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ASSAY OF ENZYMES INVOLVED IN CYTOKININ METABOLISM BY MEANS OF REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

Assay of a number of enzymes involved in cytokinin metabolism can be rapidly and conveniently carried out by using reversed-phase high-performance liquid chromatography (HPLC) to quantitate either the formation of product or the loss of substrate. The initial rates of loss of added 6-(3-methylbut-2-enylamino)purine or 6-benzylaminopurine can be determined in a variety of plant tissues. Assay of nucleosidase activity with 6-(3-methylbut-2-enylamino)-9-β-D-ribofuranosylpurine, 6-(4-hydroxy-3-methylbut-2-enylamino)-9-β-D-ribofuranosylpurine or adenosine as substrates can be accomplished by quantitating the loss of riboside or the formation of the free base by reversed-phase HPLC. Assay of enzymes catalyzing the formation of 8-OH cytokinins can be readily accomplished and the subsequent formation of 2,8-diOH cytokinin can be measured simultaneously if dual-wavelength monitoring is used. HPLC with a variety of solvent systems facilitates the assay of very small quantities of enzymes and the detection of multiple products.

INTRODUCTION

Cytokinins are N^6-substituted derivatives of adenine and are important in the regulation of a variety of metabolic processes in plant tissues[1,2]. Although considerable progress has been made in identifying the metabolites of cytokinins in plant tissues, relatively little is known about the enzymes involved in the metabolism of these compounds. This lack of knowledge is due primarily to difficulties in obtaining reasonable quantities of these enzymes and the lack of rapid and sensitive procedures for assaying them.

The use of high-performance liquid chromatography (HPLC) in enzyme assays has been recently reviewed by Sloan[3]. HPLC is particularly advantageous when the substrates and products have very similar UV absorptions, when multiple products are formed, or when the products are unknown. Assays by HPLC can have a significant advantage over traditional spectrophotometric techniques where coupling enzymes are used, because they avoid the problems that can arise due to inhibition of coupling enzymes. This inhibition can be very significant when crude extracts from plant tissues are assayed.

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Entsch *et al.*[4] utilized HPLC to assay an enzyme that glucosylates cytokinins at the 7-position with UDP-glucose. Marshall and Chism[5] studied the factors affecting the rate of metabolism of 6-(3-methylbut-2-enylamino)purine (IPA) in tomato fruits by following the loss of IPA with HPLC. Entsch *et al.*[6] examined the properties of β-(9-cytokinin)alanine synthase by means of HPLC.

In this paper we describe reversed-phase HPLC techniques for determining the activity of enzymes which hydroxylate IPA and 6-(3-methylbut-2-enylamino)-8-hydroxypurine (8-OH IPA), cytokinin nucleosidases, and adenosine nucleosidases.

EXPERIMENTAL

Materials

Cytokinins were purchased from Sigma (St. Louis, MO, U.S.A.). Xanthine oxidase was purified by the method of Mangino and Brunner[7]. Nucleosidases were extracted from tomato fruits by the method of Rolle and Chism[8]. Mung beans were germinated for 3.5 days at 28°C. Solvents were of HPLC grade.

Sample preparation

Segments (2 mm) of mung bean hypocotyls or cubes (1 g) of tomato pericarp were vacuum-infiltrated for 10 min with a solution containing 50 μ*M* cytokinin. Immediately after infiltration, 1 g of tissue was homogenized with 1 ml of methanol (−60°C) in a Brinkman Polytron. This sample was designated as "0 time". Tissue was sampled after various time intervals and treated similarly. The methanol extracts were centrifuged at 1300 *g* and the supernatant fractions were kept at −20°C overnight. The thawed extracts were recentrifuged and filtered through a 0.2-μm filter before injection into the chromatograph.

Enzyme extracts were added to buffer, containing the appropriate cytokinin. Activity in 1-ml aliquots of the reaction mixture was stopped by addition of 1 ml of methanol. This mixture was frozen, thawed, centrifuged, and filtered, as described above.

Chromatography

HPLC analyses were carried out using a Waters Assoc. apparatus, equipped with an M-6000A pump, U6K or Rheodyne Model 1725 injector, C_{18} Corasil precolumn (5 cm × 3.9 mm), μBondapak C_{18} column (30 cm × 3.9 mm) and a Model 450 variable-wavelength detector.

Hydroxylation assay

Partially purified xanthine oxidase was added to 0.1 *M* phosphate buffer (pH 7.4), containing 200 μ*M* IPA and incubated at 25°C. Aliquots of the reaction mixture were removed from the vessel at 10-min intervals. The reaction was stopped by the addition of an equal volume of methanol and treated as described above. A 20-μl aliquot was analyzed by HPLC at a wavelength of 275 μm for 8-OH IPA and at 309 nm for 2,8-dihydroxy-6-(3-methylbut-2-enylamino)purine (2,8-diOH IPA) by using 40% aqueous methanol as the eluent.

Loss of cytokinin assay

Tissue samples were prepared as described above with 50 μM IPA or 6-benylaminopurine (BA) as the substrate. Aliquots (20 μl) of the methanol extracts were separated by using 58% aqueous methanol as the eluent and monitoring at 269 nm.

Nucleosidase assays

Aliquots (250 μl) of the partially purified plant extracts were added to 750 μl of 0.1 M MES [2(N-morpholino)ethanesulfonic acid] (pH 6.0), which contained 50 μM nucleoside. The reaction mixture was maintained at 32°C in a water bath. Aliquots of 100 μl, taken at 0 and 2 h for the assay of cytokinin nucleosidases and at 0 and 20 min for the assay of adenosine nucleosidases, were mixed with an equal volume of cold (−60°C) methanol. The mixture was then frozen at −60°C, thawed, centrifuged at 1300 g, and filtered through a 0.2-μm filter. Aliquots (20 μl) of the extracts were analyzed by HPLC at 269 nm. The eluents used were: 58% aqueous methanol [6-(3-methylbut-2-enylamino)-9-β-D-ribofuranosylpurine (IPAR) as substrate], 40% aqueous methanol [6-(4-hydroxy-3-methylbut-2-enylamino)-9-β-D-ribofuranosyl-purine (ZR) as substrate], or 30% aqueous methanol (adenosine as substrate).

RESULTS AND DISCUSSION

Hydroxylation of cytokinins

Chen *et al.*[9] showed that xanthine oxidase could hydroxylate some cytokinins to form the 8-hydroxy and the 2,8-dihydroxy derivatives. These authors used a spectrophotometric assay and open-column chromatography. Reversed-phase HPLC with a μBondapak C_{18} column and 40% aqueous methanol offers a convenient alternative to these procedures. When IPA is the substrate, the two derivatives can be resolved in 16 min (Fig. 1). The loss of IPA or the formation of 8-OH IPA can be readily quantified by UV absorption at 275 nm, the λ_{max} of 8-OH IPA. In order to quantify 2,8-diOH IPA, which has an absorption maximum at 309 nm and absorbs only weakly at 275 nm, an additional chromatogram, monitored at 309 nm (Fig. 2) or dual-wavelength monitoring (275 and 309 nm) is required. Monitoring at both wavelengths provides the data necessary to correct the quantity of 8-OH IPA for the losses due to the conversion to 2,8-diOH IPA. If dual-wavelength monitoring is not available, analysis time can be reduced to 5 min for the chromatogram monitored at 309 nm (Fig. 2). 8-OH IPA and IPA do not interfere, because of their low absorptivity at 309 nm.

In mung bean hypocotyls, the presence of 8-OH IPA inhibits the metabolism of IPA (Table I). By monitoring at 309 nm as well as at 275 nm it can be shown that the effect is due to 8-OH IPA and not to a metabolite of 8-OH IPA, because the 8-OH IPA is metabolized very slowly, if at all. Because 8-OH IPA is not metabolized and no peak corresponding to 8-OH IPA appears when IPA is incubated with the mung bean hypocotyls (Fig. 3), 8-OH IPA cannot be a major metabolite of IPA in this system. The identity of the major metabolite of IPA in this system remains to be elucidated.

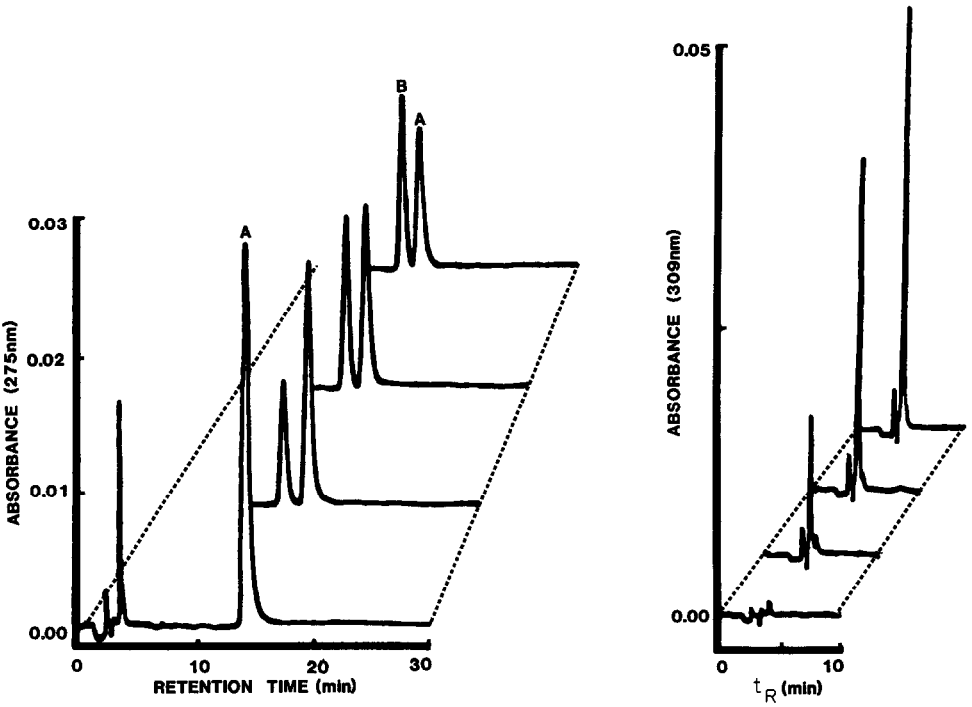

Fig. 1. Reversed-phase HPLC for monitoring the formation of 8-OH IPA from IPA. Reaction conditions are as described in the text. Aliquots (20 μl) of the stopped reaction mixture were injected into a μBondapak C_{18} column and eluted with 40% aqueous methanol at a flow-rate of 1 ml/min. IPA (peak A) is eluted after 8-OH IPA (peak B), which is absent at "0 time". The chromatograms are from aliquots taken at 0, 30, 60 and 90 min.

Fig. 2. Reversed-phase HPLC for monitoring the formation of 2,8-OH IPA from IPA. Conditions and samples are the same as described in Fig. 1.

Loss of cytokinin

The rate of metabolism of IPA or BA, added to segments of plant tissue, can readily be monitored by using reversed-phase HPLC on a μBondapak C_{18} column with 58% aqueous methanol as the eluent (Fig. 4). The analysis time is about 8 min and this facilitates kinetic studies. In this system, IPA and BA are unresolved and thus they cannot be used simultaneously, but it is suitable for either BA or IPA alone.

TABLE I

Effect of 8-OH-IPA on the loss of IPA from mung bean tissue. Mung bean hypocotyls were vacuum-infiltrated with 50 m*M* MES (pH 6.8), containing 50 μM IPA, 50 μM 8-OH IPA, or 50 μM IPA, and 50 μM 8-OH IPA. The reaction was stopped by grinding the hypocotyls in cold (−60°C) methanol.

	Rate of loss of IPA (nmoles/g/h)	*Rate of loss of 8-OH IPA (nmoles/g/h)*
IPA	3.22	–
8-OH-IPA	–	0.25
IPA and 8-OH-IPA	0.98	0.38

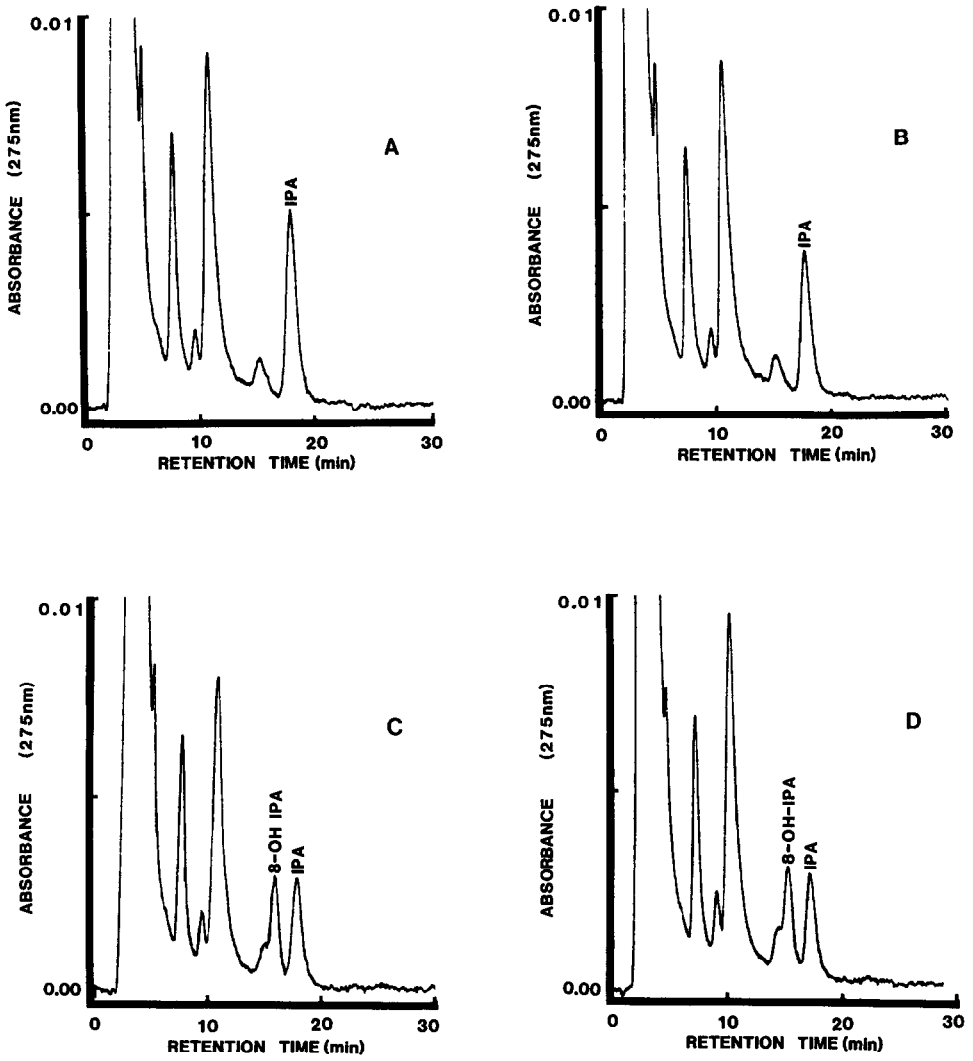

Fig. 3. Reversed-phase HPLC for monitoring the disappearance of IPA from mung bean tissue, in the presence or absence of 8-OH IPA. Chromatographic conditions are the same as described in Fig. 1. Chromatograms A and B are samples incubated with IPA alone for 0 and 1 h, respectively. Chromatograms C and D are samples incubated with both IPA and 8-OH IPA for 0 and 1 h, respectively.

Because most of the compounds present in the tissues that absorb significantly at 269 nm are more polar than BA or IPA, the analysis time can be shortened by adjusting the solvent strength so that the peak of interest is retained just long enough to be separated from the large peak containing these compounds. This system is suitable for most plant tissues we have tried (apple, cucumber, mung bean hypocotyls and cotyledons, carnation petals, bananas, and tomatoes), but green leaves often require a longer analysis time for separating the cytokinin peak. More polar cytokinins, like 6-(4-hydroxy-3-methylbut-*trans*-2-enylamino) purine (Z) require considerably longer analysis times with weaker solvents in order to resolve the components of interest.

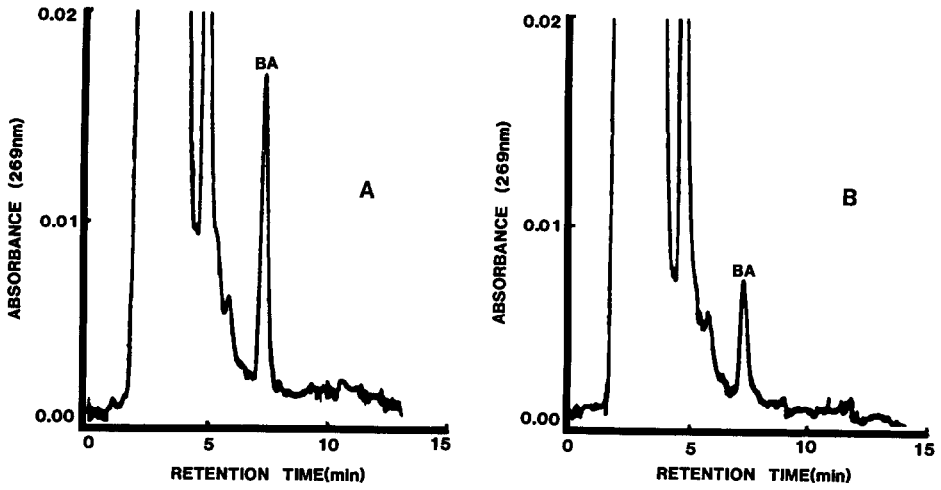

Fig. 4. Reversed-phase HPLC for monitoring the disappearance of BA from mung bean tissue. Aliquots (20 μl) of tissue extract were injected into a μBondapak C_{18} column and eluted isocratically at 1 ml/min with 58% aqueous methanol. Chromatograms A and B are for incubations of 0 and 1 h, respectively.

However, even with longer analysis times, the resolution may be inadequate. This makes kinetic studies, which require a large number of data points, more tedious.

Cytokinin nucleosidases

The importance of cytokinin nucleosidases in maintaining a pool of active cytokinins by converting the less active nucleoside forms to the more active free base forms, has been suggested by Chen[2]. Reversed-phase HPLC can readily separate the free base form of cytokinins from the more polar nucleoside forms. When IPAR is the substrate, a 10-min analysis time is needed to separate the product from the substrate (Fig. 5). The reaction rate can be followed by determining either the loss of substrate or the formation of product. Kinetic studies of extracts having as little activity as 0.35 μmoles/min/mg protein have been performed[7]. This activity can be measured in many plant tissues by adding the nucleoside directly to the tissue and extracting with methanol at various time intervals. BA and its corresponding riboside have k values very similar to IPA and IPAR, and thus they can readily be determined by means of this system (data not shown). Determination of nucleosidase activity with ZR as substrate requires the use of a less polar solvent system (40% aqueous methanol) to provide adequate separation of zeatin from its riboside (Fig. 6). This solvent system will not separate zeatin and its riboside from interfering substances when the system described for pieces of tissue is used. However, it is applicable to crude plant extracts that have been concentrated by ammonium sulfate precipitation or other techniques that also remove low-molecular-weight substances.

Adenosine nucleosidase activity can also be conveniently measured with reversed-phase HPLC. Because adenine and adenosine are considerably more polar than the compounds discussed previously, the solvent used is 30% aqueous methanol (Fig. 7). Numerous compounds in plant tissues contaminate the compounds of interest in this system, so that it is not usable with pieces of tissue or crude extracts

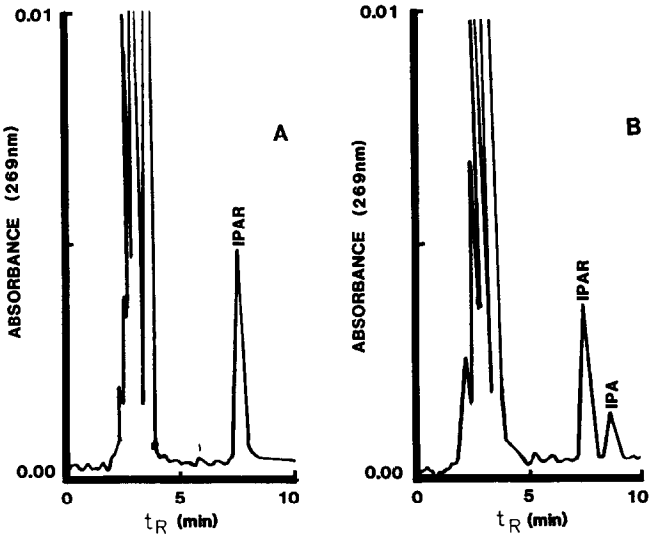

Fig. 5. Hydrolysis of IPAR, incubated with cytokinin nucleosidase, isolated from tomato fruit, for 0 (A) and 2 h (B). The samples were separated on a μBondapak C_{18} column with 58% aqueous methanol at a flow-rate of 1 ml/min.

that still contain the low-molecular-weight compounds. However, this does not generally cause problems, because extracts must be concentrated in order to obtain a reasonable level of enzyme activity, and the low-molecular-weight compounds are generally removed during the concentration steps.

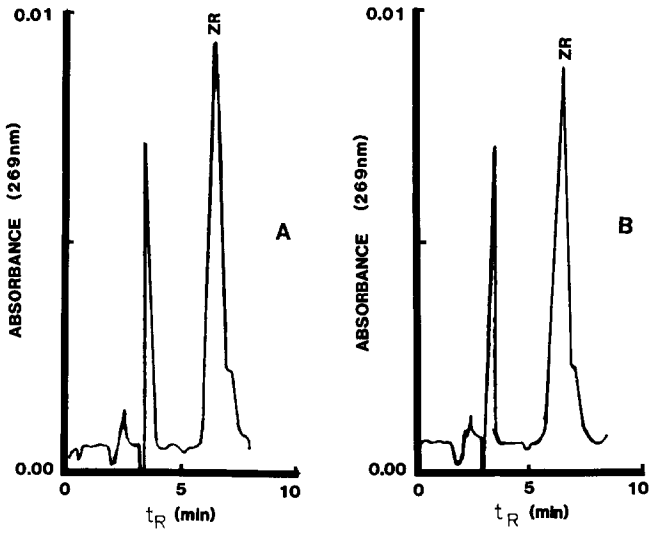

Fig. 6. Hydrolysis of ZR, incubated with cytokinin nucleosidase, isolated from tomato fruit, for 0 (A) and 2 h (B). The samples were separated on a μBondapak C_{18} column with 40% aqueous methanol at a flow-rate of 1 ml/min.

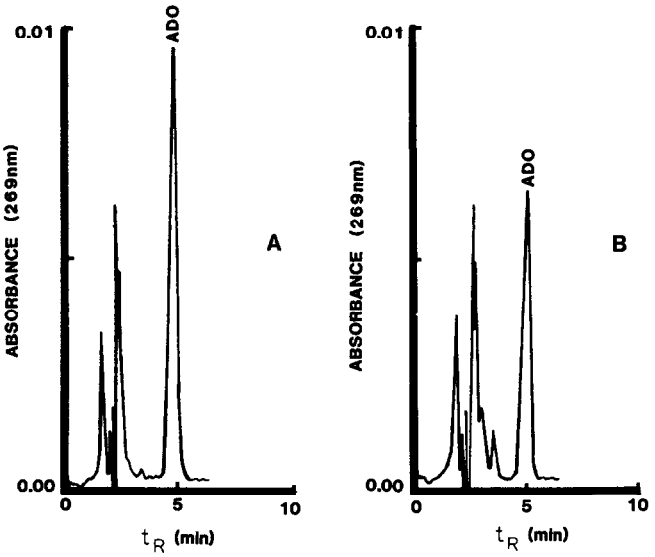

Fig. 7. Hydrolysis of adenosine, incubated with cytokinin nucleosidase, isolated from tomato fruit, for 0 (A) and 20 min (B). The samples were separated on a μBondapak C_{18} column with 30% aqueous methanol at a flow-rate of 1 ml/min.

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