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[^{3}H]Indole-3-Acetyl-*myo*-Inositol Hydrolysis by Extracts of *Zea mays* L. Vegetative Tissue[1]

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ABSTRACT

[^{3}H]Indole-3-acetyl-*myo*-inositol was hydrolyzed by buffered extracts of acetone powders prepared from 4 day shoots of dark grown *Zea mays* L. seedlings. The hydrolytic activity was proportional to the amount of extract added and was linear for up to 6 hours at 37°C. Boiled or alcohol denatured extracts were inactive. Analysis of reaction mixtures by high performance liquid chromatography demonstrated that not all isomers of indole-3-acetyl-*myo*-inositol were hydrolyzed at the same rate. Buffered extracts of acetone powders were prepared from coleoptiles and mesocotyls. The rates of hydrolysis observed with coleoptile extracts were greater than those observed with mesocotyl extracts. Active extracts also catalyzed the hydrolysis of esterase substrates such as α-naphthyl acetate and the methyl esters of indoleacetic acid and naphthyleneacetic acid. Attempts to purify the indole-3-acetyl-*myo*-inositol hydrolyzing activity by chromatographic procedures resulted in only slight purification with large losses of activity. Chromatography over hydroxylapatite allowed separation of two enzymically active fractions, one of which catalyzed the hydrolysis of both indole-3-acetyl-*myo*-inositol and esterase substrates. With the other fraction enzymic hydrolysis of esterase substrates was readily demonstrated, but no hydrolysis of indole-3-acetyl-*myo*-inositol was ever detected.

Esters of the plant growth hormone, IAA, serve as a source of IAA for both the endosperm (7, 10, 20) and vegetative tissues of *Zea mays* (5). A high concentration of esterified IAA relative to that of the free acid is characteristic of both vegetative and endosperm tissue (1, 2). According to the hormonal homeostasis theory (7), hydrolysis of esterified IAA releases free IAA to the plant as required for growth and in response to environmental stimuli. This hypothesis predicts that enzymes will be present in vegetative tissue which catalyze the hydrolysis of IAA esters.

Evidence that IAA conjugates serve as sources of free IAA consists of the observations that: (a) IAInos[3] applied to the endosperm yields free IAA in both the shoot and the root (6, 18); (b) IAA esters are formed during kernel maturation (8) and are hydrolyzed during germination (20) to yield free IAA (10); and (c) IAA conjugates serve as "slow release" sources of IAA in many tissue culture systems (14).

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[3] Abbreviation: IAInos, indole-3-acetyl-*myo*-inositol.

This paper describes some characteristics of an enzyme preparation from 4 d shoots of dark grown *Z. mays* seedlings which catalyzed the hydrolysis of [^{3}H]IAInos. Preliminary reports have appeared (12, 13).

There are six biologically distinguishable isomers of IAInos, four of which can be separated chemically. It is not known which isomers predominate *in vivo*, but it is possible that a limited number would serve as substrates for a hydrolytic enzyme. Preliminary experiments are presented which suggest preferential hydrolysis of one or more of the six possible isomers of IAInos.

IAInos hydrolytic activity from mesocotyls and from coleoptiles, including primary leaves, was compared. Since the coleoptile of a young seedling plays an important role in the auxin controlled growth of the mesocotyl, IAA esters present in the coleoptile could provide a source of IAA resulting in the rapid growth observed in the acropetal regions of the mesocotyl.

The enzymic hydrolysis of the synthetic ester, α-naphthyl acetate, was also studied to see if an enzyme fraction hydrolyzing IAInos would also hydrolyze a general esterase substrate, and, conversely, to identify other esterases which could then be examined for activity in the hydrolysis of IAInos. In this paper we demonstrate that an esterase fraction which does not hydrolyze IAInos can be separated, by chromatography over hydroxylapatite, from an esterase fraction which does hydrolyze IAInos.

MATERIALS AND METHODS

Chemicals. The following chemicals were obtained from commercial sources and were used without further purification: bovine plasma gamma globulin, BioRad; hydroxyapatite, Calbiochem; ACS Scintillation cocktail, Amersham; and enzyme grade $(NH_4)_2SO_4$, Mann Research Laboratories, Inc. 5-[^{3}H]Indolyl-3-acetyl-*myo*-inositol (29 Ci/mmol) was synthesized as reported by Michalczuk and Chisnell (17) and was a gift from J. Chisnell. Indolyl-3-acetyl-*myo*-inositol was synthesized by Cohen (19). α-Naphthyl acetate was synthesized from α-naphthol and acetyl chloride in benzene (3) and recrystallized from 2-propanol.

Protein was determined by the method of Bradford (4) using bovine plasma gamma globulin as the standard.

Preparation of Plant Material. Kernels of *Z. mays* sweet corn (Stowell's Evergreen, Burpee Seed Co.) were imbibed in running tap water overnight, then depending on the amounts required, sown in flats of Vermiculite or rolled in paper towels, and grown for 4 d in darkness at 25°C. Shoots, including mesocotyl, coleoptile, and primary leaves, were harvested under a green safe light and collected in an ice chilled beaker. After weighing the shoots, an acetone powder was prepared by grinding the shoots in chilled acetone (10 ml acetone to 1 g tissue, chilled to −5 to −10°C with Dry Ice) in a blender. Acetone was removed by filtration and the resultant powder drained, rinsed with cold diethyl ether, and drained again. The powder was dried over P_2O_5 *in vacuo* and stored at −18°C. For comparisons of activity between coleoptiles

and mesocotyls, these parts were harvested separately and used in preparing acetone powders.

Ten g of acetone powder were suspended in 150 ml of 0.05 M Tris buffer (pH 7.15), stirred for 30 min, and particulate matter removed by centrifugation at 12,000*g* for 10 min. The pellet was resuspended in 150 ml of fresh Tris buffer and again extracted for 30 min. Following centrifugation, the supernatants were combined and the protein precipitated by adding solid $(NH_4)_2SO_4$ to 90% saturation. The mixture was stirred 45 to 60 min, then centrifuged at 12,000*g* for 15 min. The pelleted protein was redissolved in 60 ml K-phosphate, 0.05 M, pH 7.15, and dialyzed against two changes of the same buffer for a total of 3 to 4 h. The extract could be frozen and would retain enzyme activity for as long as 1 year. Upon thawing, a precipitate formed which could be removed by centrifugation without loss of activity.

Assay of IAInos Hydrolytic Activity. Reaction mixtures contained plant extract plus Tris or K-phosphate in a total volume of 100 μl in a 6 × 50 mm culture tube. Five μl of [^{3}H]IAInos in 50% aqueous 2-propanol (6 pmol IAInos/5 μl, approximately 4 × 10^5 dpm) was added and a 5 μl aliquot withdrawn immediately for determination of radioactivity. The mixture was incubated at 37°C for 4 h and the reaction terminated by the addition of 100 μl of 2-propanol. A control reaction mixture without plant extract was used to determine nonenzymic hydrolysis as the rate of [^{3}H]IAInos hydrolysis was the same as that found in reaction mixtures containing boiled enzyme. Reaction mixtures were frozen if not used immediately.

Unhydrolyzed [^{3}H]IAInos does not bind to DEAE-Sephadex (acetate form in 50% aqueous 2-propanol) while the product of hydrolysis, [^{3}H]IAA does bind unless the solvent, 50% aqueous 2-propanol, is made 2 N with respect to acetic acid. Substrate and product could be separated by chromatography over a small column of DEAE-Sephadex or by centrifugation of the reaction mixture with the ion exchanger. Aliquots from both the neutral wash and the acidic elution solvent were used for determination of radioactivity in a Beckman LS 7000 liquid scintillation counter. Recovery of radioactivity averaged 85 to 90% using either method.

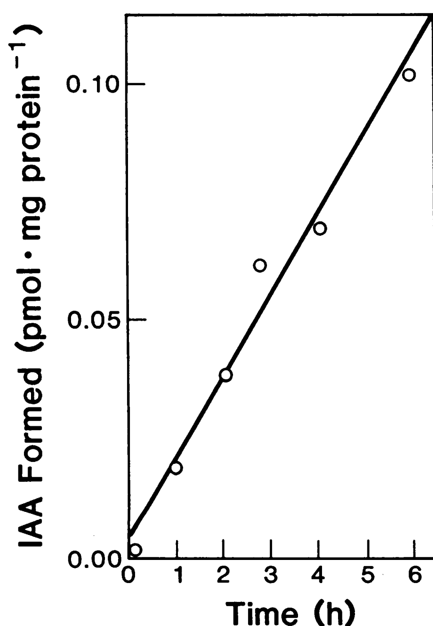

FIG. 1. Rate of [^{3}H]IAInos formation as a function of incubation time. Reaction mixtures were prepared and assayed as described in "Materials and Methods." Radioactivity present as IAA was corrected for nonenzymic hydrolysis and the line is drawn by linear regression analysis.

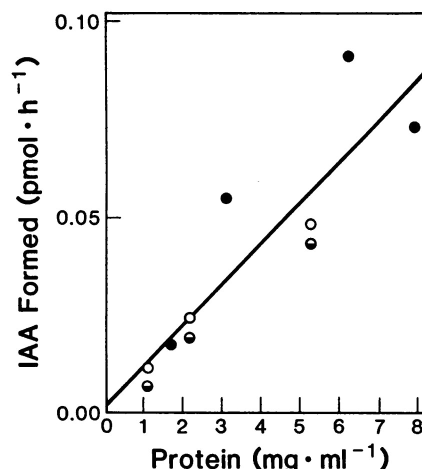

FIG. 2. The relationship between enzyme concentration and rate of [^{3}H]IAInos hydrolysis. Reaction mixtures were prepared as described in "Materials and Methods" and were incubated for 4 h. The line is drawn by linear regression analysis. The points shown (○, ⊖, ●) represent data from 3 extractions over a 12 month period.

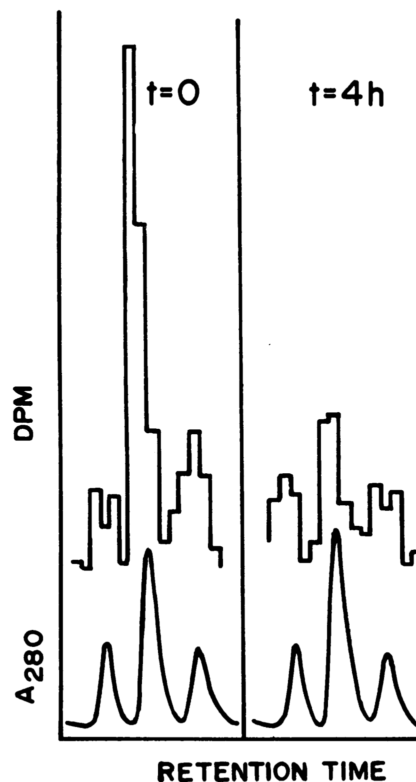

FIG. 3. Radioactivity associated with peaks of IAInos eluted from HPLC. Extracts were incubated with [^{3}H]IAInos for 0 or 4 h and the reaction terminated by the addition of an equal volume of 2-propanol. An aliquot was mixed with 1 μg chemically synthesized IAInos, then chromatographed over a Partisil 10-ODS column. Fractions of 0.5 ml were collected and the radioactivity determined in each. The relative amounts of radioactivity associated with each peak of the IAInos isomeric mixture are shown in the upper portion of each panel.

Assay of α-Naphthyl Acetate Esterase Activity. Reaction mixtures consisted of buffered plant extracts and 4.3 μmol of α-naphthyl acetate in a total volume of 3.0 ml. The mixture, excluding substrate, was incubated at 37°C in a Cary 15 Dual Beam Recording Spectrophotometer for 5 min. After addition of substrate, the extent of the reaction was observed for at least

Table I. *Comparison of Enzymic Activities of Coleoptiles and Mesocotyls*

Plant Part[a]	Substrate			
	[^{3}H]IAInos	([^{3}H]IAA produced)	α-Naphthyl Acetate	(α Naphthol produced)
	pmol·h^{-1}·mg^{-1} protein	*pmol·h^{-1}·plant part*[b]	*nmol·min^{-1}·mg^{-1} protein*	*nmol·min^{-1}·plant part*[b]
Coleoptiles	0.012 ± 0.003	0.030	47 ± 6	116
Mesocotyls	0.008 ± 0.002	0.007	48 ± 6	44

[a] Extracts were prepared as described in "Materials and Methods" except that 0.1 g acetone powder was extracted in 3.0 ml Tris buffer. [b] Rates per mg protein were converted to rates per plant part by determining that each coleoptile contains 2.5 mg protein and each mesocotyl contains 0.92 mg protein.

8 min. The rate of hydrolysis was determined by measuring the increase in A at 320 nm for the first 5 min of the reaction.

The molar extinction coefficient of α-naphthol was found to be 2496 L·mol^{-1}·cm^{-1} in agreement with the value of 2485 reported by Johnson and Ashford (15). Rates of hydrolysis are reported as nmol α-naphthol produced min^{-1}·mg^{-1} protein.

HPLC Resolution of IAInos Isomers and IAA. IAA and IAInos isomers were partially resolved by HPLC over a Partisil 10-ODS column using isocratic elution in 1% aqueous acetic acid:ethanol (95:5) at a flow rate of 1 ml·min^{-1}. Absorbance was monitored at 280 nm with a Varian UV5 selectable detector. Fractions of 0.5 ml were collected for determination of radioactivity when samples containing [^{3}H]IAInos were chromatographed. Unlabeled IAInos was mixed with radioactive samples just prior to HPLC so the resolution of the isomers and IAA could be followed by A at 280 nm.

Hydroxylapatite Chromatography. After precipitation with $(NH_4)_2SO_4$ as described above, extracts were redissolved in 10 mM K-phosphate, pH 7.15, and were dialyzed against the same buffer. Eight ml containing 8.4 mg protein·ml^{-1} loaded onto a hydroxylapatite column (1.5 × 13.5 cm). The column was washed with the starting buffer at a flow rate of 15 ml·h^{-1} and fractions of 2.8 ml were collected. After unbound protein was washed from the column, a gradient of K-phosphate (pH 7.15), increasing in molarity from 0.01 to 0.5 M in 200 ml, was applied and the remaining protein eluted.

RESULTS

Hydrolysis of [^{3}H]IAInos. IAInos hydrolysis was measured by the rate of appearance of IAA; reported rates have been corrected for nonenzymic hydrolysis occurring in buffer alone. [^{3}H]IAA was a product of hydrolysis and was demonstrated by co-chromatography of the radioactive product with unlabeled IAA on TLC and in HPLC over a Partisil 10-ODS column (data not shown).

As can be seen from Figure 1, the hydrolysis of [^{3}H]IAInos proceeds linearly with respect to time. After 4 h the extent of enzymic hydrolysis is enough greater than nonenzymic hydrolysis so that even at low enzyme concentration, hydrolysis can be measured.

Although the assay for IAInos hydrolysis does not measure initial velocity and the substrate concentration, 1.2 μM, is less than optimal, it is possible to demonstrate proportionality between the rate of [^{3}H]IAA formation and enzyme concentration. The rate of [^{3}H]IAInos hydrolysis measured after 4 h increases in a linear fashion as enzyme concentration is increased up to a protein concentration of about 5 mg·ml^{-1} (Fig. 2).

Hydrolysis of [^{3}H]IAInos by plant extracts occurs over a wide range of hydrogen ion concentrations in a variety of buffers. At higher pH values nonenzymic hydrolysis is accelerated such that the difference between experimental and control reaction mixtures cannot be accurately determined. At pH values below 5.0 some proteins precipitate in crude or partially purified extracts with variable losses in activity. Net hydrolysis is maximal between pH 7.0 and 7.5 in Tris or K-phosphate. Activity was routinely assayed at pH 7.15 because at this pH acyl migration in the substrate would be less than at a more basic or acidic pH (11).

Extracts held at 100°C for 1.5 min lost IAInos hydrolyzing activity. Very dilute (less than 50 μg·ml^{-1}) protein solutions lost activity after freezing, and the addition of 1 mg·ml^{-1} of BSA prevented this loss. Hydrolyzing activity was not observed when extracts were made 50% with respect to 2-propanol or when held at 0°C.

Preferential Hydrolysis of Isomers. IAA could be esterified to *myo*-inositol at any of six hydroxyls. It is possible to resolve some of the esters by TLC (16), GLC (9), and HPLC (5) but only the 2-*O*-IAInos ester can be matched with a spot on TLC at a particular R_F or with a retention time on GLC or HPLC.

Experiments in which aliquots of an enzymic reaction mixture were chromatographed over HPLC points to a preferential hydrolysis of some isomers. In Figure 3 the ratio of radioactivity collected to area of the peak at the corresponding retention time showed that isomers eluting under the middle peak disappeared in an enzymically catalyzed reaction. The radioactivity in this peak was decreased approximately 50%. In control reaction mixtures nonenzymic hydrolysis resulted in losses in radioactivity which were roughly equivalent for each peak (data not shown).

Hydrolysis of α-Naphthyl Acetate. α-Naphthyl acetate hydrolysis activity was found in all enzyme preparations and was readily demonstrated spectrophotometrically. The A at 320 nm of the product α-naphthol was proportional to concentration.

Esterase activity was maximal at pH 9.0, but was routinely assayed at pH 7.15 in either 0.05 M Tris or K-phosphate since this pH was used for IAInos hydrolase activity. The appearance of product was linear over the course of the reaction for the time interval measured, and the rate was proportional to the amount of extract added to the reaction mixture. Boiled enzyme was inactive.

Hydrolytic Activities in Coleoptiles as Compared to Mesocotyls. Extracts from mesocotyls were compared to those from coleoptiles with regard to the hydrolysis of [^{3}H]IAInos and α-naphthyl acetate. The results, summarized in Table I, show that extraction of coleoptiles yields more IAInos hydrolyzing activity. The specific activity is greater as is the activity per coleoptile; activity in a coleoptile is about 4 times that in a mesocotyl. The specific activity for α-naphthyl acetate hydrolysis is the same in coleoptiles and mesocotyls, but activity on a per plant part basis is 2.5 times greater for the coleoptile.

Hydroxylapatite Chromatography. After $(NH_4)_2SO_4$ precipitation, pelleted protein was redissolved in 10 mM K-phosphate buffer, pH 7.15, and was chromatographed over hydroxylapatite as described in "Materials and Methods." Following the elution of a peak of unbound protein from 25 to 42 ml, fraction 1, protein remaining bound to the hydroxylapatite was eluted with a gradient of K-phosphate, pH 7.15, of increasing molarity (0.01–0.5 M in 200 ml). A second major protein peak, fraction 2 eluted between 78 and 106 ml.

Fractions under each peak were pooled and the protein concentrated with $(NH_4)_2SO_4$, then resuspended in 0.05 M Tris, pH 7.15. The protein in fraction 1 enzymically hydrolyzed α-naphthyl acetate at rates greater than those of the similarly treated fraction 2 protein (452 mol·min^{-1}·mg protein^{-1} as compared to 51 nmol·min^{-1}·mg protein respectively). Both fractions were also active in the hydrolysis of the methyl esters of IAA and naphthylene acetic acid (data not shown).

No [^{3}H]IAInos hydrolytic activity of fraction 1 protein was ever detected. This is in contrast to the protein in fraction 2 which enzymically hydrolyzed both [^{3}H]IAInos and α-naphthyl acetate.

DISCUSSION

Results presented in this paper show that it is possible to prepare extracts from shoots of *Z. mays* which catalyze the hydrolysis of [^{3}H]IAInos to yield [^{3}H]IAA. Our enzyme preparations catalyzed the reaction at rates which were proportional with time and with the amount of extract. Activity was lost when extracts were boiled or mixed with 2-propanol.

[^{3}H]IAInos was chosen as the substrate for *in vitro* analysis of IAA ester hydrolytic activity because: (a) IAInos is present in endosperm (16) and in shoots of 4 d dark grown *Z. mays* seedlings (5); (b) IAInos constitutes almost 20% of the ester pool in both endosperm (10) and vegetative tissue (5); (c) IAInos is transported from seed to shoot in quantities sufficient to support normal elongation growth in a young corn shoot (18); and (d) IAInos was available in substrate amounts and as a radioactive compound prepared by both enzymic (17) and chemical (19) syntheses.

Although the rates of [^{3}H]IAInos hydrolysis that we were able to measure in our extracts were low (less than 1 pmol·h^{-1}·mg^{-1} protein), in part perhaps because substrate concentration in routine assays was less than optimal, an enzyme specifically hydrolyzing IAInos would not be expected to be a very abundant protein as only μM amounts of IAA are required for growth. Three kinds of data suggest that the observed *in vitro* activity has relevance to *in vivo* hydrolysis. First, the extracts preferentially utilized one, or possibly some, of the 6 possible stereoisomers of IAInos and it could be argued that such specificity would be unexpected of a general esterase. Second, the IAInos hydrolyzing activity we have studied is present in greater amounts in the coleoptile tissue, and this is where physiological data would suggest hydrolysis occurs. Third, not all of the many esterases present in maize vegetative tissue can hydrolyze IAInos, and thus the IAInos hydrolyzing fraction resolved by hydroxylapatite chromatography appears distinct in that it has this additional capability. However, since the rate of α-naphthyl acetate hydrolysis exceeds rates observed for the hydrolysis of IAInos in this fraction, further purification is required to determine if α-naphthyl acetate hydrolyzing activity can be resolved from IAInos hydrolyzing activity. It is significant however, that some esterases present in *Z. mays* lack the more specific activity towards IAInos, for this reinforces the hypothesis that esters of IAA such as IAInos play an important role in regulating the level of free IAA available to a growing seedling.

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