra of the products showed that they were derivatives of the parent compound with this structure intact. Identification was not completed. The product from compound 5 had spectral characteristics like the parent compound in neutral solution.

Compound number	Group substitution on purine	Relative rate of glucoside formation	Ratio of glucoside products (7/9)	Cytokinin activity
1		100	1.65	high
2		49	6.8	high
3		145	1.0	low
4		22	3.1	none
5		11	one product (position unknown)	moderate
6		no product detectable	—	none
7		150	one product at position 7	low
8		96	24	none

that *cis*-zeatin is much less active biologically than zeatin [25]. The dihydroxyzeatin molecule (see Table III) contains an asymmetric carbon in the side chain. Both enantiomers were attacked by the enzyme, giving two 7-glucoside products which were resolved by liquid chromatography. A large polar group on the end of the N^6 side chain (zeatin-*O*-glucoside) did not prevent activity. This substrate is active in bioassays and occurs as an endogenous cytokinin [26].

Another group of purines which are of considerable importance to the study of specificity is listed in Table IV. These compounds (assigned Arabic numbers 1—8) with a benzyl or related substituent are very insoluble in H_2O, and so were studied with the enzyme at 0.20 mM to be sure of homogeneous solutions. This concentration is lower than that used in Table III, and should have accentuated differences in V and K_m between the compounds. However, this was balanced by consumption of up to 50% of a substrate in the reaction. For comparison of Tables III and IV, it should be noted that enzyme activity is approximately 15% higher for 6-benzylaminopurine than for zeatin at 0.20 mM. It is clear that some of the compounds in Table IV are just as effective as substrates as the best in Table III; generally the proportion of 9-glucoside formed is greater than that formed by the compounds of Table III. When an aromatic group was attached to the N^6 of adenine, the enzyme was active. However, when that group was possibly too bulky, activity was lower (Table IV, No. 4). An exocyclic nitrogen at position 6 was not essential, though clearly the nature of the attachment at 6 was important (cf. Nos. 3 and 5). It would be interesting to study the effect of an alkyl group with no heteroatom at position 6. The enzyme accepted a second side chain at position 2 (No. 7), but a benzyl group at position 3 (No. 6) abolished activity. Glucosylation of No. 8 suggests that a side chain at position 2 is just as acceptable to the enzyme as at position 6.

Compounds II and III (materials) have re-arrangements of the atoms in the purine ring. No product was found from incubations similar to those described in Table IV. A compound, *o*-hydroxybenzyl alcohol, which could represent a simplification of the purine ring structure was incubated with the enzyme. The one product formed was identical in ultraviolet absorbance and chromatography to the compound, salicin, which has a β-D-glucosyl moiety attached to the original phenolic oxygen. The enzyme reaction was studied for dependence on substrate concentration at saturating UDPglucose, and an estimate obtained for K_m of the phenol — 0.9 to $1.0 \cdot 10^{-3}$ M. It was found that the maximum rate of the enzyme reaction at saturating substrate concentration was 40% higher than for zeatin under the same conditions. It was clear that there was no absolute requirement for the purine ring, but there was definitely specificity for the substituted purines, as evidenced by the K_m factor.

Discussion

Previously, a brief report from this laboratory [14] described the discovery of two enzymes in radish which catalysed the formation of 7- and 9-glucosides of 6-benzylaminopurine. The most active preparations were found in expanded radish cotyledons. The combined activity accounted for the principal metabolites formed when this cytokinin was supplied to radish tissues. It is now clear

that the enzymes also produce the analogous 7-glucoside of zeatin, which has been extracted from radish and shown to be endogenous [3]. The partial purification of the more abundant enzyme activity is described here. The preparation had to conserve enzyme as much as possible, while producing a product suitable for quantitative study. Although the enzyme was in trace amounts, a conventional fractionation was effective in achieving the goals. The only difficulty with the product used was a loss of activity after standing at room temperature for a few hours. An improvement may be possible if a highly specific affinity chromatography step was applied to a much larger preparation.

Without the recent technology of high-performance liquid chromatography, an effective quantitative study of this and similar enzymes would not be possible. Trace quantities of water-soluble products with strong ultraviolet absorption were readily quantified and enzyme assays were flexible and accurate. The products separated by HPLC were adequately purified for chemical characterization.

The study of specificity showed that the enzyme had an almost absolute requirement for UDPglucose as substrate. The analysis was not comprehensive but a prcise requirement for a nucleotide sugar is a common observation [21].

Essential to the physiological role of the enzyme is the specificity it exhibits towards the recognized features of cytokinin structure. The structural features required for high cytokinin activity are an intact purine ring with an appropriate substituent at position 6. The substituent may be aliphatic or cyclic [2]. The choice of potential substrates was prejudiced in favour of substrates related to cytokinins, but this appeared justified by the results. The enzyme did not have a precise structural requirement for the acceptor molecule. However, it did demonstrate (Tables III and IV) a clear preference for an adenine ring with an alkyl or aryl side chain at position N^6. In the case of N^6-alkyl adenines, significant cytokinin activity [2] and also ability to function as an effective enzyme substrate both require an N^6-substituent with at least three carbon atoms. Although a benzyl group at N-3 of adenine greatly enhances alkylation at N-7 in chemical reactions [27,28], 3-benzyladenine (compound 6, Table IV) was not a substrate for the glucosylating enzyme.

With an aliphatic side chain at position 6 of adenine (the structure of zeatin and related hormones), the enzymic product was predominantly the 7-glucoside (Table III), which was also the only glucoside metabolite formed in appreciable amounts when zeatin was supplied exogenously to radish seedlings [4,29]. In contrast, 6-benzylaminopurine yielded the 7- and 9-glucosides in the ratio of about 2 : 1, both when supplied to radish cotyledons [7] and when glucosylated enzymically in vitro (Table IV).

When the enzyme was studied in detail (as here), it was a surprise to find that the glucosides were not formed by separate enzymes. With the small size of the enzyme (46 000 daltons), it is a reasonable assumption that there is only one active site per molecule which is capable of forming the two glucoside products. Simple explanations such as steric hindrance of the most favoured site of reaction (position 7) or two different orientations of binding do not fit the results. The dominance of 7-glucoside formation is an unusual reaction when the free base in solution is most susceptible to alkylation at positions 3 and 9 [27,28]. It is possible that the enzyme induces tautomerization as has

been found with hypoxanthine and xanthine oxidase [30]?

Although a trace enzyme, the glucosyl transferase studied could exert a regulatory role in metabolism since its substrates (cytokinins) occur in trace amounts, evoke key responses at the sub-nanomolar level, and in some tissues [4] do not appear to turn over rapidly. With the discovery of zeatin-7-glucoside as a natural metabolite in radish seed [3], there can be no doubt about the occurrence of the enzyme reaction in vivo. The enzyme has not been described before and would probably fit in section EC 2.4.1 of the classification of enzymes. We propose the trivial name of 'cytokinin 7-glucosyltransferase'. The enzyme has potential use in the synthesis of purine 7-glucosides which are difficult to prepare chemically [1]. Enzymic synthesis would be of particular value in preparation of labelled cytokinin glucosides of high specific activity to determine the metabolic fate of such compounds. Inhibitors of cytokinin 7-glucosyltransferase merit study, as a stable, effective, and specific inhibitor in vivo could be a valuable physiological tool and a means of elevating endogenous free cytokinin levels by suppressing formation of the very weakly active 7-glucosides. The enzyme studies presented herein support the proposition that cytokinin 7-glucosides formed endogenously, are the products of an interesting enzyme system potentially capable of regulating plant development. Thus, formation of zeatin 7-glucoside and the oxidation of zeatin to adenine by cytokinin oxidase [13] may provide alternative metabolic mechanisms for lowering endogenous cytokinin levels.

References

1 Cowley, D.E., Duke, C.C., Liepa, A.J., MacLeod, J.K. and Letham, D.S. (1978) Aust. J. Chem. 31, 1095—1111

2 Letham, D.S. (1978) in Phytohormones and Related Compounds — a Comprehensive Treatise, Vol. 1 (Letham, D.S., Goodwin, P.B. and Higgins, T.J., eds.), pp. 205—263, Elsevier/North Holland, Amsterdam

3 Summons, R.E., MacLeod, J.K., Parker, C.W. and Letham, D.S. (1977) FEBS Lett. 82, 211—214

4 Parker, C.W. and Letham, D.S. (1973) Planta 114, 199—218

5 Fox, J.E., Cornette, J., Deleuze, G., Dyson, W., Giersak, C., Niu, P., Zapata, J. and McChesney, J. (1973) Plant Physiol. 52, 627—632

6 Gawer, M., Laloue, M., Terrine, C. and Guern, J. (1977) Plant Sci. Lett. 8, 267—274

7 Wilson, M.M., Gordon, M.E., Letham, D.S. and Parker, C.W. (1974) J. Exp. Bot. 25, 725—732

8 Laloue, M. (1977) Planta 134, 273—275

9 Letham, D.S., Parker, C.W., Duke, C.C., Summons, R.E. and MacLeod, J.K. (1977) Ann. Bot. (Lond.) 41, 261—263

10 Parker, C.W., Letham, D.S., Gollnow, B.I., Summons, R.E., Duke, C.C. and MacLeod, J.K. (1978) Planta 142, 239—251

11 Miller, L.P. (1973) in Phytochemistry, Vol. 1 (Miller, L.P., ed.), pp. 297—375, Van Nostrand Reinhold, New York

12 Montgomery, J.A. and Thomas, H.J. (1965) J. Am. Chem. Soc. 87, 5442—5447

13 Whitty, C.D. and Hall, R.H. (1974) Can. J. Biochem 52, 789—799

14 Entsch, B. and Letham, D.S. (1979) Plant. Sci. Lett. 14, 205—212

15 Letham, D.S., Summons, R.E., Entsch, B., Gollnow, B.I., Parker, C.W. and MacLeod, J.K. (1978) Phytochemistry 17, 2053—2057

16 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265—275

17 Warburg, O. and Christian, W. (1941) Biochem. Z. 310, 384—391

18 Karger, B.L., Gant, J.R., Hartkopf, A., and Weiner, P.H. (1976) J. Chromatogr. 128, 65—78

19 MacLeod, J.K., Summons, R.E. and Letham, D.S. (1976) J. Org. Chem. 41, 3959—3967

20 Leonard, N.J., Carraway, K.L. and Helgeson, J.P. (1965) J. Heterocyclic Chem. 2, 291—297

21 Kelleher, W.J. (1965) J. Pharm. Sci. 54, 1081—1109

22 Andrews, P. (1964) Biochem. J. 91, 222—233

23 Cleland, W.W. (1970) in The Enzymes (Boyer, P.D., ed.), Vol. II, 3rd edn., pp. 1—66, Academic Press, New York
24 Segel, I.H. (1975) Enzyme Kinetics, pp. 560—570, John Wiley and Sons, New York
25 Vreman, H.J., Schmitz, R.Y., Skoog, F., Playtis, A.J., Frihart, C.R. and Leonard, N.J. (1974) Phytochemistry 13, 31—37
26 Peterson, J.B. and Miller, C.O. (1977) Plant Physiol. 59, 1026—1028
27 Robins, R.K. (1967) in Heterocyclic Compounds (Elderfield, R.C., ed.), Vol. 8, pp. 162—442, Wiley, New York
28 Lister, J.H. (1971) in The Chemistry of Heterocyclic Compounds (Brown, D.J., ed.), Vol. 24, Part 2, pp. 342—348, Wiley-Interscience, New York
29 Gordon, M.E., Letham, D.S. and Parker, C.W. (1974) Ann. Bot. (Lond.) 38, 809—825
30 Bergmann, F. and Levene, L. (1976) Biochim. Biophys. Acta 429, 672—688