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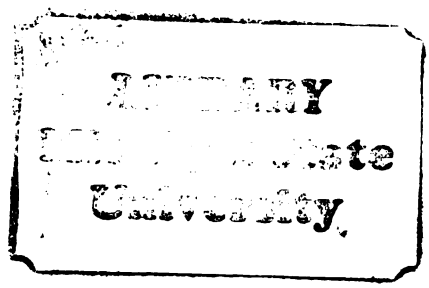

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THE IDENTIFICATION AND CHARACTERIZATION OF INDOLE-3-ACETYL-MYO-INOSITOL HYDROLASE FROM VEGETATIVE TISSUE OF ZEA MAYS L.

presented by

Prudence J. Hall

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Robert S. Bandurski
Major professor

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THE IDENTIFICATION AND CHARACTERIZATION OF INDOLE-3-ACETYL-MYO-INOSITOL HYDROLASE FROM VEGETATIVE TISSUE OF ZEA MAYS L.

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Prudence J. Hall

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ABSTRACT

THE IDENTIFICATION AND CHARACTERIZATION OF INDOLE-3-ACETYL-MYO-INOSITOL HYDROLASE FROM VEGETATIVE TISSUE OF ZEA MAYS L.

By

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The presence of conjugates of the plant growth hormone indole-3-acetic acid (IAA) in vegetative tissue of plants in excess of the free acid is postulated to play a role in maintenance of endogenous hormone levels. In shoots of Zea mays L. seedlings, esters of IAA predominate and it is believed that these esters are enzymatically hydrolyzed to provide free IAA to the growing shoot. One ester has been isolated from corn shoots and was identified as indole-3-acetyl-myo-inositol (IAInos). This dissertation describes the partial purification and characterization of an enzyme from corn shoots which hydrolyzes myo-inositol esters of IAA.

The enzyme was partially purified by column chromatography using ω-aminohexyl agarose, hydroxylapatite, and Sephadex G-100. In addition to the hydrolysis of IAInos, the enzyme preparation hydrolyzed the methyl esters of IAA and naphthyleneacetic acid, and the synthetic ester, α-naphthyl acetate. The rate of enzymatic hydrolysis was unaffected by added cofactors, sulfhydryl reagents, and metal cations, except for Mn^{2+} which was somewhat inhibitory. Glycerol appeared to inhibit the appearance of free IAA, but other experiments demonstrated the possibility of a transesterification reaction.

The hydrolysis of IAInos can be demonstrated in extracts from seeds and roots in addition to shoots. Additional data is presented

demonstrating the localization of enzymatic activity in the coleoptilar portion of the shoot. A calculation based on published data showed that the relative amount of esterified IAA in coleoptiles as opposed to mesocotyls parallels the distribution of enzymatic activty.

IAA esterified to myo-inositol is present in substrate preparations as chemically and biologically resolvable isomers. Examination of substrate in reaction mixtures before and after enzymatic hydrolysis demonstrated that the chemically resolvable isomers disappeared at different rates. This leads to the suggestion that the endogenous rate of hydrolysis of IAInos is controlled in part by the abundance of some isomers relative to others.

While the presence of hormone conjugates in plant tissues is recognized, and many studies have been published on their characterization and synthesis, the data presented in this dissertation constitutes the first report of an enzyme hydrolyzing a conjugate of IAA.

To George T. Jones, Professor of Botany, Oberlin College

and to Rich, Ethan, Eric and Kitty

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LIST OF ABBREVIATIONS

BA	benzoic acid
BSA	bovine serum albumin
D	Daltons
DEAE	diethylaminoethyl
EDTA	ethylenediaminetetraacetic acid
GLC	gas liquid chromatography
HPLC	high performance liquid chromatography
IAA	indole-3-acetic acid
IAInos	indole-3-acetyl-*myo*-inositol
Kphos	potassium phosphate buffer
Me-BA	benzoic acid, methyl ester
Me-IAA	indole-3-acetic acid, methyl ester
Me-NAA	naphthylene acetic acid, methyl ester
Me-PAA	phenylacetic acid, methyl ester
NAA	naphthyleneacetic acid
PAA	phenylacetic acid
TLC	thin layer chromatography
Tris	Tris (tris(hydroxymethyl)aminomethane) chloride buffer

GENERAL INTRODUCTION AND LITERATURE REVIEW

Indole-3-acetic acid (IAA) is a growth regulating hormone in plants. Many higher plants, during early non-photosynthetic growth, may be considered to be limited in their growth by IAA since removing the plant's source of IAA causes a cessation of growth while supplying additional IAA increases the rate of growth (62). Curiously, plants in which growth is limited by IAA (in the sense that exogenous IAA will increase the rate of growth) contain large amounts of IAA, but most of the IAA is in a covalently conjugated, and apparently, inactive form (4). In this situation the rate of conjugate hydrolysis may well determine the rate of plant growth. Most of the IAA in seedlings of Zea mays is present as esterified IAA; some is present as myo-inositol esters. The corn seedling thus provides an opportunity to study the enzymology of hydrolysis of an IAA conjugate. The purpose of this dissertation is to describe the enzyme which hydrolyzes the myo-inositol esters of IAA in vegetative tissue of Z. mays.

Three projects in the laboratory of Robert S. Bandurski made this work possible. First, Lech Michalczuk demonstrated the synthesis of indolyl-3-acetyl-myo-inositol (IAInos) by enzymes in the endosperm of developing Z. mays kernels (40). Michalczuk and John Chisnell (42) extended this work by biologically synthesizing radioactive IAInos in

quantities sufficient for use in other experiments. Second, Janusz Nowacki and Jerry Cohen chemically synthesized IAInos (45). Third, John Chisnell identified IAInos as an endogenous IAA ester in shoots of Z. mays (12).

At the outset of this work the overriding questions were:

1) Can enzymatic hydrolysis of IAInos be demonstrated in extracts from Z. mays vegetative tissue?
2) Will this enzyme hydrolyze other ester substrates, most notably other IAA esters, esters of IAA analogues, and artificial esters?
3) Is this enzyme an esterase? If so, will any esterase be able to hydrolyze IAInos? Is there only one enzyme which hydrolyzes IAInos?
4) Is this enzymatic activity localized within the plant?
5) What controls the activity of the enzyme? Can we observe changes in activity of the enzyme in response to perturbations in the environment?

The roles of endogenous and exogenous esters of IAA in plant tissues are first discussed followed by a brief discussion of esterases - why plant biologists have been interested in them, and some things that have been learned about them. The main body of the dissertation is composed of two papers to be submitted for publication: (1) Partial Purification of IAA-myo-inositol Hydrolase from Zea mays L. Vegetative Tissue, and (2) Characterization of IAA-myo-inositol Hydrolase as Isolated from Zea mays L. Vegetative Tissue. A discussion section follows in which some problems encountered during the course of these studies are raised, and some suggestions for their solution are offered. Results of experiments are discussed and interpreted with respect to the questions asked at the

outset. Appendices are included which 1) report the results of experiments too incomplete to be included in the main text, and 2) provide detailed experimental procedures for the benefit of others interested in continuing these studies.

Conjugates of IAA: roles and metabolism

Why are plant physiologists interested in hydrolysis of conjugates of IAA?

The chemical identification of IAA as an auxin was published by Kogl *et al* in 1934 (36). The source of the IAA was urine, but this "heteroauxin" was active in promoting growth in plant tissues. "Heteroauxin" was isolated by Thimann (59) from *Rhizopus suinus* and it too was chemically identified as IAA.

At about the same time several investigators, in the interests of increasing yields of IAA, tried different methods for extracting auxins from plant tissues. Alkali extraction was found to increase yields of auxin as much as 20 fold. In 1941 Hatcher and Gregory (32) reported that a maximal amount of auxin could be extracted with water or by diffusion into agar from developing grains of rye 5 to 6 weeks after anthesis, and that no auxin activity could be extracted from the dry grain. The same year Avery *et al* (1) published an extensive report concerning the increased amount of auxin that could be extracted from corn using water at pH 9 to 10. These workers (1) made the distinction between free auxin and an auxin precursor and speculated about the nature of the auxin precursor. The ease of releasing "free" auxin from the precursor by alkaline hydrolysis suggested to them that the precursor was an ester. They hesitated, however, to definitely class it as such because the

precursor was insoluble in several organic solvents that a simple ester of IAA should be soluble in, it was inactive in growth tests whereas the methyl and ethyl esters of IAA were active. They were relatively certain it was not an amide. The success of Avery and co-workers in using alkaline extraction, however, prompted Hatcher to repeat his experiments. In 1943 Hatcher (31) reported that an inactive auxin derivative continued to accumulate in developing grains of rye even after free auxin was no longer detectable, and that alkaline extraction yielded up to 20 times as much active auxin as the grain ripened.

Meanwhile Haagen-Smit (27) established that the auxin released from a precursor in corn kernels was indeed IAA. He characterized the IAA primarily on the basis of its melting point and the fact that there was no change in the melting point when the IAA was mixed with authentic IAA. Berger and Avery (8) confirmed the identity of the auxin released from the precursor as IAA.

At that time the primary method used to determine whether auxin was present was by bioassay. In 1935 Cholodny (13) described experiments in which he used sections of endosperm from Avena kernels to induce negative curvature in Avena coleoptiles, that is, the coleoptiles grew faster on the side to which the block of endosperm was applied. He sliced dry, unimbibed endosperm, moistened it and placed it on the side of a decapitated, etiolated Avena coleoptile. A negative curvature of 30 to 40° developed in 1.5 h at 20 to 21°C. On the basis of two experiments Cholodny ruled out the possibility that free auxin present in the endosperm caused the curvature. First, he showed that dry seeds lost growth promoting ability when heated to 100°C. He knew that auxin was heat stable, so he concluded that whatever growth promoter was present in

the endosperm was not auxin in its usual form. Second, he showed that no growth promoting substance was extracted by 96% ethanol, as auxin would be. However, if seeds were soaked for 12 h, then extracted with water, auxin activity was found in the water extract. Furthermore, the substance in the water was alcohol and ether soluble, as auxin would be. So seeds which had begun to germinate could produce the growth promoting substance. Germination was not required, however, as seeds in which the embryo had been killed could still induce curvature. His basic observations were that some compound in the endosperm could induce curvature if moistened. The compound was not itself auxin, but auxin seemed to come from it. Cholodny concluded that the Avena endosperm hormone is liberated from a precursor by hydrolyzing enzymes (13, p. 298). He further suggested that since the endosperm was a rich source of auxin precursor, that it should be possible to determine the structure of the growth hormone (by chemical means) becuase there was so much of it in the endosperm (13, p. 306).

Although Cholodny's work did not receive the attention it deserved at the time (19), Cholodny did set the stage for later studies of the hydrolysis of conjugates of IAA and the role of conjugates in hormone physiology. The realization that seeds contained large quantities of a precursor of this important hormone was to have repercussions in the study of IAA effects for years.

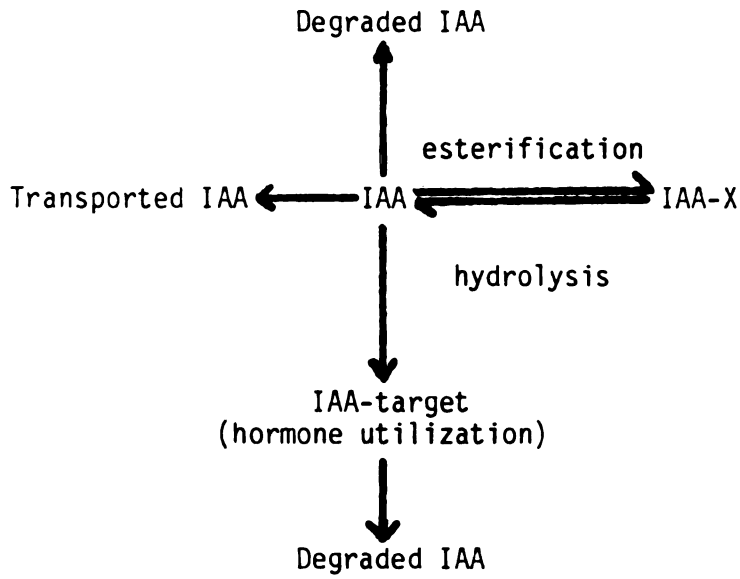
Interest in esters of auxins and auxin analogs had already emerged in the mid-1930's. Zimmerman and his associates (63) at the Boyce Thompson Institute synthesized a series of esters of IAA, naphthalene acetic acid (NAA), and phenylacetic acid (PAA) among others, and studied their effectiveness when applied in lanolin in causing bending in tomato

shoots and root initiation in Kalanchoe and tomato. They also studied aerial roots in Cissus where auxin applied in the region of elongation would inhibit elongation, and promote bending and branching. The roots would bend towards the side of application which contrasted with the direction of bending observed in shoots. They studied the effects of auxins and their synthetic esters on tomato plants by injection into the stem or by uptake from the soil. The methyl and ethyl esters of IAA were consistently more effective in all test systems as compared to other auxin esters, and methyl IAA was more effective than ethyl IAA, which was in turn more effective than butyl IAA. The methyl esters of NAA and PAA were slightly less effective than their respective free acids. Avery et al (2) studied synthetic auxins using the Avena curvature assay. They concluded that IAA and indolebutyric acid and their derivatives, the methyl esters and the potassium salts, were more active than other analogs tested. As shown later by Sell et al (57) the methyl and ethyl esters of IAA were 100 times more effective than free IAA in stimulating parthenocarpy in tomatoes.

The search for endogenous esterified auxins began shortly thereafter (8,9) and continues until the present (12,33,49) although a catalog of IAA esters has been achieved only for Z. mays endosperm. After Hamilton et al (28) demonstrated that half of the esterified IAA was insoluble and the other half soluble in water, a series of papers by Bandurski and coworkers appeared which elegantly identified and characterized the water-soluble low molecular weight esters. Labarca (39) identified the IAInos and IAInos-arabinoside esters. Ueda (61) added IAInos-galactose to the list. Ehmann (20,21) studied IAA-glucose and di- and tri-IAInos.

At the present time our understanding of how the plant uses its conjugated auxin is incomplete, although the importance of its role is very apparent (3,4,5,14). From studies of esterified IAA in Z. mays endosperm and shoot tissue, the notion of conjugates of IAA playing a major role in a homeostatic mechanism for control of hormonal levels emerged (3,4,5,37).

This hypothesis proposes that IAA conjugates are formed and released in plant tissue as a way to control the levels of free hormone. The hypothesis rests in part on the assumption that the level of free hormone for a particular growth state is critical to the plant for integrating growth and development. This control is thought to work in the following way:



A steady state concentration of IAA is maintained in one part of the plant that is appropriate to the environment of that particular part. A change in the environment requiring an alteration in growth results in resetting the homeostatic mechanism such that a new appropriate level of IAA concentration is achieved. The major precursor of free hormone is the pool of conjugated hormone. To remove hormone from the pool of free hormone four routes are envisioned: a conjugate may be synthesized; the

hormone may be degraded; the hormone may be transported out of the tissue; or the hormone may be used in growth promotion.

A major cornerstone of the hypothesis is the observation that all plant tissues so far examined contain IAA in the conjugated state in excess of that in the free state. Bandurski and Schulze (6) examined the free and bound IAA of a number of different plant tissues and found no tissue which had more free IAA than bound. It also appeared that, except for Avena vegetative tissue in which an amide conjugate predominates, esterified IAA was the predominant bound form in the cereal grain plants.

Another important observation contributing to the hypothesis is that of Ueda and Bandurski (60). They measured the disappearance of IAA conjugates in germinating Z. mays kernels. The amount of free plus ester IAA recovered after alkaline hydrolysis of entire plants decreased during the first 96 h of germination from nearly 70 mg IAA/kg dry weight to about 7 mg/kg. The disappearance had also been studied by Corcuera (15). The use of the auxin reserves of the seed during germination again emphasized their importance in hormone balances.

Utilization of seed auxin reserves also implies that a mechanism exists for releasing free hormone from conjugates. The most obvious way for hydrolysis to occur in vivo is by enzymatic hydrolysis. In fact Kogl et al (36) had employed biological digestion as a way to obtain large amounts of IAA. Haagen-Smit et al (27) did similar experiments and found that they could obtain 6.9 mg IAA/kg wheat (dry kernels) as compared to 0.2 mg/kg by water extraction. Chymotrypsin at pH 7 only liberated 0.76 mg/kg.

Although enzymatic hydrolysis of IAA esters is implicated in the endosperm, the homeostatic hypothesis assumes operation of the homeostatic mechanism in all parts of the plant.

One system which readily lends itself to study of the homeostatic mechanism is the shoot of a dark grown Z. mays seedling. The shoot responds to environmental stimuli by altering its growth pattern. For example, a seedling laid on its side will grow so that the shoot bends and eventually grows vertically again (62). Or, a shoot exposed to a brief flash of bright light grows at a reduced rate (22). Two observations about hormone levels in shoot tissue greatly enhance the appeal of homeostasis as an explanation. First, IAA conjugates are present in vegetative tissues of the 4 day corn shoot (6,48) and second, the ratio of free IAA to esterified IAA changes in response to an environmental stimulus - photoinhibition of growth causes a reduction in free IAA and an increase in esterified IAA (7).

The enzymatic formation of esterified IAA in developing Z. mays kernels has been studied extensively and the enzymes synthesizing all the low molecular weight esters have been described. Michalczuk (40,41) showed that IAA-β-D-glucose is synthesized from IAA and UDP-glucose by one enzyme. A second enzyme catalyzes the acylation of myo-inositol resulting in the products IAInos and glucose. Corcuera et al (17) studied the enzymatic synthesis of IAInos-galactose from IAInos and UDP-galactose. Corcuera and Bandurski (16) then described the two step synthesis of IAInos arabinose from IAInos and UDP-xylose, presumably involving a 4'-epimerase. These reactions account for the synthesis of all the known low molecular weight esters of IAA in Z. mays.

The use of IAA esters by plants has also been studied. Besides studies described above, Seeley et al (56) used paper chromatography as a tool to demonstrate the hydrolysis of exogenous synthetic IAA esters. They knew that Me-IAA was active in the Avena extension assay and in the pea curvature assay. They floated segments of wheat shoots in solutions containing Me-IAA, then concentrated the solution and chromatographed an aliquot using n-butanol:ammonia:water as the solvent system. Two Ehrlich positive spots were observed, one at the Rf of Me-IAA, the other at the Rf of IAA. Other plants yielded different products. For instance, peas appeared to metabolize Me-IAA to IAA and indoleacetonitrile.

IAA amide conjugates have been shown to be active in several plant tissue culture systems by Hangarter et al (30). They felt the conjugates served as slow release forms of the hormone. Hangarter and Good (29) demonstrated metabolism of the conjugates by applying [1-^{14}C]IAA-L-alanine and [1-^{14}C]IAA-glycine to pea stem segments. IAA was presumably hydrolyzed from the conjugate, and they measured the release of $^{14}CO_2$ resulting from decarboxylation of IAA. Since the D-isomers of amino acids were not active, the decarboxylation was assumed to be enzymatic.

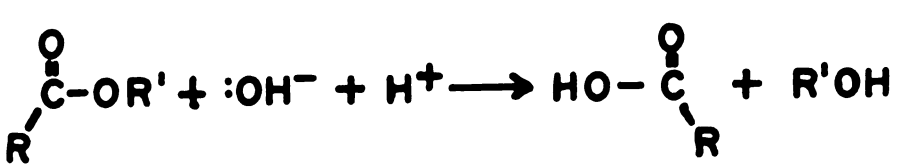
Hydrolysis of IAInos in the shoot was considered by Nowacki and Bandurski (44) in their study of transport of IAInos. If they applied ^{14}C-IAInos to the cut endosperm of a 4 day Z. mays seedling, they could reisolate radioactivity from the shoot. Of the radioactivity appearing in the shoot, 7% was free IAA, 56% was IAInos, 29% was in esters other than IAInos, and 8% was unaccounted for. The question was whether the ^{14}C-IAA recovered from the shoot arose from the hydrolysis of ^{14}C-IAInos in the endosperm or in the shoot. They knew how much ^{14}C-IAInos had been

applied to the endosperm, the pool size of IAInos and IAA, and the rate of IAInos hydrolysis in the endosperm. They reasoned that since nearly all of the IAA, free and esterified, occurs in the endosperm, that if hydrolysis of the ^{14}C-IAInos occurred in the endosperm, the resulting ^{14}C-IAA would be greatly diluted by endogenous IAA, and consequently any ^{14}C-IAA which was transported from the endosperm to the shoot would be virtually undetectable. They concluded that since they could detect labeled IAA in the shoot, hydrolysis of IAInos must have occurred in the shoot.

Esterases: activities and identification in plants

If the hydrolysis of IAInos is enzymatically catalyzed, what kind of enzyme might be expected to catalyze the reaction? Because the substrate is an ester of a carboxylic acid, catalysis by a carboxylic acid esterase (EC 3.1.1) is likely.

In general, the hydrolytic enzymes (EC 3) catalyze a nucleophilic displacement reaction in which a nucleophile forms a bond with an electron deficient carbon (or phosphorus) atom, displacing some other atom, such as oxygen, nitrogen or sulfur. The leaving group is the displaced atom plus its bonding electrons and anything else attached. When the electron deficient carbon is a carbonyl and the displaced atom is an oxygen, then the enzyme is an esterase. The reaction for ester hydrolysis can be diagrammed as follows:



The animal carboxyester hydrolases have been subdivided into various categories (38). There is at least some agreement that esterases might be subdivided on the basis of their catalytic activity in the presence of different organophosphorus inhibitors. The A-esterases hydrolyze aromatic esters and are not inhibited by diethyl p-nitrophenyl phosphate (Paraoxon) or diisopropylfluorophosphate (DFP). In fact, an A-esterase will hydrolyze Paraoxon to give p-nitrophenol and a diethyl phosphate anion. Many A-esterases are also stimulated by Ca^{2+} and are inhibited by chelating agents (43). The B-esterases or serine hydrolases have also been called the non-specific esterases. They are stoichiometrically inhibited by organophosphorus compounds without hydrolyzing them.

The role of serine at the active site of serine hydrolases allows a wide variety of enzymes to be classified as serine hydrolases. These enzymes include cholinesterases and chymotrypsin.

Esterases enjoy a wide distribution in animals and plants. Esterases have been reported in citrus fruits, carrots, radish seedlings, nearly all cereal grains, etc. One common way to establish the presence of esterases in a plant is to electrophoretically fractionate a crude extract in a starch gel, then assay the enzymatic activity using a simple ester such as α-naphthyl acetate as the substrate. If the assay is done in the presence of a diazonium salt, such as Fast Violet B, then the product of hydrolysis α-naphthol, reacts with the salt to form an azo compound at the site of the reaction, and a colored band results. In crude plant extracts the number of esterase bands can be overwhelming!

One early investigation of esterase activity in plants was reported by Jansen et al (35) who characterized a citrus acetylesterase with respect to several substrates. Schwartz, in 1960 (55), picked endosperm

of developing maize kernels as the system in which he could study mutant genes and their transmission by looking at the gene products. In addition to providing a material for genetic analysis which had a short generation time, in which mating could be controlled, and in which large numbers of progeny could be studied, maize was considered excellent for cytogenetic analysis. Schwartz used starch gel electrophoresis to separate esterases from crude (he squeezed the kernels with a pliers, then collected the exudate on filter paper wicks to apply to the gel) extracts. He stained for esterases using α-naphthyl acetate as the substrate and visualized the bands by coupling with Fast Blue RR. His findings opened up a new area of interest for plant biologists. His corn lines were highly inbred and well defined. Three forms of the enzyme could be found in his parental stocks - they were defined on the basis of migration rates towards the cathode. From crossing studies he found hybrids which showed both parental types of esterase plus a hybrid esterase which was intermediate in its migration rate. Not only had he devised a simple method to assay gene products, but he had also demonstrated some potentially interesting features about inheritance of specific gene products in plants. Although he said there was "no easily detectable phenotype alteration associated with these mutant genes" (55, p. 1212), he suggested the possibility that the formation of hybrid enzymes could be a factor in hybrid vigor. The study of plant isozymes exploded (51,52,53). Esterase isozyme variants are still being studied today (54; Q. Yi, personal communication). Unlike animal esterase isozyme studies, those in plants, that I am aware of, have not really added to any understanding of the role of esterases in growth and development. Although esterase isozyme studies in animal tissues also

concentrate on breeding and transmission of hybrid traits, some functions of the enzymes are known (11,25).

Not only did geneticists and plant breeders study esterases, but so did plant histochemists. For example, Gahan and McLean (24) wanted to determine the subcellular localization of esterases in Vicia faba root tips. Biochemical studies had shown that most hydrolytic activities were associated with the vaculoles in plant cells, but cytochemical studies had shown two locations for esterases, one in association with acid phosphatases, the other in particles adjacent to the cell wall. Gahan and McLean reported that esterase activity was located at several places intracellularly. They could only guess at the roles esterases might be playing. There are a few biochemical studies on non-specific esterases from plant cells (10,34) in which the esterases are characterized with respect to different artificial substrates.

Studies in which an esterase is characterized with respect to an endogenous substrate include that by Jaffe and coworkers who published a series of papers in which they described the isolation and characterization of cholinesterases from mung bean roots (23,50). Goodenough and Entwistle (26) isolated an esterase from Malus pumila fruit. The enzyme, purified to homogeneity, hydrolyzed a series of endogenous volatile esters. These esters had been observed to increase as fruits ripened and were thought to "enhance the appeal of the fruit to animals likely to eat it and thus distribute the seeds" (26, p. 145). The specific activity of the enzyme increased as the fruit increased in size.

Two papers (46,58) from Strack and coworkers are of special interest in studies of plant esterases. These workers looked at the hydrolysis of sinapine (sinapoylcholine), a naturally occurring ester in seeds of most

Brassicaceae. Sinapine is degraded during germination but it was not known if the degradation was catalyzed by non-specific esterases or by an esterase specific for sinapine. Strack et al (58) isolated enzyme activity from cotyledons of Raphanus sativus which turned out to be quite specific for sinapine, and was only weakly inhibited by the organophosphorus serine esterase inhibitors. Neostigmine, which Riov and Jaffe (53) reported to be specific for plant cholinesterases was also only weakly inhibitory. Strack concluded that sinapine esterase is not a cholinesterase and is not related to cholinesterase from Phaseolus aureus as described by Jaffe. Enzyme activity was localized in the cotyledons and increased during early germination. Esterase activity hydrolyzing indophenyl acetate was maximal in dry seeds; only trace amounts were hydrolyzed at 48 hr when sinapine hydrolysis was maximal. Zymograms of cotyledon extracts at various stages of germination showed several bands stained for α-naphthyl acetate esterase activity. None of these bands corresponded in location to the single band which stained for sinapine esterase activity.

The implication of Strack's experiments is that studying esterolytic activity may be far from straightforward. To further compound things, some other well-characterized enzymes also have esterolytic activity. Perhaps it is not too surprising that some proteolytic enzymes will hydrolyze esters of amino acids. For example, a protease from Staphylococcus aureus (18) hydrolyzes the phenyl ester of a peptide substrate.

A better studied example of a multi-functional enzyme is 3-phosphoglyceraldehyde dehydrogenase. Park et al (47) described the hydrolysis of p-nitrophenyl acetate by crystalline NAD-free enzyme. The

enzyme also catalyzed the oxidation of acetaldehyde to acetyl phosphate, the transfer of the acetyl group to inorganic phosphate, arsenate, Coenzyme A and other sulfhydryl acceptors, and the hydrolysis of acetyl phosphate to acetate and inorganic phosphate. A study of the mechanism of the latter reaction led to their study of esterase activity.

The literature reviewed in this section was selected for the purpose of laying the ground work for consideration of the experiments to be described in the next section. I hope I have convinced readers that an attempt to demonstrate the hydrolysis of IAInos was a worthwhile undertaking. Although its characteristics may, in the end, make it an atypical IAA ester, its existence in the shoots of corn seedlings is acknowledged. The two related questions - can any esterase hydrolyze IAInos (or, are there any that don't?) and, is IAInos hydrolase an esterase? - seem appropriate in view of what is known about esterases.

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EXPERIMENTAL I

PARTIAL PURIFICATION OF IAA-*MYO*-INOSITOL HYDROLASE FROM *ZEA MAYS* L. VEGETATIVE TISSUE

ABSTRACT

An enzyme which hydrolyzes IAA-myo-inositol (IAInos) was observed in extracts of shoots of dark grown seedlings of Zea mays L. This enzyme activity, which we call IAInos hydrolase, was partially purified by chromatography over ω-aminohexyl agarose, hydroxylapatite and Sephadex G-100, and has an apparent molecular weight of 45,000 D. The enzyme preparation can also hydrolyze α-naphthyl acetate. A second esterase was also partially purified from extracts of maize seedlings. This esterase could not catalyze the hydrolysis of IAInos but did hydrolyze α-naphthyl acetate. The hydrolysis of both IAInos and α-naphthyl acetate can be detected in extracts of roots and of germinating kernels as well as shoots.

INTRODUCTION

Esters of the plant growth hormone indole acetic acid (IAA) serve as one source of the hormone in both endosperm (10,31) and vegetative tissues of Zea mays (7). High concentrations of esterified IAA relative to that of the free acid have been observed in both vegetative and endosperm tissue (1,2). According to the hormonal homeostasis theory (8), hydrolysis of esterified IAA in any tissue releases free IAA which is then available to the plant as required for growth and response to environmental stimuli. This hypothesis predicts that an enzyme (or enzymes) will be present in vegetative tissue which catalyzes the hydrolysis of esters of IAA.

This paper reports the partial purification of an enzyme which catalyzes the hydrolysis of IAInos. A preliminary report has appeared (12). IAInos was chosen as substrate for in vitro analysis of IAA ester hydrolase activity for the following reasons. First, IAInos is present not only in the endosperm of Z. mays kernels (19) but is also found in shoots of 4 d dark grown Z. mays seedlings (6). Second, IAInos constitutes almost 20% of the ester pool of both endosperm (10) and vegetative tissue (6). Third, experiments measuring the transport of radioactive IAInos indicate that IAInos is transported from seed to shoot in quantities sufficient to support normal elongation growth in a young corn shoot (22). Finally, IAInos was available as a radioactive compound (20) and by chemical synthesis (23).

Plants contain many esterolytic enzymes (4,11,13,15,17,18,24,26, 29,30), but not much is known about esterase specificity. For example, it was not known whether any of the several esterases reported in Z. mays (28), would be able to hydrolyze IAInos. Very often esterases have been characterized on the basis of their ability to hydrolyze synthetic substrates (4,14,15,27). In our work the hydrolysis of a synthetic ester, α-naphthyl acetate, was studied to see if an enzyme fraction hydrolyzing IAInos was also active in the hydrolysis of a general esterase substrate, and, conversely, to identify other esterases which could then be examined for activity in the hydrolysis of IAInos. If all esterases were capable of catalyzing the hydrolysis of IAInos then the problem of how such activities are regulated would indeed be complex. If only one or a few enzymes catalyze IAInos hydrolysis, then it is more feasible to look at the mechanisms underlying regulation of the activity.

Our purification of esterase activities demonstrated that not every esterase has IAInos hydrolyzing activity. We report that at least one esterase can be partially purified from shoots of Z. mays seedlings, and that it cannot hydrolyze IAInos.

We also report that IAInos hydrolase activity is not unique to shoot vegetative tissue, but is also present in seed and root tissue.

This work is one of few reports in the plant biology literature (11,17,18,24,26,29,30) in which an esterase hydrolyzing an endogenous ester substrate has been purified and characterized and is to our knowledge, the first report of the partial purification of an enzyme catalyzing hydrolysis of an IAA conjugate.

MATERIALS AND METHODS

Chemicals

The following chemicals were obtained from commercial sources and were used without further purification: Fast Violet B, Coomassie Brilliant Blue R and ω-aminohexyl agarose were from Sigma; Bio-Gel HTP and bovine plasma gamma globulin from BioRad; Sephadex G-100 superfine from Pharmacia; ACS Scintillation cocktail from Amersham; and $(NH_4)_2SO_4$, enzyme grade from Mann Research Laboratories, Inc. [5-^{3}H]-indolyl-3-acetyl-myo-inositol (29 Ci/mmol) was synthesized as reported by Michalczuk and Chisnell (20) and was a gift from J. Chisnell. α-Naphthyl acetate was synthesized from α-naphthol and acetyl chloride in benzene (3) and recrystallized from 2-propanol.

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 of enzyme required, either sown in vermiculite in flats 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, an acetone powder was prepared from the shoots by grinding in a blendor in acetone (10 ml of acetone to 1 g tissue) chilled to -5 to -10°C with dry ice. The acetone was removed by filtration and the

residual powder rinsed with cold diethyl ether. The resulting powder was dried over P_2O_5 *in vacuo* and stored at -18°C. Root extracts were obtained by grinding primary roots in cold Tris. Liquid endosperm was collected in chilled Tris by nicking, then squeezing kernels of 4 d seedlings. An acetone powder made from whole kernels of 4 d seedlings was kindly provided by H. Nonhebel.

Buffers

Tris chloride (Tris) and potassium phosphate (Kphos), both at 0.05 M and pH 7.15 were used except as noted.

Protein Concentration

Protein was determined by the method of Bradford (3a) using bovine plasma gamma globulin as the standard.

Polyacrylamide Gel Electrophoresis

Electrophoresis was conducted on slab gels 2 mm thick using a discontinuous buffer system (25). The stacking gel (4.5% acrylamide) was in 0.75 M Tris, pH 7.0, and the separating gel (8% acrylamide) was in 3 M Tris, pH 8.9. The reservoir buffer was Tris-glycine (2.4 mM Tris, 24 mM glycine), pH 8.3 (9). Proteins were stained with 0.1% (w/v) Coomassie Brilliant Blue R in acetic acid:methanol:water (5:20:75) and destained in the same solvent.

Esterase Activity Staining

Following electrophoresis gels were rinsed with Tris. α-Naphthyl acetate (40 mg in 4 ml of 50% aqueous acetone) was added to a chilled

mixture of 200 ml of Tris containing 100 mg of Fast Violet B. After mixing the solution was poured over the drained gel and incubated at room temperature until bands of the desired intensity developed (approximately 10 to 20 min). In some cases the gel was soaked 20 to 30 min in 0.5 M boric acid prior to rinsing in Tris since this reduced background discoloration of the gel. After staining, the gels were rinsed with fresh Tris and photographed. Gels could be stored in the cold for 3 to 4 days, or for more permanent storage, gels were dried onto Whatman 3MM filter paper.

Assay of IAInos Hydrolase Activity

Reaction mixtures contained plant extract plus Tris or Kphos in a total volume of 100 µl in a 6 x 50 mm culture tube (Kimble). Five µl of ^{3}H-IAInos in 50% aqueous 2-propanol (6 pmol IAInos/5 µl, approximately 3 x 10^{5} dpm) was added and a 5 µl aliquot withdrawn immediately for counting. 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 non-enzymatic hydrolysis. Reaction mixtures were frozen if not analyzed immediately.

In early experiments the extent of hydrolysis was determined by chromatographing the reaction mixture over approximately 1.2 ml DEAE-Sephadex (acetate form in 50% aqueous 2-propanol) in a disposable Pasteur pipette. Unhydrolyzed ^{3}H-IAInos was not bound to the DEAE and was eluted with 4.0 ml of 50% aqueous 2-propanol. The product, ^{3}H-IAA, was then eluted with 5.0 ml of acidified (2N acetic acid) 50% aqueous 2-propanol. Aliquots of 0.5 ml from both the neutral and acidic fractions were used for determination of radioactivity in a Beckman LS

7000 liquid scintillation counter using ACS solution as the scintillant. In later experiments the reaction mixture was mixed with approximately 0.5 ml of DEAE-Sephadex in a 1.5 ml polypropylene tube (Cole-Parmer) and the unreacted substrate removed by a series of centrifugations in a Beckman Microfuge B. After each centrifugation the supernatant solution was removed for determination of radioactivity; the exchanger was mixed with 0.3 ml of 50% aqueous 2-propanol and the cycle repeated 6 times. The product, ^{3}H-IAA, was then removed by 5 cycles of centrifugation in a total volume of 1.5 ml acidified 50% aqueous 2-propanol. Recovery of radioactivity averaged 85 to 90% using either method.

Assay of α-Naphthyl Acetate Esterase Activity

The production of α-naphthol was followed by measuring the increase in absorbance at 320 nm. Reaction mixtures included buffered plant extracts and 4.3 μmol α-naphthyl acetate in a total volume of 3.0 ml. The mixture, excluding substrate, was incubated at 37°C in the specimen chamber of a Cary 15 Dual Beam Recording Spectrophotometer for 5 min. After addition of substrate, the reaction was incubated and observed for at least 8 min. The rate of hydrolysis was determined by measuring the increase in absorbance for the first 5 min of the reaction.

The molar extinction coefficient of α-naphthol in this reaction mixture was determined to be 2496 L/mol/cm. This agrees with the value reported earlier by Johnson and Ashford (16). Rates of hydrolysis are reported as nmol α-naphthol produced/min/mg protein.

RESULTS

Purification

The following steps were used in the purification of IAInos hydrolase and were conducted at 4°C.

Step 1. Preparation of crude extract. Ten g of acetone powder was suspended in 150 ml of Tris with gentle stirring for 30 min. Particulate matter was removed by centrifugation at 12000 g for 10 min, the resulting pellet resuspended in the same volume of fresh buffer and extracted an additional 30 min. Following centrifugation, the supernatant fluids were combined and designated Stage I enzyme.

Step 2. Ammonium sulfate precipitation. Solid ammonium sulfate was added to Stage I enzyme to 90% saturation, the mixture stirred 45 to 60 min, then centrifuged at 12000 g for 15 min. The pelleted protein was dissolved in 60 ml Kphos. The protein solution was dialyzed against 2 changes of the same buffer for a total of 3 to 4 h. The resulting extract, Stage II enzyme, could be frozen and would retain hydrolase activity for as long as 1 year. Upon thawing a precipitate formed which could be removed by brief centrifugation with no loss of activity.

Step 3. Hydrophobic interaction chromatography. Clarified Stage II enzyme was chromatographed over an ω-aminohexyl agarose column equilibrated with Kphos. The column was washed with the same buffer until protein was no longer detected by absorbance at 280 nm in the effluent. Protein bound to the column was eluted with the starting

buffer containing 0.5 M NaCl. The column was regenerated by washing with the starting buffer.

Most of the IAInos hydrolase activity was not retained on the column, and appeared in the first protein peak (Figure 1). Approximately 70% of the IAInos hydrolase activity applied was recovered between 11 and 40 ml. These fractions were pooled and ammonium sulfate added to 90% saturation. Precipitated protein was resuspended in 35 ml 0.01 M Kphos, pH 7.15, dialyzed against 2 changes of the same buffer and frozen. Hydrolase in the frozen suspension remained active at least 2 months. After thawing, a precipitate was observed which was removed by centrifugation with no loss of activity. This fraction was designated Stage III enzyme.

Step 4. Hydroxylapatite chromatography. Thirty ml of clarified Stage III enzyme was chromatographed over hydroxylapatite prepared in 0.01 M Kphos. Unbound protein was eluted with the same buffer until the absorbance of the eluate at 280 nm decreased to less than 0.1; protein bound to the column was eluted with a gradient of Kphos from 0.01 M to 0.5 M, pH 7.15. IAInos hydrolyzing activity eluted after the gradient was applied. Tubes containing hydrolase activity were pooled and stored at -18°C. This fraction was designated Stage IV enzyme. When the column was overloaded, some enzyme activity appeared tailing the peak of unbound protein. The purification by hydroxylapatite chromatography was only 1 to 3 fold, and the recovery of total activity was about 50%. The unbound protein fractions were active in the hydrolysis of α-naphthyl acetate. These fractions were pooled as above, and were designated as Stage IVE enzyme.

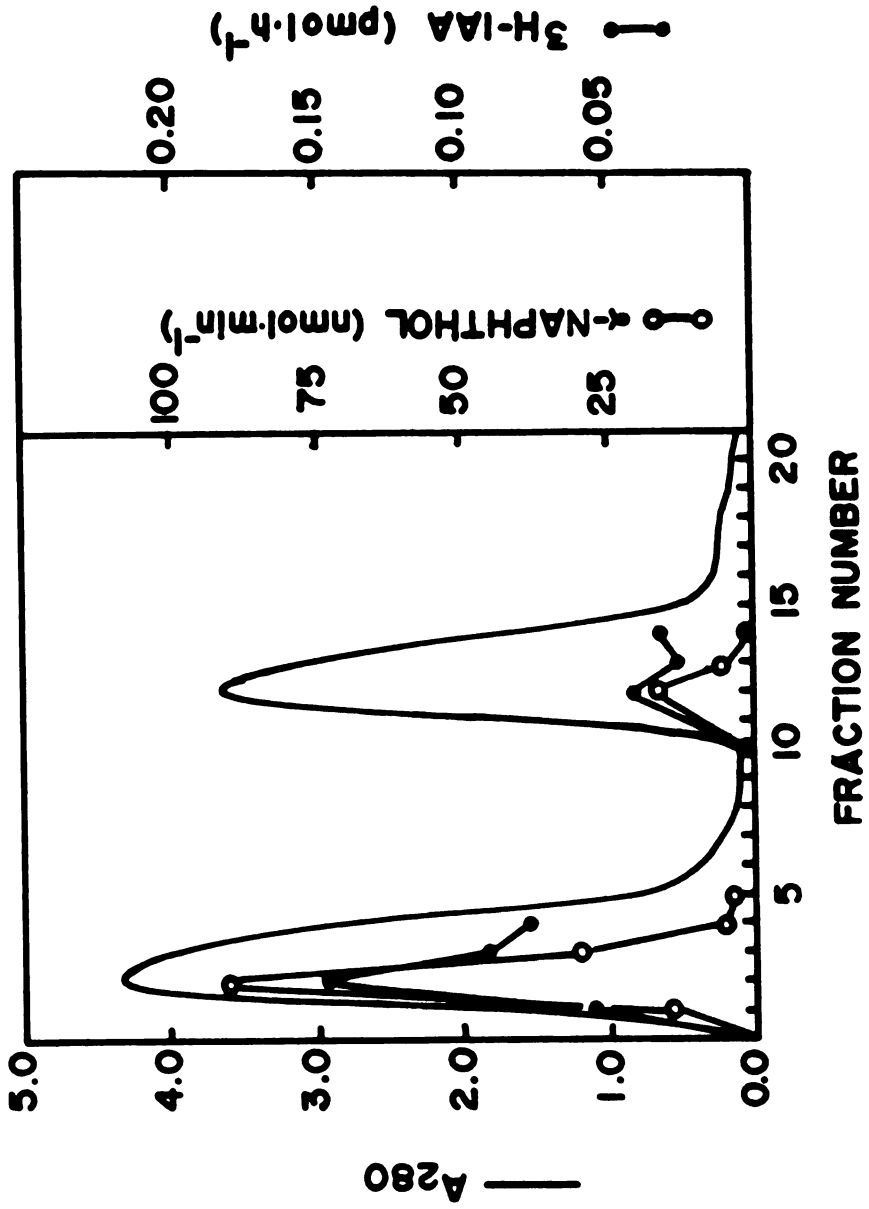

Figure 1. Hydrophobic interaction chromatography of Stage II enzyme. The elution pattern of enzymatic activities of Stage II enzyme from ω-aminohexyl agarose column (column size, 1.8 x 5.2 cm; flow rate 0.75 ml/min; fraction size, 10 ml/fraction) is shown. At fraction 10 protein bound to the column was eluted with 0.5M NaCl in Kphos buffer. Fractions were assayed for hydrolase (●) and esterase (o) activity as indicated, using 0.1 ml extract/assay. Fractions 2 to 4 were pooled for further purification.

Step 5. Gel filtration chromatography. Stage IV and Stage IVE enzyme preparations could be further fractionated by chromatography over Sephadex G-100. Chromatography of approximately 12 g of protein of Stage IV enzyme over Sephadex G-100 (column size 1.6 x 33 cm, flow rate 10 ml/h, 1 ml/fraction) resolved the mixture as shown in Figure 2. Fractions between 26 and 32 ml were pooled and stored at -18°C for up to one month with no loss of activity. This fraction was designated IAInos hydrolase.

Stage IVE enzyme was concentrated by precipitation with $(NH_4)_2SO_4$ and 15 mg was chromatographed over Sephadex G-100 (column size 1.5 x 23 cm, flow rate 10 ml/h, 1 ml/fraction). Figure 3 shows the protein and esterase activity elution profiles. Fractions 10 to 25 were pooled and stored at -18°C. This preparation was designated as estersae. A portion of this esterase was rechromatographed over a calibrated Sephadex G-100 column and was used in some experiments. It is designated esterase R.

A flow chart for the purification of IAInos hydrolase and esterase activities is shown in Figure 4. Table 1 summarizes the purification of IAInos hydrolase and Table 2 summarizes the esterase purification.

Polyacrylamide Gel Electrophoresis

Duplicate samples from different stages of purification were separated electrophoretically. The gel was divided in two and one portion stained with Coomassie Blue, the other portion with α-naphthyl acetate in the presence of Fast Violet B. In this way the mobilities of bands of protein could be compared with mobilities of bands of esterase activity. Prior to chromatography on hydroxylapatite there are up to 12 protein and 8 esterase bands. Not each of the 8 esterase bands has a

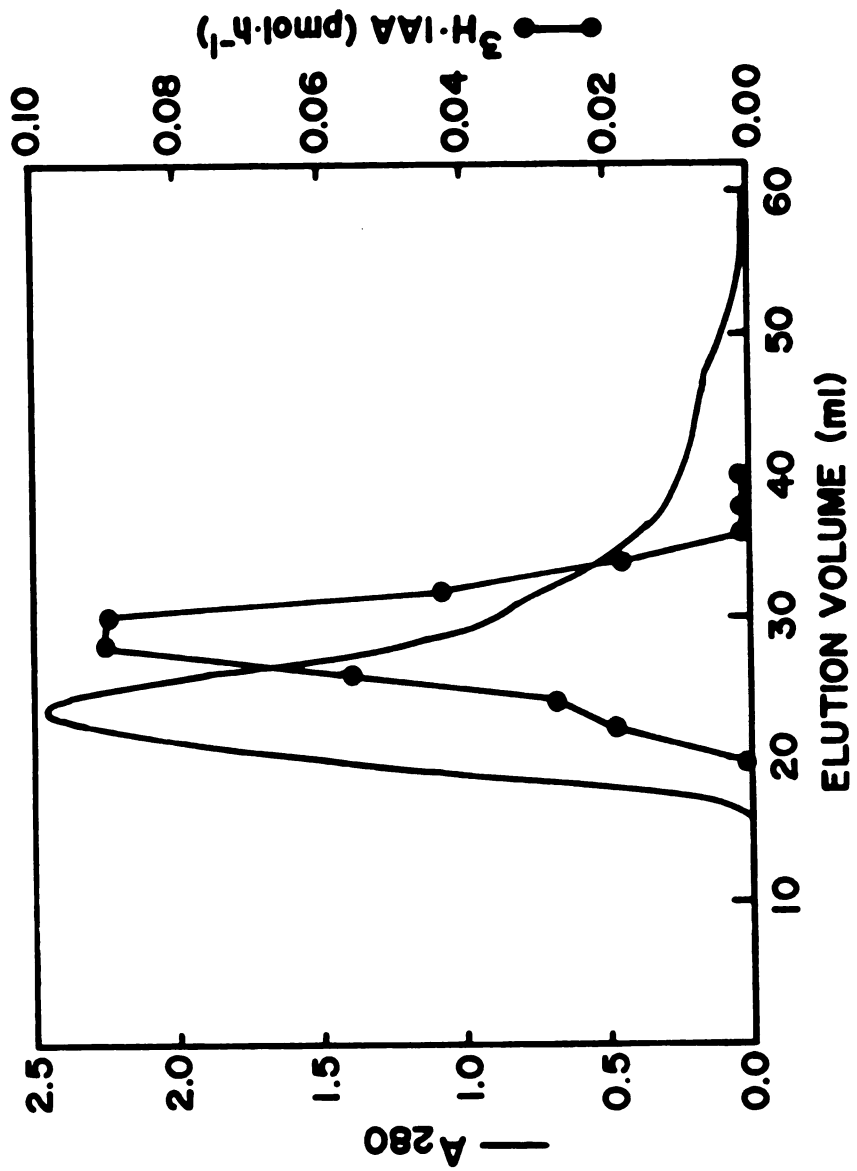

Figure 2. Gel filtration chromatography of Stage IV enzyme. The elution pattern of IAInos hydrolase activity of Stage IV enzyme from Sephadex G-100 (column size 1.6 x 33 cm; flow rate 10 ml/h, 1 ml/fraction) is shown. Fractions were assayed for ^{3}H-IAInos hydrolysis as indicated. Fractions 26-32 were pooled for further experiments.

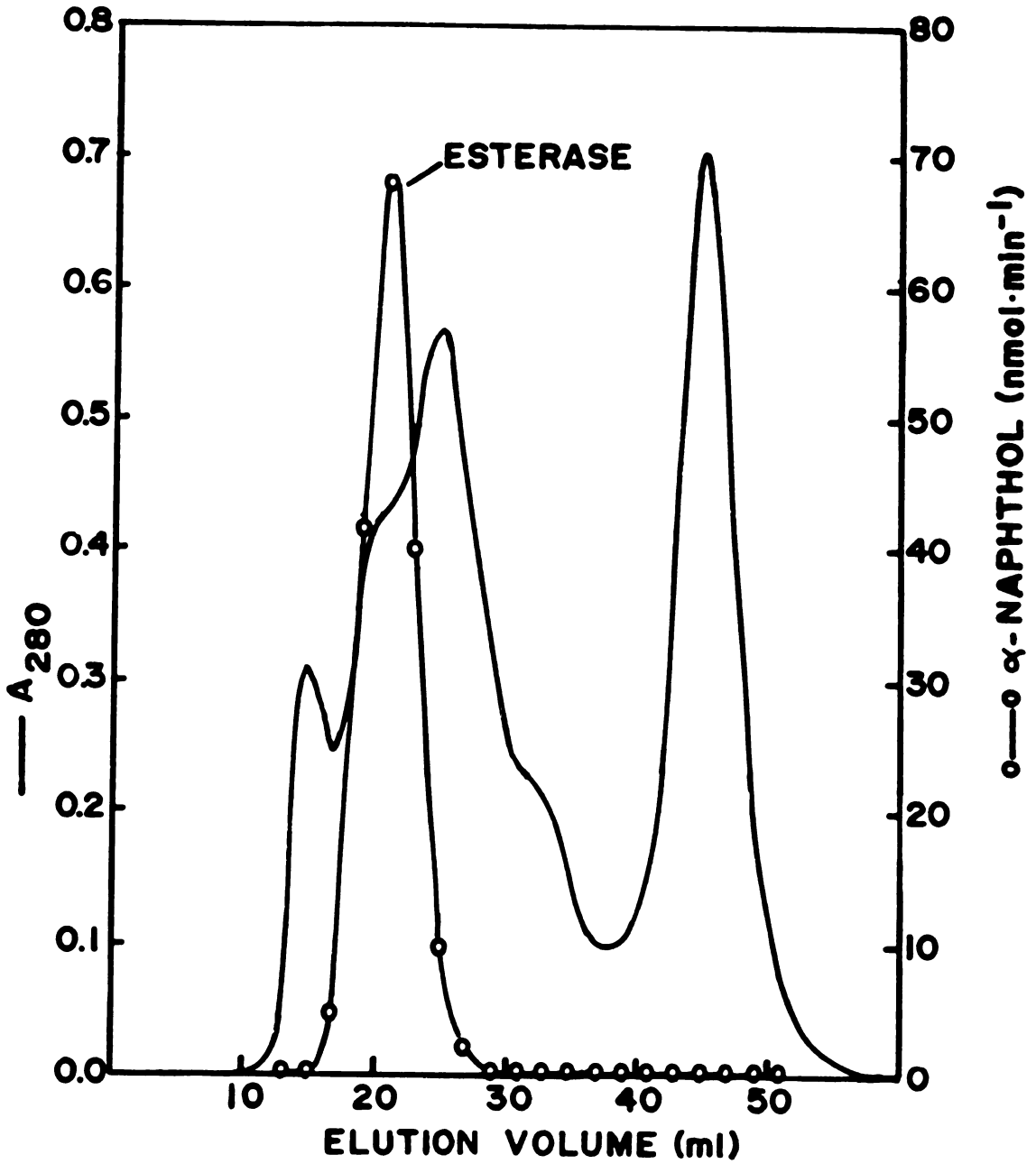

Figure 3. Gel filtration chromatography of Stage IVE enzyme. The elution pattern of Stage IVE esterase from Sephadex G-100 column (column size 1.5 x 23 cm, flow rate 10 ml/h, 1 ml/fraction) is shown. Fractions were assayed for α-naphthyl acetate hydrolysis as indicated. Fractions 10-25 were pooled for further experiments.

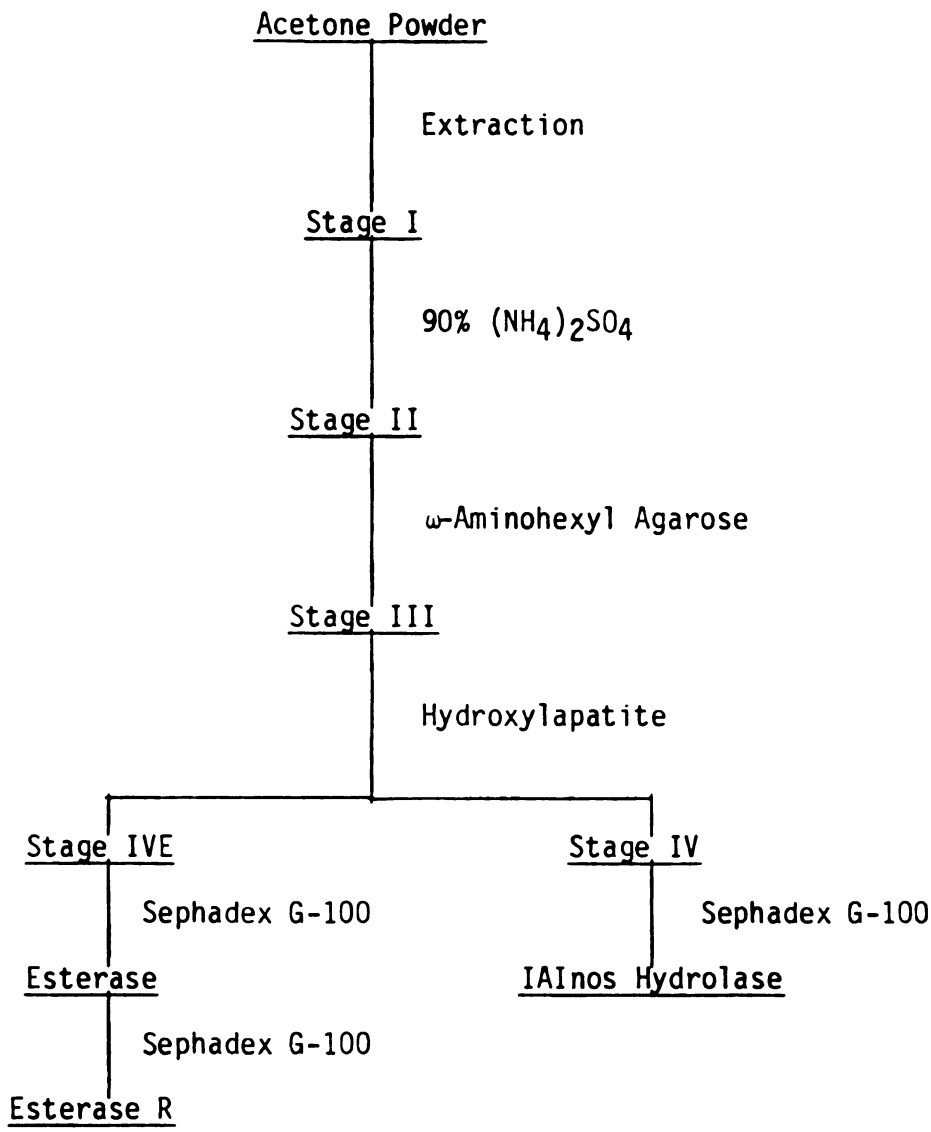

Figure 4. Procedures for the partial purification of esterase and IAInos hydrolysis. This flow chart shows the procedures used to partially purify the esterase and IAInos hydrolase as described in the text.

TABLE 1. Purification of IAInos hydrolase.

Fraction	Treatment	Volume (ml)	Total Protein (mg)	Specific Activity[a]	Total Units of Activity[b]	Purification[c]	Recovery[d] (%)
I	Acetone Powder	224	1994	0.098	195	1	100
II	90% $(NH_4)_2SO_4$	61	1445	0.070	101	1	52
III	ω-Aminohexyl agarose	35	570	0.155	88	1.6	45
IV	Hydroxylapatite	58	203	0.138	28	1.4	14
IAInos Hydrolase	Sephadex G-100	28	96	0.380	37	3.9	19

[a]Units of activity - pmol/h/mg protein; assayed in the presence of 6 pmol ^{3}H-IAInos.
[b]Specific Activity X Total Protein.
[c]Specific Activity ÷ Specific Activity of Stage I enzyme.
[d]Total Units of Activity ÷ Total Units of Activity of Stage I enzyme.

TABLE 2. Purification of esterase.

Fraction	Treatment	Volume (ml)	Total Protein (mg)	Specific Activity[a]	Total Units of Activity[b]	Purification[c]	Recovery[d] (%)
I	Acetone Powder	224	1994	70	139,580	1	100
II	90% $(NH_4)_2SO_4$	61	1445	89	128,605	1.3	92
III	ω-Aminohexyl agarose	35	570	98	55,860	1.4	40
lVE	Hydroxylapatite	26	31	401	32,303	5.7	23
Esterase	Sephadex G-100	14	11	528	5,808	7.5	4
Esterase R	Sephadex G-100	22	6	600	3,600	8.6	3
IV	Hydroxylapatite	58	203	55	11,165	0.8	8
IAInos Hydrolase	Sephadex G-100	28	96	48	4,608	0.7	3

[a]Units of activity - nmol/h/mg protein; assayed in the presence of 4.3 μmol α-naphthyl acetate.
[b]Specific Activity X Total Protein.
[c]Specific Activity ÷ Specific Activity of Stage I enzyme.
[d]Total Units of Activity ÷ Total Units of Activity of Stage I enzyme.

corresponding protein band but the 5 major bands of esterase activity do have the same relative mobility as that of 5 bands of protein. After hydroxylapatite chromatography and gel filtration chromatography there are 5 bands with esterase activity which are associated with the hydrolase enzyme (Figure 5). Four of the bands are quite faint. The fifth is the only one for which a corresponding band of protein can be seen. Its relative mobility is approximately 0.17. The esterase enzyme has two distinct bands of esterase activity and corresponding protein bands can be detected. These two bands appear as very faint bands in the hydrolase preparation. The relative mobilities are 0.46 and 0.55.

Molecular Weight Estimates

The molecular weights of the esterase and the hydrolase were estimated by comparing their retention volumes with those of the standard proteins, chymotrypinsinogen A, ovalbumin, and bovine serum albumin, on Sephadex G-100. The hydrolase activity eluted at nearly the same elution volume as ovalbumin thus indicating a molecular weight of 45,000 D. The esterase eluted immediately preceding chymotrypsinogen 4, indicating a molecular weight of 27,000 D. Figure 6 shows the semi-logarithmic plot of molecular weight vs. relative retention volume used in making these estimates.

Hydrolytic Activities

The hydrolase fraction actively hydrolyzes ^{3}H-IAInos. Hydrolase activities of IAInos hydrolase at various stages of purification of shown in Table 1. That ^{3}H-IAA is a product of hydrolysis was demonstrated by co-chromatography of the radioactive product with unlabeled IAA on TLC

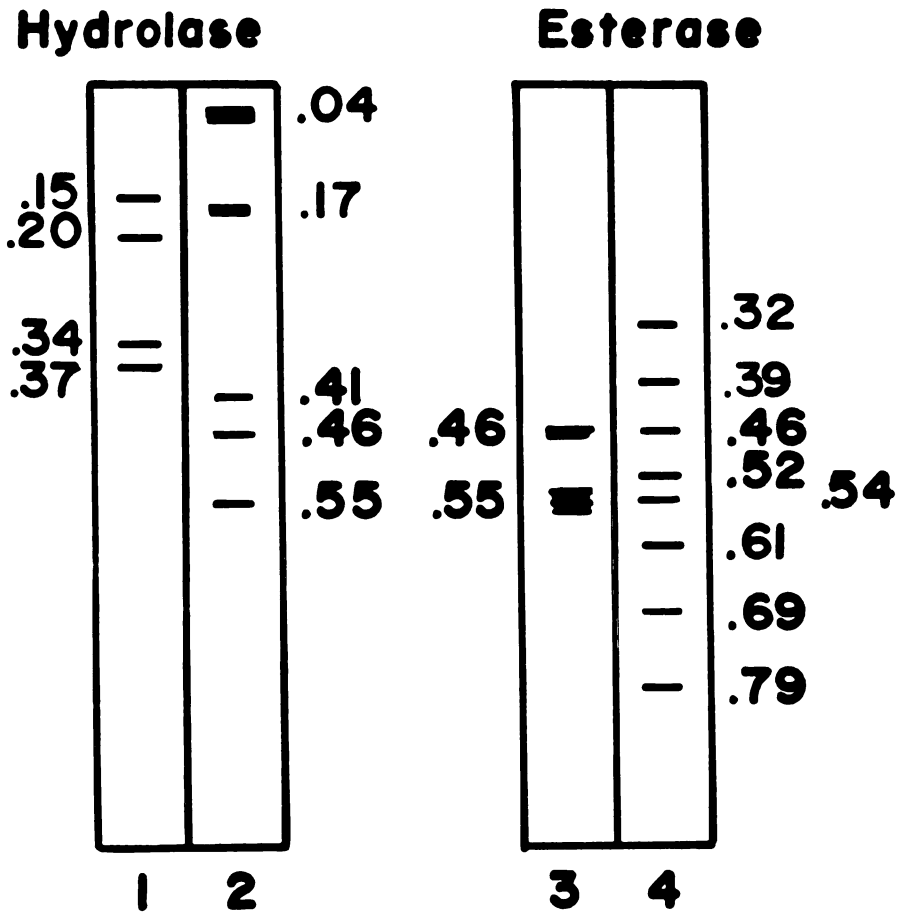

Figure 5. Gel electrophoresis of IAInos hydrolase and esterase. These tracings of polyacrylamide gels show the electrophoretic separation of proteins present in partially purified IAInos hydrolase and esterase preparations. Lanes 1 and 4 were stained with Coomassie Blue. Lanes 2 and 3 were stained for α-naphthyl acetate esterase activity. The numbers indicate the distance of migration of a particular protein relative to the dye front (the bottom of the gel).

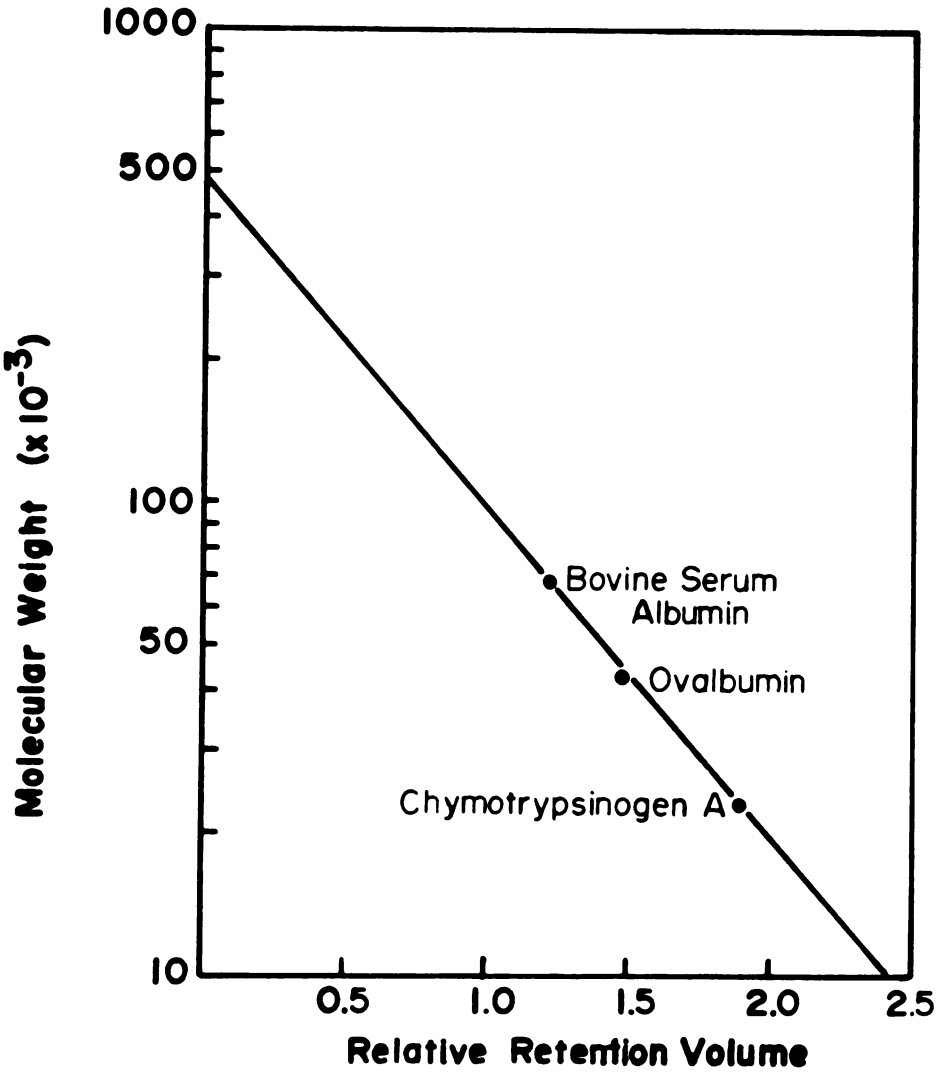

Figure 6. Calibration of Sephadex G-100 gel filtration column. The relative retention volume represents the retention volumes of the standard proteins to the retention volume of Blue Dextran.

and by HPLC over a Partisil 10-ODS column (data not shown). We are unable to measure any hydrolysis of ^{3}H-IAInos with the esterase.

The extent of IAInos hydrolysis is reported as the rate of appearance of IAA. Non-enzymatic hydrolysis was measured by incubating substrate in buffer alone. Reported rates of enzymatic hydrolysis have been corrected for non-enzymatic hydrolysis.

α-Naphthyl acetate esterase activity is present in all enzyme preparations at all stages of purification. This esterase activity is readily demonstrated spectrophotometrically and as bands on acrylamide gels. Table 2 summarizes esterase activities for the esterase and the hydrolase at various stages of purification.

Denaturation

Extracts which have been held at the temperature of boiling water for longer than 1.5 min fail to exhibit IAInos hydrolyzing activity. Very dilute (less than 50 μg/ml) protein solutions lost activity after freezing. The addition of 1 mg/ml BSA prevented this loss. Hydrolyzing activity is not observed when extracts are made 50% with respect to 2-propanol or when held at 0°C.

Other Sources of IAInos Hydrolase Activity

A Tris buffer extract of *Z. mays* roots had IAInos hydrolase activity. The activity remained in the supernatant solution after centrifugation. Following resuspension of the pelleted material and a second centrifugation, the pooled supernatant fractions were precipitated with $(NH_4)_2SO_4$ at 90% saturation. The recovered protein hydrolyzed

^{3}H-IAInos at about the same rate as stage I enzyme from shoot actone powders. Hydrolysis of α-naphthyl acetate was also similar.

Liquid endosperm obtained from kernels of 4 d seedlings exhibited only slight IAInos hydrolyzing activity. In contrast, an enzyme preparation from an acetone powder of kernels (endosperm plus seed coat) of 4 day seedlings hydrolyzed ^{3}H-IAInos at rates about twice that observed for stage I enzyme from shoot acetone powders. The hydrolysis of α-naphthyl acetate by this extract is comparable to that of shoot acetone powders. Activities of the various extracts are summarized in Table 3.

TABLE 3. Comparison of enzymatic activities from crude extracts of Z. mays.

Source of Enzyme	IAInos Hydrolyzed pmol/h/mg protein	α-Naphthyl Acetate Hydrolyzed nmol/min/mg protein
Stage I - acetone powder from shoots	0.098	70
Buffer extract of roots	0.090	65
Buffer suspension of liquid endosperm	-	64
Acetone powder from seeds	0.259	56
Buffer extract of shoots	0.100	20

DISCUSSION

The results presented here demonstrate that it is possible to partially purify an extract from acetone powders of Z. mays vegetative tissue which hydrolyzes ^{3}H-IAInos to yield ^{3}H-IAA. As indicated by Table 1 and Figure 5 the protein (or proteins) responsible for this activity has not been purified to homogeneity. An obvious consequence of this is that it is not possible to determine which band of protein on an acrylamide gel is the one responsible for the observed activity.

Extractions of shoot tissue into cold buffer with or without Triton X-100 yield fractions with hydrolase activity comparable to that of acetone powders, but activity was lost within a few days at 4°C or at -18°C. β-Mercaptoethanol at 10 mM did not affect stability and slightly decreased activity.

Several different purification steps were attempted. Fractionation with $(NH_4)_2SO_4$ yielded active fractions from 40 to 60% saturation. Losses in activity were excessively high; 7 to 8% of the total activity was recovered with only slight purification. Such high loss of activity often indicates that the mechanism and control of the enzymatic activity are poorly understood. Ion exchange chromatography on DEAE-Cellulose or DEAE-Sephadex using Kphos or Tris (at pH 8.0) gave no clear cut purification of hydrolase activity. It was hoped that preparative starch gel electrophoresis would yield a more homogeneous active fraction, however, recovery was too slight to make the technique useful.

The observation that IAInos hydrolase activity is present in root and seed extracts correlates with observations by Momonoki et al (21) that esterified IAA concentration decreases slightly in the root during germination, and with studies on the disappearance of esterified IAA from the endosperm of germinating corn kernels (31). The virtual absence of activity in liquid endosperm from 4 day germinating seedlings contrasts with high activities recovered from extracts of whole kernels at the same stage of germination. Studies of cereal seeds indicate that most hydrolytic enzymes originate in the aleurone layer, although some are associated with membrane bound bodies in the endosperm (5). Esterases occur in the endosperm (5,28), but it is not always clear that the extracts exclude seed coat layers. The disappearance of esters of IAA from the endosperm can result from two processes. First is the hydrolysis of conjugates in the endosperm. The second is the transport of conjugates from the endosperm to other parts of the seedling as has been reported by Chisnell and Bandurski (7) and by Nowacki and Bandurski (22).

Because we expected that rates of hydrolysis in vivo would be low, we chose high specific activity ^{3}H-IAInos as the substrate, for any product formed would be radioactive enough to make it detectable. At the low substrate levels used during purification the extent of hydrolysis ranged from 3 to 20% above controls in 4 h, depending on the protein concentration and the degree of purification. This is equivalent to several thousand dpm which is adequate for measurement. This assay is by necessity an endpoint assay; measurement of the amount of product formed is not straightforward nor easily accomplished. In addition, the substrate, IAInos, either radiolabeled or unlabeled, is available only in limited amounts.

The final yield of active protein, 5%, suggests that the total amount of protein in a 4 d corn shoot that is involved in this reaction is slight. At the same time losses of units of hydrolase activity are high. Recovered activity is only about 20% of that present in Stage I enzyme. If the final recovery of units of hydrolase activity had been 100%, then undoubtedly the recovery of protein would have been greater, perhaps as high as 26%. We would not expect the IAInos hydrolase to be a very abundant protein. In fact, it may be a very minor component in the mixture of proteins present after partial purification. If so, then our reported values for specific activity of the hydrolase are underestimated.

The partial purification of an enzyme with esterolytic activity but which does not hydrolyze IAInos is of interest to our study of IAInos hydrolase. We were concerned that hydrolysis of the inositol ester of IAA might be a property of any esterase and apparently it is not. Still to be resolved are the questions of whether or not one specific hydrolase is responsible for IAInos hydrolysis, and if the hydrolase is also an esterase.

The extent of purification of the esterase is only 8 to 9 fold as indicated by the specific activity for α-naphthyl acetate. However, if the esterase activity in crude fractions is the sum of activities of several enzymes, then the purification of this particular enzyme may, in fact, be much greater, because the specific activity in crude fractions would be overestimated. If protein hydrolyzing α-naphthyl acetate represents a very small proportion of the total protein, even in purified samples, the specific activity would be underestimated.

The observation of 2 bands of esterase activity on acrylamide gels with relative mobilities of 0.46 and 0.55 (Figure 5) prompted us to compare them with bands of esterase observed after electrophoresis of IAInos. Two of the five bands observed for IAInos hydrolase migrate the same distance as the two bands in the esterase; consequently, these two bands may be discounted as esterases capable of hydrolyzing IAInos.

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EXPERIMENTAL II

CHARACTERIZATION OF IAA-*MYO*-INOSITOL HYDROLASE AS ISOLATED FROM *ZEA MAYS* L. VEGETATIVE TISSUE

ABSTRACT

IAA-*myo*-inositol (IAInos) hydrolase activity, partially purified from 4 d dark grown *Zea mays* L. shoots, has been measured using ^{3}H-IAInos, the tritiated analog of an endogenous IAA ester. The activity was measured at pH 7.15 and was not stimulated by any of several potential effectors: cations, glutathione, and EDTA. Glycerol reduced the amount of ^{3}H-IAA released as product although it stimulated the hydrolysis of α-naphthyl acetate; it had no effect on the hydrolysis of the methyl ester of ^{14}C-IAA (Me-IAA). IAInos hydrolase activity is compared to other esterolytic activities for the same enzyme preparation. The esterolytic activities of IAInos hydrolase are contrasted with those of an esterase, partially purified from 4 d dark grown *Z. mays* shoots, which is unable to hydrolyze IAInos. Possible mechanisms for *in vivo* regulation of IAInos hydrolase activity are considered.

INTRODUCTION

This paper describes the characteristics of IAInos hydrolase activity as isolated from 4 d shoots of Zea mays (17). IAInos is an endogenous IAA ester in corn endosperm (21) and in vegetative tissue (6), and accounts for approximately 20% of the esterified IAA found in shoots (6). Previous studies from this laboratory have demonstrated the importance of esterified IAA in plant IAA biochemistry and physiology (8). Cohen and Bandurski (9) showed that conjugates such as IAInos are resistant to peroxidative attack. Studies on the turnover of IAA compounds in the endosperm of Z. mays (12,18) established esters as storage forms of the hormone. Esters of IAA are transported within the plant (7,25). Changes in the ratio of free to conjugated IAA in vegetative tissue occur during normal growth (23) and as a result of environmental perturbations (3,4).

Specifically we have looked for effectors that affect the rate of hydrolysis or the affinity of the enzyme for its substrate. We have asked if the enzyme hydrolyzing this reaction is specific or promiscuous with regards to substrate. Besides characterizing the enzymic activity with respect to pH, time of incubation, and substrate concentration, we investigated various compounds as positive effectors or as inhibitors. We have also studied other esters as substrates for hydrolase activity.

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 region of the mesocotyl. Accordingly, activity of IAInos hydrolase from mesocotyls as compared to coleoptiles, including primary leaves, was studied.

Esterification of IAA to myo-inositol means that there are possibly 6 biologically distinguishable isomers of IAInos. It is not known if any isomers predominate in vivo, but it is possible that a limited number serve as substrates for a hydrolytic enzyme, and this suggests one way for the plant to control the level of free hormone. Preliminary experiments are presented which suggest preferential hydrolysis of some of the 6 possible isomers of IAInos.

MATERIALS AND METHODS

Chemicals

[1-^{14}C]-indolyl-3-acetic acid, 59 mCi/mmol, was obtained from New England Nuclear. Other commercially obtained materials have been described previously (17). [5-^{3}H]-indolyl-3-acetyl-myo-inositol, 29 Ci/mmol, was a gift from J. Chisnell. Indolyl-3-acetyl-myo-inositol was synthesized by J.D. Cohen (26).

Buffers

Tris chloride and potassium phosphate, both at pH 7.15 and 0.05 M, were used except as noted.

Plant Extracts

IAInos hydrolase activity was partially purified from 4 d dark grown corn shoots as described prevously (17). The purification procedure is summarized in Figure 1. In some experiments stage I or stage II enzyme preparations were used. In addition, stage II enzyme which had been fractionated with $(NH_4)_2SO_4$ at 40 to 50% saturation was used in some experiments. For comparisons of activity between coleoptiles and mesocotyls, these parts were harvested separately and used in preparing acetone powders. Esterase activity was partially purified from shoots as described previously (17).

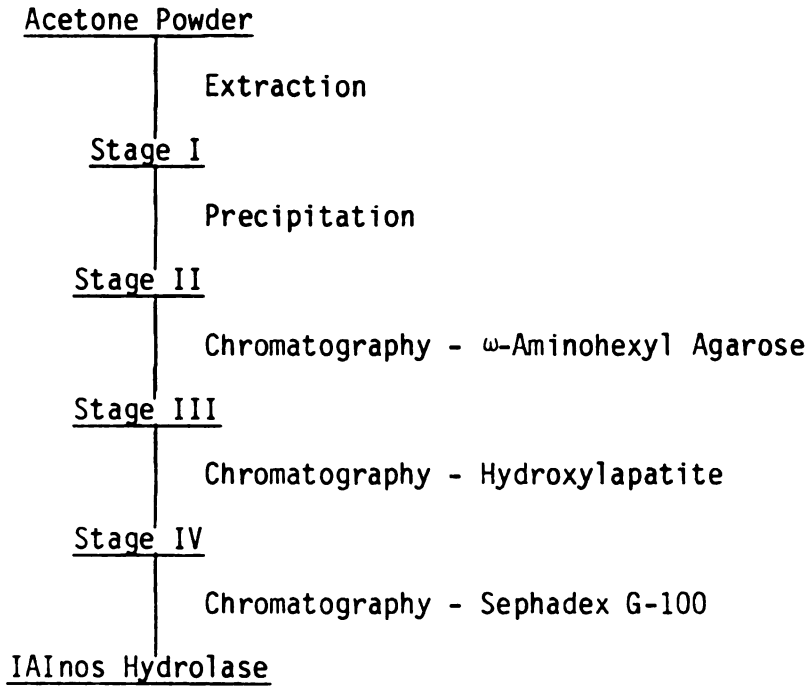

Figure 1. Summary of steps used in the partial purification of IAInos hydrolase.

Protein Concentrations

Protein concentration was determined by the method of Bradford (5) using bovine plasma gamma globulin as the standard.

IAInos Hydrolase Activity

The hydrolysis of ^{3}H-IAInos was measured as described previously (17). To provide higher levels of substrate in some experiments, ^{3}H-IAInos was diluted by the addition of unlabeled, chemically synthesized IAInos. The absorbance at 280 nm was measured and the concentration of IAInos calculated using a molar extinction coefficient of 6060. The unlabeled IAInos was prepared in 50% aqueous 2-propanol such that an addition of 5 µl yielded the desired concentration of substrate in addition to 6 pmol ^{3}H-IAInos. A 5 µl aliquot was immediately withdrawn from the reaction mixture for determination of radioactivity.

Hydrolysis of α-Naphthyl Acetate

General esterase activity was measured spectrophotometrically as the production of α-naphthol (17).

Preparation of Methyl Esters

Methyl esters of ^{14}C-IAA, IAA, naphthylene acetic acid (NAA), phenylacetic acid (PAA) and benzoic acid (BA) were prepared by reaction with ethereal diazomethane (31). The methyl esters were purified by chromatography over DEAE-Sephadex (acetate form) in 50% aqueous 2-propanol. Esters eluted with this solvent while the free acids were bound to the exchanger.

Hydrolysis of Methyl IAA (Me-IAA)

Hydrolysis of the methyl ester of IAA was followed by measuring the production of ^{14}C-IAA from ^{14}C-Me-IAA. The reaction volume was 100 µl, and 5 µl of ^{14}C-Me-IAA (approximately 46 pmol) was added. A 5 µl aliquot was immediately withdrawn for counting. The reaction mixture was incubated at 37°C for 2 h, then the sample was processed as described for the assay of ^{3}H-IAInos hydrolysis (17). To obtain greater substrate concentrations, ^{14}C-Me-IAA was diluted by the addition of unlabeled Me-IAA.

Hydrolysis of Methyl Esters of NAA, PAA and BA

The desired methyl ester was suspended in 50% aqueous 2-propanol at a concentration such that disappearance of substrate and any production of product could be followed by UV absorbance during HPLC of the reaction mixture. Reaction mixtures consisted of 5 µl (approximately 20 nmol) of substrate in 100 µl of buffered extract or 100 µl of buffer. After incubation product and unreacted substrate were separated by chromatography over a 0.46 x 25 cm Partisil-10 ODS column with a CO:Pell ODS precolumn in a Varian 5000 Liquid Chromatograph. The mixture was chromatographed isocratically in ethanol:acetic acid:water (40:1:59). The presence of a peak at the retention time of the free acid was taken as evidence of hydrolysis.

HPLC Resolution of IAInos Isomers and IAA

IAA and IAInos isomers were partially resolved by HPLC by isocratic elution in 1% aqueous acetic acid:ethanol (95:5) at a flow rate of 1 ml/min. Fractions of 0.5 ml were collected for determination of

radioactivity when ^{3}H-IAInos was chromatographed. Unlabeled IAInos was mixed with radioactive samples just prior to HPLC so the resolution of the isomers and IAA could be followed by absorbance at 280 nm.

RESULTS

Characterization of IAInos Hydrolase

pH optimum. 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 non-enzymic hydrolysis of the substrate is accelerated to the point where the difference in hydrolysis between experimental and control reaction mixtures is too small to allow accurate determination of the enzymic rate. At pH values below 5 some proteins precipitate in crude or partially purified extracts with variable losses in activity. Consequently the true optimum for IAInos hydrolysis is not known. Net hydrolysis is maximal between pH 7.0 to 7.5 in Tris or potassium phosphate buffer. We chose to assay the activity at pH 7.15. At this pH it was anticipated that acyl migration in the substrate would be less than at a more basic or acidic pH (17).

Time course. The hydrolysis of ^{3}H-IAInos proceeds linearly with respect to time. After 4 h the extent of enzymic hydrolysis is enough greater than non-enzymic hydrolysis so that even at low enzyme concentration, hydrolysis can be measured.

Enzyme concentration. The proportionality between the rate of ^{3}H-IAA formation and enzyme concentration (mg of protein) is difficult to establish, in part because the assay does not measure initial velocity, and in part because the amount of substrate present is less than optimum. The rate of ^{3}H-IAInos hydrolysis measured after 4 h

doubles with a doubling of enzyme concentration up to a protein concentration of about 5 mg/ml (Figure 2).

Effect of additions to the reaction mixture. The rate of hydrolysis of substrate by extracts at various stages of purification was unaffected by the presence of reduced glutathione or p-chloromercuriphenylsulfonic acid at a concentration of 50 mM and 0.1 mM, respectively. Divalent cations, Ca^{2+}, Mg^{2+}, and Zn^{2+} at 100 μM, and EDTA at 10 mM were also without effect on the reaction rate while 100 μM Mn^{2+} decreased activity to about half that of controls. (Data not shown.)

Glycerol reduced radioactivity appearing in the acidic fraction in proportion to the amount added from 2.5 to 15%. Glycerol at greater than 25% did not further inhibit the appearance of radioactivity (Figure 3).

Effect of added substrate. ^{3}H-IAInos (60 nM) diluted with unlabeled IAInos from 46 μM to 1.3 mM was used to measure the rate of hydrolysis as a function of substrate concentration. The 40 to 50% $(NH_4)_2SO_4$ fraction of stage II enzyme showed typical Michaelis-Menten kinetics. The maximum velocity was 1 x 10^{-9} mol/h/mg protein and the apparent K_m was 270 μM. After Sephadex chromatography, IAInos hydrolase catalyzed the hydrolysis at a maximum rate of 2.3 x 10^{-9} mol/h/mg protein and had an apparent K_m of 210 μM as determined from a Lineweaver-Burk double reciprocal plot. Different treatments of the data yielded similar values for the K_m and maximum velocities.

Preferential hydrolysis of isomers. The structure of myo-inositol would allow for esterification of IAA to myo-inositol at any of the 6 available hydroxyls. Difficulty in separating the esters by physical and chemical means is compounded by migration of the acyl group from one position to another under incompletely understood circumstances

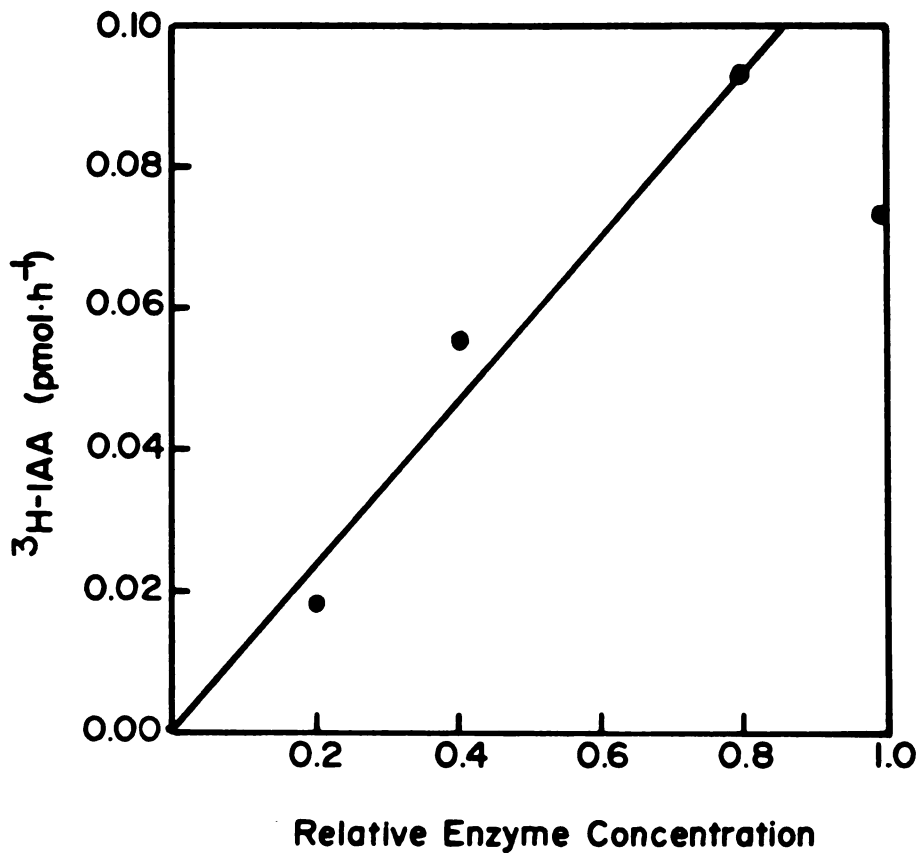

Figure 2. The effect of enzyme concentration on the rate of hydrolysis of ^{3}H-IAInos. Stage IV enzyme at 6 mg/ml was diluted with Kphos buffer and hydrolysis was assayed as described in the Methods section.

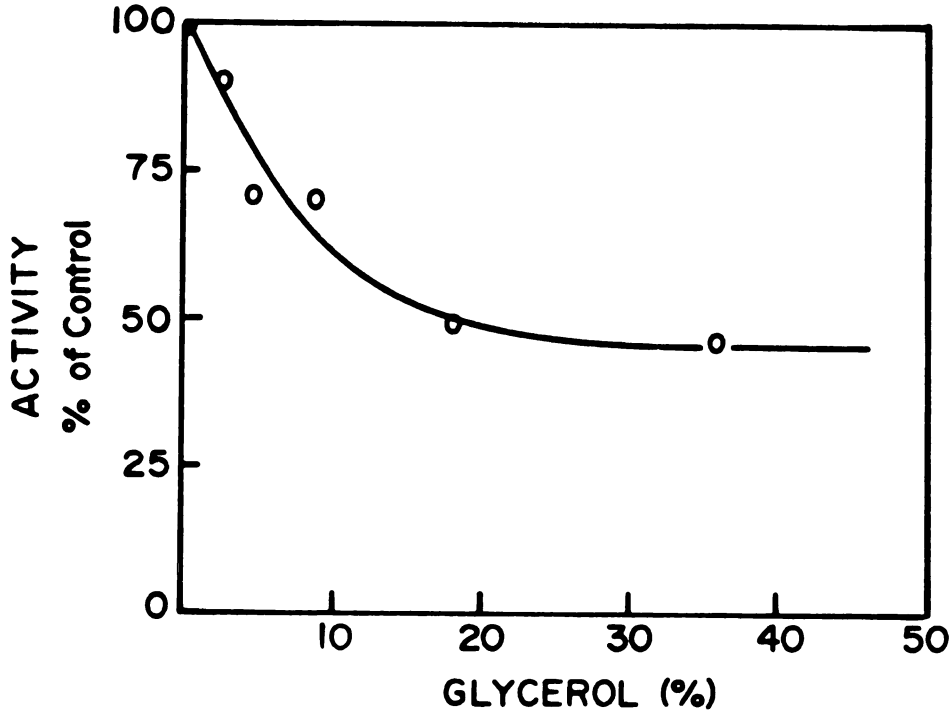

Figure 3. The effect of glycerol on ^{3}H-IAInos hydrolysis. Fifty µl of Stage II enzyme (clarified 40 to 50% $(NH^4)^2SO^4$ fraction) was mixed with Kphos and glycerol to give the final concentrations of glycerol indicated in a total volume of 100 µl.

(2,6,11,14,21,24,35). It is possible to resolve some of the esters by TLC, GLC and HPLC (6,11,21) but only for the 2-O-IAInos ester has a particular structure been confirmed (24,34). On TLC with a solvent system composed of ethyl acetate, methyl ethyl ketone, ethanol and water (50:30:10:10), two Ehmann positive spots (10) can be resolved (11). In this particular solvent system the slower migrating spot is the 2-O-IAInos or axial ester (24). The 2 position is the only axial position in myo-inositol and IAA esterified to it confers different solubility and reactivity properties on the compound as compared to the other 5 isomers. GLC (34) will resolve an isomeric mixture into 4 peaks, but only the axial ester can be definitely assigned to a particular peak. HPLC in ethanol:water (5:95) resolves an isomeric mixture into 3 major peaks (Figure 4a). IAInos eluting under each peak can be collected, dried and chromatographed on TLC. The first HPLC peak yields an even mixture of axial and equatorial esters on TLC. The amount of axial ester in the second peak is diminished while only the equatorial esters are present in the third peak (Figure 4b).

Since the hydrolysis of IAInos at endogenous substrate levels was slow and unaffected by additions to the reaction mixture, we thought that the hydrolase might be hydrolyzing one isomeric form in preference to another. That this was in fact the case can be seen from the data of Figure 5. Comparison of the ratio of radioactivity collected to the area of the peak at the corresponding retention times shows that isomers eluting under peak 2 disappear in an enzymatically catalyzed reaction. The radioactivity in this peak is decreased approximately 50%. This suggests that the hydrolase cannot hydrolyze each of the 6 esters, but requires certain configurations for its activity. A determination of at

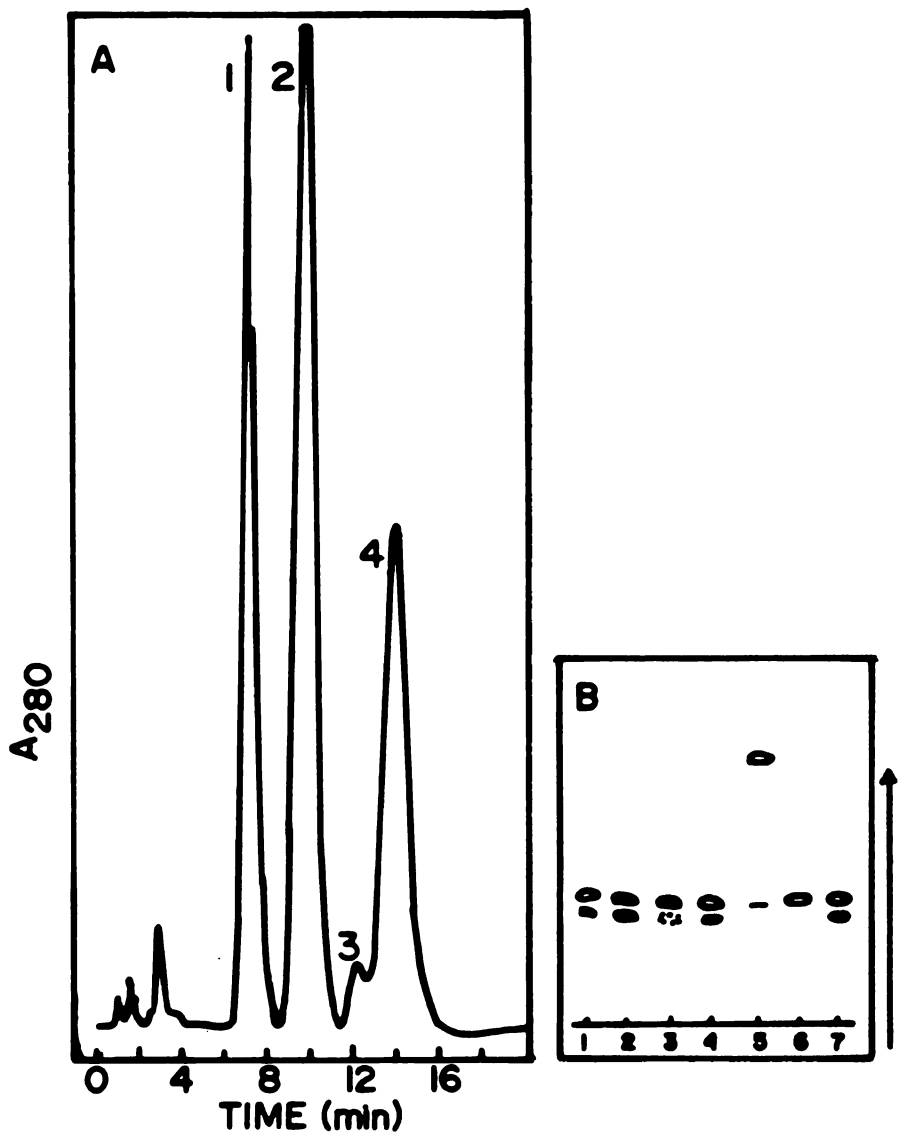

Figure 4. Separation of isomers of IAInos. A. Chemically synthesized IAInos was chromatographed over a Partisil-10 ODS HPLC column. The flow rate was 2 ml/min, the solvent was ethanol:water (5:95). Fractions eluting under each of the 4 peaks were pooled, dried in vacuo, and resuspended in a small volume of 50% aqueous 2-propanol. Aliquots from each were chromatographed in a TLC system using methylethyketone:ethyl acetate: ethanol:water (3:5:1:1) as the solvent. B. TLC of standard IAInos and samples from HPLC. Lanes 1, 4, 6, standard IAInos; Lane 2, sample from peak 1; Lane 3, sample from peak 2; Lane 5, sample from peak 3; Lane 6, sample from peak 4.

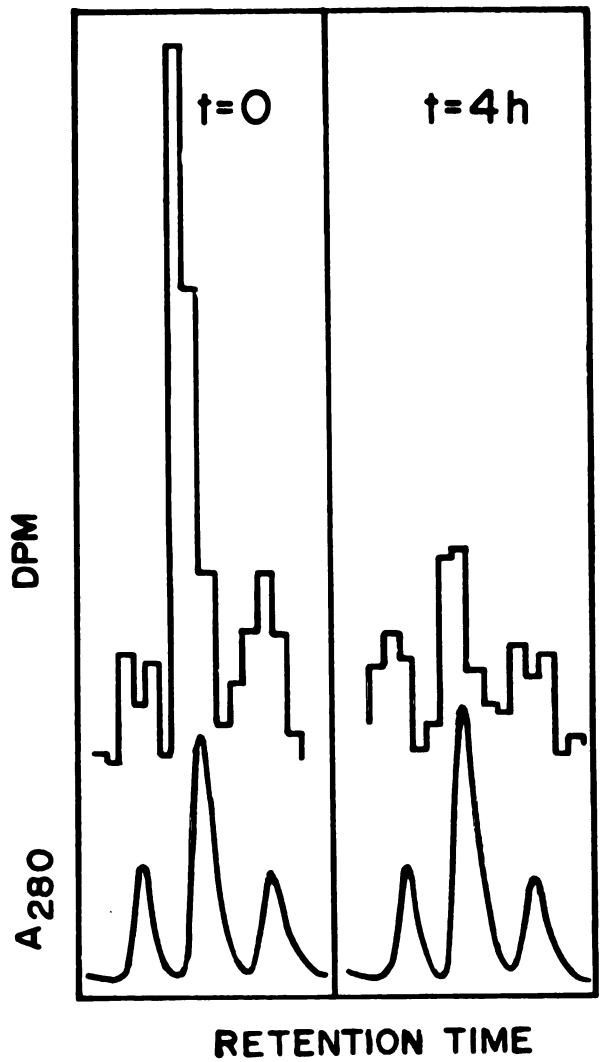

Figure 5. Radioactivity associated with peaks of IAInos eluted from HPLC. Stage II enzyme (clarified 40 to 50% $(NH_4)_2SO_4$ fraction) was mixed with ^{3}H-IAInos and the reaction mixture incubated 0 or 4 h. The reaction was terminated by the addition of an equal volume of 2-propanol. An aliquot was mixed with one µg chemically synthesized IAInos prepared in 50% aqueous 2-propanol, 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 are shown in the upper portion of each panel.

least some of the active isomers might be made using a combination of HPLC and GLC. Useful data might also be provided by allowing the reaction to go to completion.

Esterolytic Activities of IAInos Hydrolase and Esterase

Since the hydrolysis of IAInos involves the breaking of an ester bond, it was expected that the hydrolase might catalyze the hydrolysis of other esters. Other ester substrates were studied partly to determine specificity of the hydrolase, and also in hopes of finding a substrate which would be easier to obtain and easier to assay. If such a substrate existed then it might be used in studied of mechanisms. It was also important to determine if the hydrolysis of other esters by the hydrolase was different in any way from the hydrolysis of the same esters by the partially purified esterase. The ester α-naphthyl acetate and the methyl esters of IAA, NAA, PAA and BA were used for these studies.

Hydrolysis of α-naphthyl acetate. α-Naphthyl acetate esterase activity is present in all enzyme preparations at all stages of purification. Hydrolysis of α-naphthyl acetate by IAInos hydrolase is 40 times faster than hydrolysis of IAInos. The reaction, as measured spectrophotometrically, proceeds with a maximum velocity of 1.6×10^{-9} mol/min/mg protein as compared to 2×10^{-9} mol/h/mg protein for IAInos hydrolysis. The apparent K_m for α-naphthyl acetate is 45 μM. The reaction rate observed in stage I enzyme preparations is unaffected by 30 mM Na^+, K^+, or NH_4^+, 3 mM Ca^{2+}, Mn^{2+}, or Co^{2+}, BSA (1 mg/ml) or 2 mM glutathione. About 50% inhibition in the rate of hydrolysis is observed in the presence of 5 mM p-chloromercuribenzoic acid.

Upon gel filtration chromatography IAInos hydrolase retains esterase activity (Figure 6). The peak of α-naphthyl acetate esterase activity eluted at 25 ml while hydrolase activity reached a maximum between 28 and 30 ml. As described earlier (17), hydroxlyapatite chromatography resolves an esterase from IAInos hydrolase. This esterase was further purified by gel filtration chromatography (17, see especially Figure 3), and the peaks of α-naphthyl acetate esterase activity eluted at 21 ml on a calibrated Sephadex G-100 column (17).

Hydrolysis of α-naphthyl acetate by the esterase is readily demonstrated. The maximum rate observed for the esterase is 2.4×10^{-8} mol/min/mg protein, and the apparent K_m is 100 μM.

Hydrolysis of Me-IAA. In the presence of 46 pmol ^{14}C-Me-IAA, IAInos hydrolase produces ^{14}C-IAA at a rate of 37 pmol/h/mg protein. This compares to 2 pmol IAA produced/h/mg protein in the presence of 46 pmol ^{3}H-IAInos. The maximal rate of Me-IAA hydrolysis is 1.3×10^{-8} mol/h/mg protein and the apparent K_m is 1.2 mM. The esterase hydrolyzes Me-IAA at a maximal rate of 8×10^{-8} mol/h/mg protein and has an apparent K_m of 500 μM.

Hydrolysis of other esters. As shown in Figure 7, IAInos hydrolase hydrolyzed the methyl ester of NAA (Me-NAA) to a slight degree in 4 h. We could not observe any hydrolysis of the methyl esters of PAA (Me-PAA) or BA (Me-BA). Although this assay is not quantitative, we estimate that 4% of the Me-NAA was hydrolyzed and appeared at the retention time of NAA. The esterase was more active in the hydrolysis of Me-NAA. Approximately 14% of the added Me-NAA was hydrolyzed. The esterase did not hydrolyze Me-PAA or Me-BA.

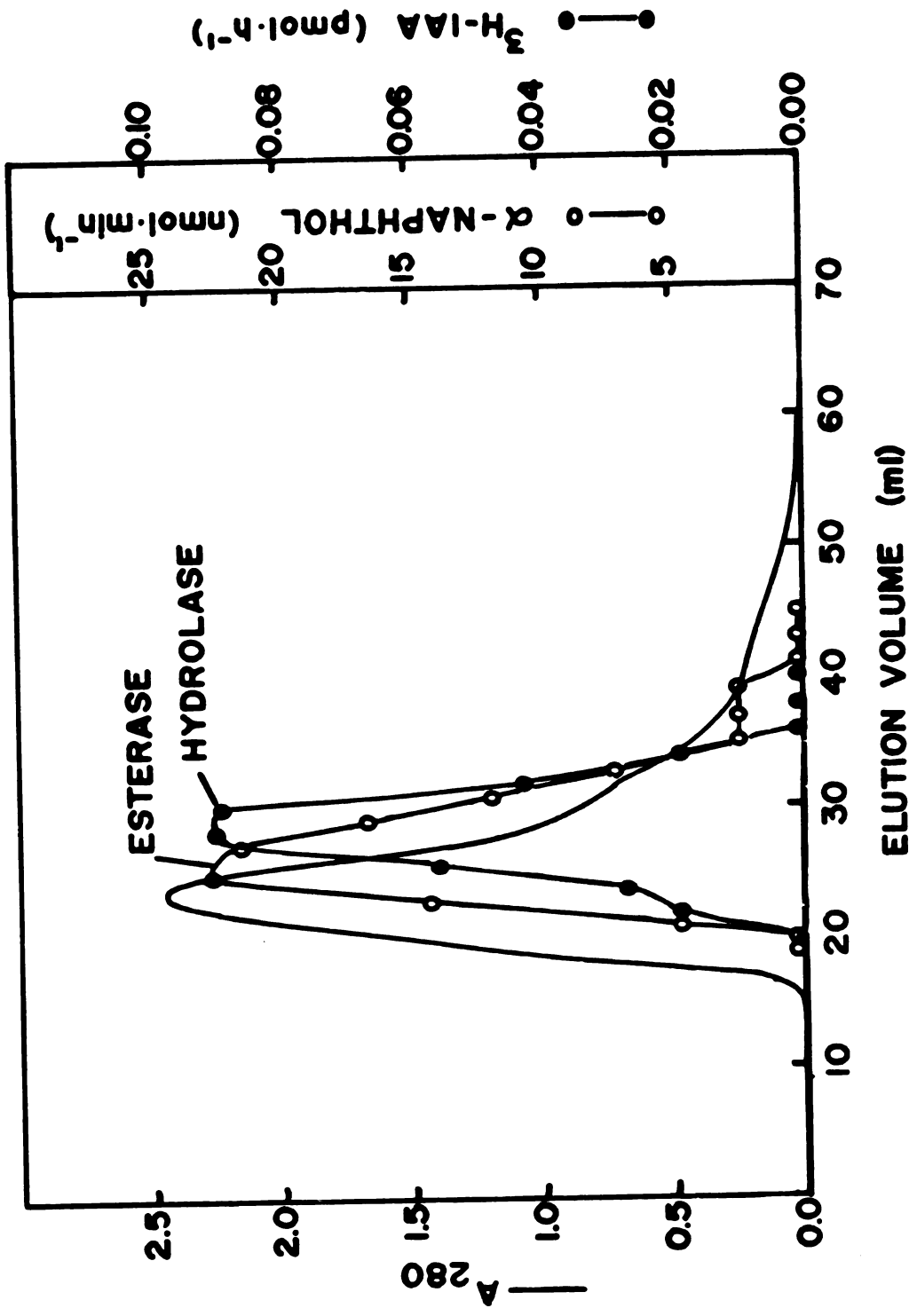

Figure 6. Gel filtration chromatography of Stage IV enzyme. The sample was chromatographed over a calibrated Sephadex G-100 column (1.6 x 33 cm) in potassium phosphate buffer. Fractions of one ml were collected. Aliquots of 100 µl were used for assays of ^{3}H-IAInos hydrolase or α-naphthyl acetate esterase as indicated.

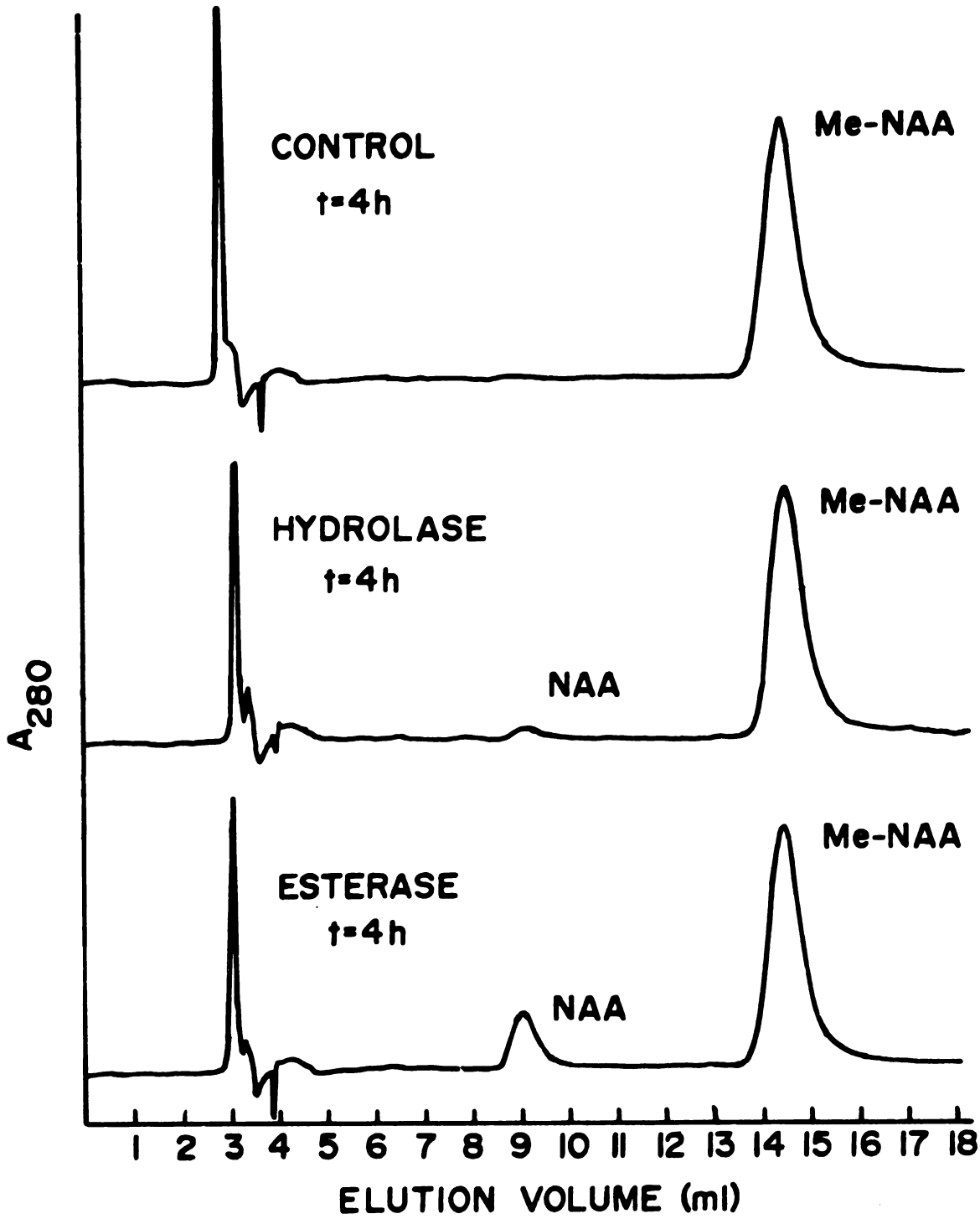

Figure 7. Enzymatic hydrolysis of Me-NAA. Hydrolase or esterase preparations were incubated with 20 nmol Me-NAA. Control reactions contained no enzyme. An aliquot of each reaction mixture was chromatographed by HPLC (Partisil-10 ODS; ethanol:acetic acid:water (40:1:59), one ml/min) to separate product from substrate. Me-NAA elutes at 15 ml while NAA elutes at 9 ml.

Effects of glycerol. Because we suspected that hydrolytic activity might diminish upon freezing, we examined the effect of glycerol on hydrolytic activities. The hydrolysis of ^{14}C-Me-IAA was unaffected by glycerol. As mentioned above, a decrease in IAInos hydrolysis was observed which was proportional to the percentage of glycerol present in the reaction mixture up to 15%. In contrast, the rate of appearance of α-naphthol is increased by as much as 3 fold in a reaction mixture with 10% glycerol.

This discrepancy suggested the hydrolase was able to act as a transferase, as was observed in the case of phosphatases by Axelrod (1). With α-naphthyl acetate the acetic acid might be transesterified to glycerol, allowing for the increased release of α-naphthol. For IAInos hydrolysis it seemed possible that IAI would be transesterified with glycerol resulting in a product that would be retained on DEAE-Sephadex. A preliminary attempt to locate a new radioactive product by TLC and HPLC was unsuccessful, perhaps because of the small amounts of substrate employed.

Other alcohols, methanol, thanol, n-propanol, and 2-propanol, slightly decrease the rate of α-naphthol release while tert-, sec-, iso-, and n-butanol all increase activity in that order (Table 1).

TABLE 1. Effect of butyl alcohols on esterase activity.

Addition	Rate of Appearance of α-naphthol nmol/min/mg protein
None	32
tert-butanol	35
sec-butanol	40
iso-butanol	80
n-butanol	110

(The reaction mixture consisted of 0.1 ml extract, 0.2 ml alcohol, 2.6 ml KPO_4 plus 0.1 ml α-naphthyl acetate added after 5 min.)

Isolation and identification of the products of transesterification is necessary to demonstrate transferase activity. In addition, for α-naphthyl acetate hydrolysis, the rate of appearance of acetic acid in the presence or absence of an acceptor alcohol should be measured.

Hydrolytic activities in coleoptiles as compared to mesocotyls. Stage I enzyme from mesocotyls was comapred to that from coleoptiles in the hydrolysis of ^{3}H-IAInos and α-naphthyl acetate. The results, summarized in Table 2, show that extraction of coleoptiles yields more IAInos hydrolase activity. The specific activity of IAInos hydrolase is greater as is the activity per coleoptile; hydrolase 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 greater.

TABLE 2. Comparison of enzymatic activities of coleoptiles and mesocotyls.

Plant Part[a]	IAInos Hydrolase Activity		α-Naphthyl Acetate Activity	
	pmol/h/mg protein	pmol/h/plant part[b]	pmol/h/mg protein	pmol/h/plant part[b]
Coleoptiles	0.012 ± 0.003	0.030	47 ± 9	116
Mesocotyls	0.008 ± 0.002	0.007	48 ± 6	44

[a]Stage I enzyme was prepared as described (17) 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 there is 1 coleoptile/2.5 mg protein and 1 mesocotyl/0.92 mg protein.

DISCUSSION

Some characteristics of IAInos hydrolase are summarized in Table 3. They include: 1) in the presence of saturating amounts of substrate the rates of hydrolysis of IAInos and α-naphthyl acetate differ nearly 40 fold; 2) the affinity for α-naphtyl acetate is 5 times greater than that for IAInos; 3) neither activity is affected by glutathione, although the sulfhydryl inhibitor p-chloromercuribenzoate inhibited α-naphtyl acetate hydrolysis by about 50% in Stage II enzyme preparations; and 4) the methyl ester of IAA is hydrolyzed.

Does the same enzyme catalyze all these reactions? There are some precedents for enzymes catalyzing multiple reactions (13,19,20,22,27,32). If an enzyme preparation is homogeneous, as judged by such techniques as electrophoresis and ultracentrifugation, then that would be sufficient to permit the assertion that the enzyme carried out more than one reaction. It is possible that even a homogeneous enzyme preparation may respond to effectors or inhibitors in different ways for different substrates, but unless it is established that the enzyme is homogeneous, any observations on differential effectors or inhibitors should be regarded cautiously.

In the case of IAInos hydrolase activity, there are 4 observations which raise some doubts that the same enzyme is catalyzing the hydrolysis of all these substrates. First, this enzyme preparation is not homogeneous. Second, sulfhydryl inhibiting reagents had different effects on the hydrolysis of IAInos and α-naphthyl acetate. Third,

TABLE 3. Summary of properties of IAInos hydrolase.

Substrate	V_{max}[a]	K_m[b]	Effects of		Glutathione	SH Inhibitors
			Cations	Glycerol		
IAInos	2.3×10^{-9}	2.1×10^{-4}	+	+	0	0
Me-IAA	1.3×10^{-8}	1.2×10^{-3}	nd	0	nd	nd
α-Naphthyl acetate	9.8×10^{-8}	4.1×10^{-5}	0	+	0	0

[a]moles of product/h/mg protein
[b]moles/L

+ effect observed on rate of hydrolysis
0 no effect observed on rate of hydrolysis
nd not determined

glycerol did not affect the hydrolysis of Me-IAA, but did affect IAInos and α-naphthyl acetate hydrolysis. A fourth observation is that when chromatographed over Sephadex G-100, the peak of α-naphthyl acetate activity eluted just before the peak of IAInos hydrolase (Figure 6). Greater resolution of the proteins is necessary before this question can be answered. We would also like to examine this enzyme preparation for ability to catalyze other kinds of reactions - can it act as a protease, glycosidase, fatty acid esterase, dehydrogenase?

Since we can partially purify an enzyme which retains esterolytic activities but is unable to hydrolyze IAInos, we conclude that hydrolysis of inositol esters of IAA is not a property of all esterases. The properties of the esterase differ from those of IAInos hydrolase as shown in Table 4. The retention volumes on Sephadex G-100 differ for the two enzymes. The esterase eluted at 21 ml while the esterase activity associated with the hydrolase eluted at 25 ml. The maximum rates of hydrolysis of Me-IAA and α-naphthyl acetate are greater for the esterase than for the hydrolase and the apparent affinities for the substrates also differ.

An enzyme which catalyzes a critical reaction in a biological system must itself be subject to regulation. Feedback inhibition by IAA does not seem to play a role as levels of IAA required to inhibit hydrolysis of IAInos in an _in vitro_ assay system are much greater than would occur in plant tissue (data not shown). It is possible that some effector or cofactor might act to increase the reaction rate or decrase the K_m _in vivo_. None of the potential effectors we have tried so far have acted to increase the rate of hydrolysis.

TABLE 4. Comparison of properties of esterase and IAInos hydrolase.

Substrate	Extract MW Estimate	Esterase 27,000		Hydrolase 45,000	
		V_{max}[a]	K_m[b]	V_{max}[a]	K_m[b]
^{3}H-IAInos		-	-	0.2	2.1
^{14}C-Me-IAA		7.8	5.3	1.3	12.0
α-Naphthyl acetate		144.0	1.0	9.8	0.4
Me-NAA		3 nmol/4 h		1 nmol/4 h	
Me-PAA		-		-	
Me-BA		-		-	

[a] 10^{-8} mol/h/mg protein
[b] 10^{-4}/L

Another way in which IAInos hydrolysis might be controlled is by the physical separation of the enzyme from its substrate. Although we are beginning to accumulate data about the distribution of IAA and its esters in different tissues, neither the intracellular distribution of the enzyme nor that of substrate is known at this time. Higher levels of enzyme activity in coleoptiles than in mesocotyls suggest that the enzyme is not uniformly distributed.

The observed K_m for IAInos is high compared to the expected endogenous levels of substrate. The maximum rate of IAInos hydrolysis _in vitro_ is observed only at levels of IAInos which far exceed levels measured in corn shoots, and the rate of hydrolysis at _in vivo_ concentrations of IAInos is lower than is thought to be required to maintain free hormone at levels required for normal growth (18). However, several considerations and corrections discussed below indicate that the endogenous rate could be much higher.

In our assay Stage I enzyme hydrolyzes ^{3}H-IAInos at a rate of 0.098 pmol/h/mg protein. Protein extracted in making an acetone powder is 1.9 mg/shoot. Therefore, the _in vivo_ rate of hydrolysis becomes:

0.098 pmol/h/mg protein X 1.9 mg protein/shoot = 0.19 pmol/h/shoot.

This is about one-twentieth the rate of 5 to 9 pmol/h/shoot calculated to be required for maintenance of endogenous pools of IAA in a shoot (18).

We suspect that hydrolytic activity is underestimated during extraction and purification. The loss of units of activity coupled with the slight increase in purification reported (17), suggest that our understanding of the nature of the enzyme is incomplete and that we are recovering as little as 10% of the enzyme actually present _in vivo_.

In addition, the following points should be considered in any assessment of the rate of hydrolysis:

1) the endogenous concentration of IAInos in shoot tissue and its localization;
2) the proportion of esterified IAA that is present as IAInos in a shoot;
3) the existence of six isomers of IAInos and evidence of preferential enzymatic hydrolysis.

The concentration of IAInos in corn shoot tissue is 74 nmol/kg fresh weight (6). Using an estimate of 6 shoots/g fresh weight, we calculated that there are 12 pmol IAInos/shoot. The concentration of ^{3}H-IAInos in the in vitro assay is 6 pmol but the rate of hydrolysis is doubled in the presence of 12 pmol of ^{3}H-IAInos. Therefore, the endogenous rate appears to be increased:

0.19 pmol/h/shoot/6 pmol IAInos X 12 pmol IAInos = 0.38 pmol/h/shoot.

This is about one-tenth of the expected rate.

Although there is no information on the intracellular distribution esters of IAA, we suspect that these compounds are in the cytoplasm, as opposed to the vacuole, and that the cytoplasm may occupy as little as 10% of the total volume of the cell, thereby effectively increasing the local concentration of esters.

Data on the distribution of IAInos in different parts of a corn shoot added to our understanding of how the hydrolytic rate might be affected. Calculations presented elsewhere (15), which are based on data for the amounts and concentrations of esterified IAA in whole shoots (28) and in mesocotyls (29), strongly suggest that the concentration of esterified IAA in coleoptiles is more than three times that in

mesocotyls. Pengelly et al (28) reported that there is 2.1 µg free plus ester IAA/g dry weight while free IAA is 0.6 µg/g dry weight. The difference, 1.5 µg/g dry weight, is the ester IAA, which is approximately 140 pmol/shoot:

$$1.5\ \mu g\ IAA/g\ dry\ weight \times 0.1\ g\ dry\ weight/g\ fresh\ weight \times 1\ g\ fresh\ weight/6\ shoots = 0.025\ \mu g\ IAA/shoot$$

$$0.025\ \mu g\ IAA/shoot \times 1\ \mu mol/175\ \mu g = 1.43 \times 10^{-4}\ \mu mol/shoot = 143\ pmol/shoot$$

Using the data of Pengelly et al (29) for the concentrations of IAA in mesocotyls, we have calculated (15) that 120 pmol of the esterified IAA may be concentrated in the coleoptile. If even 20%, or 24 pmol, of the ester fraction is present as IAInos (6), then the anticipated rate of hydrolysis approaches 0.76 pmol/h (the increase in rate is proportional to the amount of substrate present). We have assumed that 80% of the esters are not inositol esters of IAA. The enzyme activity needs to be assayed using other endogenous IAA esters as substrates as they are identified, since these esters may constitute other in vivo sources of IAA.

Preferential hydrolysis of one ester over another (Figure 5) may mean that in an in vitro assay the apparent K_m is much higher than would be the case in vivo. For example, if the 2-O-IAInos isomer was the preferred substrate, but was present in ^{3}H-IAInos or chemically synthesized IAInos at a level much less than in Z. mays tissue, the in vitro assay with increasing levels of substrate would overestimate the concentration of IAInos required for the reaction to proceed at maximum velocity. If the substrate supplied in the assay offered only one-sixth of the isomers in the preferred form, then the rate of hydrolysis should be corrected by a factor of six:

$$0.76\ pmol/h \times 6 = 4.5\ pmol/h$$

This rate is thus equivalent to the expected rate of 5 to 9 pmol/h/shoot.

The enzyme catalyzing IAInos hydrolysis must be further purified in order to properly study its specificity, for its specificty is important in any study of its role. Localization of the enzyme and substrate should be determined. Although specificty and localization will be important in a proper assessment of the physiological role of IAInos hydrolase, the data reported in this and the accompanying paper (17) strongly suggest its importance and offer some methods for its study.

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CONCLUDING DISCUSSION

In this work I have accomplished the first demonstration and partial purification of an enzyme capable of hydrolyzing a conjugate of the plant growth hormone, IAA. Studies of this enzyme, IAInos hydrolase, and a second, partially purified esterase, permit the following conclusions:

1) There is an enzyme in vegetative tissue of corn which hydrolyzes IAInos, α-naphthyl acetate, and the methyl esters of IAA and NAA. It is uncertain whether IAInos hydrolase or a contaminating protein in the enzyme preparation is responsible for hydrolysis of α-naphthyl acetate, Me-IAA and Me-NAA.
2) An esterase, separable from the hydrolase, will hydrolyze α-naphthyl acetate, Me-IAA and Me-NAA, but not IAInos.
3) The hydrolase shows specificity for one or possibly more of the 6 possible isomers of IAInos.
4) The hydrolase is concentrated mainly in the coleoptile portion of a young corn seedling, and is present in lesser amounts in the mesocotyl.
5) The hydrolase is also present in root and seed tissue.

In addition, data have been presented which pertain to the affinity of the hydrolase and the esterase enzymes for their substrates, the pH ranges in which the reactions proceed, and the effects of certain inhibitors and metal cations on the reactions.

A calculation has been made showing that the hydrolase activity extracted approaches the rate which is adequate to provide the amount of IAA required in a corn shoot for normal extension growth.

Discussion of the data pertaining to these conclusions is presented below.

Extracts from shoots, roots and germinating seeds are all active in causing the disappearance of radioactivity associated with ^{3}H-IAInos, and in causing the appearance of radioactivity associated with ^{3}H-IAA. Boiled or alcohol-precipitated extracts are inactive in promoting this reaction. A protein which catalyzes this hydrolysis can be partially purified from shoot acetone powders by chromatography over -aminohexyl agarose, hydroxylapatite, and Sephadex G-100. The partially purified extract is also active in catalyzing the hydrolysis of α-naphthyl acetate, Me-IAA, and Me-NAA, but not of Me-PAA and Me-BA. The IAInos hydrolytic activity is characterized by an estimated molecular weight of 45,000 daltons, and an affinity for IAInos in the range of 10^{-4}M. The activity is neither promoted nor retarded by Ca^{2+}, Mg^{2+}, Zn^{2+}, EDTA, NAD, NADP, p-chloromercuriphenylsulfonic acid or glutathione. Some inhibition is observed in the presence of either Mn^{2+} or glycerol. This protein preparation is not homogeneous as demonstrated by acrylamide gel electrophoresis. It contains several proteins and at least 5 bands of esterase activity.

The hydrolysis of esters other than IAInos by partially purified IAInos hydrolase preparations may be due to other esterases which are contaminants. These contaminating esterases may not hydrolyze IAInos. The esterase activities and the hydrolase activity differ in several ways. Hydrolysis of α-naphthyl acetate is partially inhibited by

p-chloromercuribenzoic acid, while another sulfhydryl reagent, p-chloromercuriphenylsulfonic acid, does not inhibit IAInos hydrolysis. In the presence of glycerol the production of α-naphthol from α-naphthyl acetate is enhanced. With respect to IAInos hydrolysis, the disappearance of radioactivity from IAInos-associated fractions is inhibited by glycerol, but as discussed earlier, this may reflect transesterification. Glycerol does not affect the hydrolysis of Me-IAA.

The data which provides the strongest support for distinguishing between proteins with esterase activity and that with hydrolase activity in hydrolase preparations is the observation that the enzymatic activities elute at different volumes during gel filtration chromatography. The hydrolase activity is maximal at 28 to 30 ml which corresponds to a molecular weight of 45,000 daltons. The esterase activity is maximal at 25 ml, suggesting a molecular weight of 60,000 daltons. Estimation of molecular weights by gel filtration chromatography is not definitive. The relative retention volume depends in part on the molecular configuration of the protein; carbohydrate associated with a protein will also interfere with molecular sieving on Sephadex. However, neither of these observations can discount the fact that the two enzymatic activities behave differently during gel filtration chromatography. Greater resolution of these two activities might be achieved in any of several ways: use of a longer gel filtration column, chromatography using an ion exchanger, or separation of proteins on acrylamide or starch gels. Although the latter method is technically more difficult, it might allow one to establish which protein hydrolyzes IAInos. I suggest that IAInos hydrolase will eventually be separated from the esterases which have so far co-chromatographed with it.

Because IAInos hydrolase is only partially purified, it is not possible to say if the hydrolase is an esterase, or if it will hydrolyze other ester substrates. And it is not known whether only one enzyme hydrolyzes IAInos.

What does the enzymatic activity require in a substrate other than an ester bond? Comparison of the structures of IAInos or any of the methyl esters of IAA, NAA, PAA and BA with the structure of α-naphthyl acetate reveals that the acid moiety of α-naphthyl acetate is a small aliphatic acid, acetic acid, while the alcohol part of the molecular is the rather bulky α-naphthol. The other substrtaes all have acids which contain benzene rings; with the exception of inositol, the alcohol moiety of each is methanol. Inositol is admittedly more complex, both in size and in the presence of 6 hydroxyl groups instead of one.

IAA, NAA, and PAA are all active as auxins; BA is not. The methyl ester of IAA is also active. In some bioassays it is more active than the free acid. The activities of Me-PAA and Me-NAA in the bioassays are similar to or less than those of the corresponding free acids. Part of the activity in any bioassay may just reflect the permeability of the compound being tested.

The second conclusion, that there is an esterase which cannot hydrolyze IAInos, is supported by the isolation and partial purification of a protein fraction that is active in hydrolyzing various esters, but not IAInos. This esterase will hydrolyze α-naphthyl acetate, Me-IAA and Me-NAA. The apparent molecular weight is 23,000 daltons. By several criteria it is different from the esterase which co-chromatographs with IAInos hydrolase. The most obvious difference is in their chromatographic properties. The esterase is not retained by hydroxylapatite, and

its retention volume on a calibrated Sephadex column is maximal at 35 ml. Esterase activity associated with IAInos hydrolase elutes at 25 ml on the same column. In addition, the two activities differ in rates and affinities for α-naphthyl acetate and Me-IAA. Comparison of acrylamide gels stained for esterase activity of the two samples shows that there are at least 2 esterase bands associated with IAInos hydrolase which do not appear in the electrophoretically separated esterase sample. Two bands apear in both samples, although they are the only bands in the esterase, they are very minor bands in the hydrolase samples.

In an in vitro enzyme assay system the lack of well purified enzyme preparations make it hard to draw conclusions regarding specificity. In spite of not having well purified enzymes some general trends are evident in comparing IAInos hydrolase and the esterase. Both hydrolyze α-naphthyl acetate, Me-IAA and Me-NAA, neither hydrolyzes Me-PAA or Me-BA. The esterase has a greater affinity for Me-IAA than does the hydrolase. The maximum rate of hydrolysis is 6 times greater. The affinity of both enzymes for α-naphthyl acetate is greater than that for Me-IAA, 5 times greater for the esterase, 30 times greater for the hydrolase. The affinity of the hydrolase for α-naphthyl acetate is 5 times greater than that for IAInos. Simple esters are more rapidly hydrolyzed. The esterase hydrolyzes α-naphthyl acetate 18 times faster than it does Me-IAA, the relative rate is 7 to 1 for the hydrolase.

Perhaps it can be said that a relatively large more hydrophilic molecule such as IAInos is an impossible substrate for an esterase. Its preferred substrates may be smaller, more hydrophobic molecules.

These comparisons are summarized in the table below.

TABLE 1. Comparison of hydrolytic activities of esterase and IAInos hydrolase.

Substrate	Esterase		Hydrolase	
	V_{max}	K_m	V_{max}	K_m
IAInos	-	-	0.23	2.1
Me-IAA	7.8	5.3	1.3	12.0
α-Naphthyl Acetate	144.0	1.0	9.8	0.4

V_{max} units are 10^{-8} mol/h/mg; K_m units are 10^{-4} mol/l

Data have been presented which indicate that the rate of IAInos hydrolysis is controlled, in part, by the isomeric form of the substrate. Experiments in which reaction mixtures are co-chromatographed with unlabeled IAInos show that radioactivity disappears more rapidly from one IAInos peak than from the other two. As discussed earlier, calculations of enzymatic rates at endogenous substrate levels approach what are believed to be *in vitro* rates if one supposes only one isomer is being hydrolyzed.

This idea is made more appealing by examining the relative amounts of IAA and esterified IAA in coleoptiles *versus* mesocotyls. To determine the amount of IAA (free, free + ester, and ester) in coleoptiles we can use data presented in two papers by Pengelly *et al* (14,15). The first of the two papers reports the amounts of IAA in whole shoots as determined

by a radioimmunoassay (RIA) and validated by gas chromatography-selected ion monitoring-mass spectrometry (GC-MS). The second paper uses both techniques to measure the amounts of IAA in the cortex and stele of Z. mays mesocotyls. In this paper the RIA was used to measure the IAA in mesocotyl cortex and stele which had been further subdivided into basal and apical portions. The data which can be used from these papers is:

1) Free IAA in whole Z. mays shoots = 0.6 µg/g dry weight (14).
2) Free + ester IAA in whole shoots = 2.1 µg/g dry weight (14).
3) Free IAA in cortex + stele as determined by GC-MS = 14.4 pmol/mesocotyl (15).

   Free IAA in basal + apical portions of mesocotyl + stele as determined by RIA = 17 pmol/mesocotyl (15).
4) Free + ester IAA in cortex + stele as determined by GC-MS = 30.9 pmol/mesocotyl (15).

   Free + ester IAA in cortex + stele, apical + basal portions, as determined by RIA = 54.9 pmol/mesocotyl (15).

These values differ from values determined by Iino and Carr (10) for field corn.

If two approximations are made, the data in 1) and 2) can be converted to pmol IAA/shoot. The first approximation is that there are 6 shoots in one gram of fresh tissue, and the second is that 0.1 g dry weight is equal to 1.0 g fresh weight. If the IAA in a shoot less the IAA in a mesocotyl is equal to the IAA in a coleoptile, then the pmol of IAA in a coleoptile can be calculated. The data and calculations are shown in the table below.

From these calculations we see that the amount of esterified IAA (IAInos plus other unidentified esters) is about 120 pmol/coleoptile. It

TABLE 2. Esterified IAA in whole shoots, coleoptiles and mesocotyls.

	Reference	µg/g dry weight	ng/shoot[a]	pmol/shoot[b]	pmol/mesocotyl	method of determination	pmol/coleoptile
Free IAA	(14)	0.6	10	57			
	(15)				14.4	GC-MS	42.6
	(15)				17.0	RIA	40.0
Free + Ester IAA	(14)	2.1	35	200			
	(15)				30.9	GC-MS	169.1
	(15)				54.9	RIA	145.1
Ester IAA	(14)			143			
	(15)				16.5	GC-MS	126.5
	(15)				37.3	RIA	105.7

[a] µg/g dry wt x 0.1 g dry wt/g fresh wt x 1 g fresh wt/6 shoots x 10^3 ng/µg
[b] ng IAA/shoot x nmol/175 ng x 10^3 pmol/nmol

has been reported (4) that about 20% of the esterified IAA in vegetative tissue is IAInos. If 20%, or 24 pmol, of the esterified IAA in a coleoptile is IAInos, then the rate can be adjusted as follows:

Stage I enzyme catalyzes the hydrolysis of ^{3}H-IAInos at a rate of 0.098 pmol/h/mg protein in the presence of 6 pmol of substrate. The rate of hydrolysis is proportional to substrate concentration at non-saturating levels, so:

0.098 pmol/h/mg protein/6 pmol IAInos x 24 pmol IAInos

= 0.39 pmol/h/mg protein

Each shoot contains 1.9 mg protein which is extracted into an acetone powder, thus:

0.39 pmol/h/mg protein x 1.9 mg protein/shoot = 0.74 pmol/h/shoot. Since not all isomers of IAInos are substrates for the enzyme, and since the isomeric form of IAInos in the plant is known, we assume that only one isomer is hydrolyzed, and that it is the only one present. The rate of hydrolysis is then increased to approximately 3.5 pmol/h. This is approximately the rate thought to be required to maintain IAA levels in a shoot (9).

It is interesting that data presented in the second manuscript shows the activity of IAInos hydrolase of coleoptiles is greater than that of mesocotyls by a factor of 3 to 4. The amount of esterified IAA in coleoptiles is greater than that in mesocotyls by a factor of approximately 4 to 6, according to the calculations presented above. Perhaps this points to a localization of substrate and enzyme in a physiologically important part of the plant.

The demonstration of both IAInos hydrolase and general esterase activities in extracts of roots and seeds should be emphasized, first,

because studies by other workers (12,16) demonstrate the physiological roles of esters of IAA in growth, and second, because either roots or seeds might provide better sources of IAInos hydrolase for further studies. Activity can be extracted from roots into buffer, and the extracts do not contain some of the difficult contaminating compounds that are found in shoots. The high specific activity of Stage I enzyme found in seeds suggests that seeds may be a rich source of hydrolase enzyme.

A working hypothesis has been advanced for a mechanism by means of which the growth of a corn shoot is controlled by the tip of the coleoptile and is supplied with IAA from the endosperm (1,2,3). The hypothesis includes the following elements:

1) Conjugates of IAA, synthesized during seed maturation and stored in the endosperm, move from the endosperm into the shoot during germination.
2) Upon leaving the endosperm, conjugates are assumed to enter the vascular tissues of the seedling. The hormone, either as the conjugate or as free IAA, leaves the vascular tissues, and enters the cortical tissues of the plant.
3) Conjugated IAA in the plant will be hydrolyzed in response to stimuli related to the balance between free and conjugated IAA, either as a consequence of normal growth or of environmental perturbations.
4) Free IAA in the tip moves downward into the growing regions of the shoot.
5) Following growth promotion, IAA is catabolized.

Since it is the free hormone which promotes growth and since most of the hormone is present in conjugated form, an understanding of the

hydrolysis of conjugates of IAA is necessary for understanding growth according to this hypothesis.

The hypothesis is based in part on demonstrations of the appearance of conjugates in the endosperm during maturation (7), their disappearance during germination (6,8,16), and their transport from endosperm to shoot (5,13). Many workers have presented data which demonstrates that IAA moves from the tip of a coleoptile into lower parts of the shoot. The role of conjugated IAA as a source of this free IAA is disputed by some (10,11).

Data presented in this dissertation contributes directly to the hypothesis, as presented, in the following way. In corn shoots one of the esters of IAA is known to be IAInos. An enzyme which hydrolyzes this ester is also present in shoot tissue. Studies on the relative distribution of the enzyme show that more activity can be extracted from coleoptiles than from mesocotyls of plants growing "normally" in the dark. Thus, this supports the idea that the coleoptile is important in auxin regulated growth.

In assessing the hypothesis one question that further studies should address is that of localization of the enzyme. Further investigation is required to determine if more enzyme is present at the scutellar end of the kernel, if the enzyme is present in the stele and cortex of the mesocotyl in different amounts, and if more enzyme is associated with the tip of the coleoptile or the coleoptilar node.

Other questions of special interest are those that pertain to regulation of IAInos hydrolase at the cellular level and at the whole plant level. It is not known if IAInos hydrolase activity increases in plants in which elongation growth is accelerated. Two conditions which

might be especially useful are growth in red light and growth of plants released from anaerobic conditions. In the first case, mesocotyl elongation is inhibited and coleoptile elongation is accelerated. Since enzyme activity is greater in coleoptiles perhaps de novo synthesis or activation of the enzyme might be responsible. In the second case, plants returned to aerobic conditions, after flooding for instance, undergo a growth spurt which is claimed to be accompanied by increased amounts of IAA. Either situation might provide a better source of material for extraction of IAInos hydrolase.

The studies on the purification and activity of IAInos hydrolase as presented in this dissertation describe methods by which future studies can assess the physiological significance of this enzyme.

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APPENDICES

APPENDIX A

pH Optima for Hydrolyses

pH Optimum for ^{3}H-IAInos Hydrolysis

The extract used in these experiments had been purified by $(NH_4)_sSO_4$ fractionation at 30 to 40% saturation and chromatography over DEAE-Cellulose, Sephadex G-200 and hydroxylapatite. The specific activity was 0.5 pmol IAInos hydrolyzed/hr/mg protein. Aliquots of the extract were dialyzed against the following buffers (all at 0.05 M) at the pH listed:

Citric acid-phosphate, pH 4.0, 5.0, 6.0

Tris-maleate, pH 6.0, 6.5, 7.0, 7.5

Tris, pH 7.5, 8.0

Samples were assayed as described earlier. Figure 1 shows net velocities at the different pH's.

pH Optimum for α-Naphthyl Acetate Hydrolysis

The extract used in these experiments was stage I enzyme. Acetone powders were extracted in the buffers listed below:

Citric acid-phosphate, pH 3.0, 4.0, 5.0, 6.0

KPO_4, pH 5.0, 6.0, 6.5, 7.0, 7.5, 8.0

Glycine-NaOH, pH 8.6, 8.8, 9.0, 9.2, 9.4, 9.6

Tris, pH 7.5, 8.0, 8.4, 8.6, 8.8, 9.0

General esterase activity was assayed as described earlier. Figure 2 shows the net velocities at the different pH's.

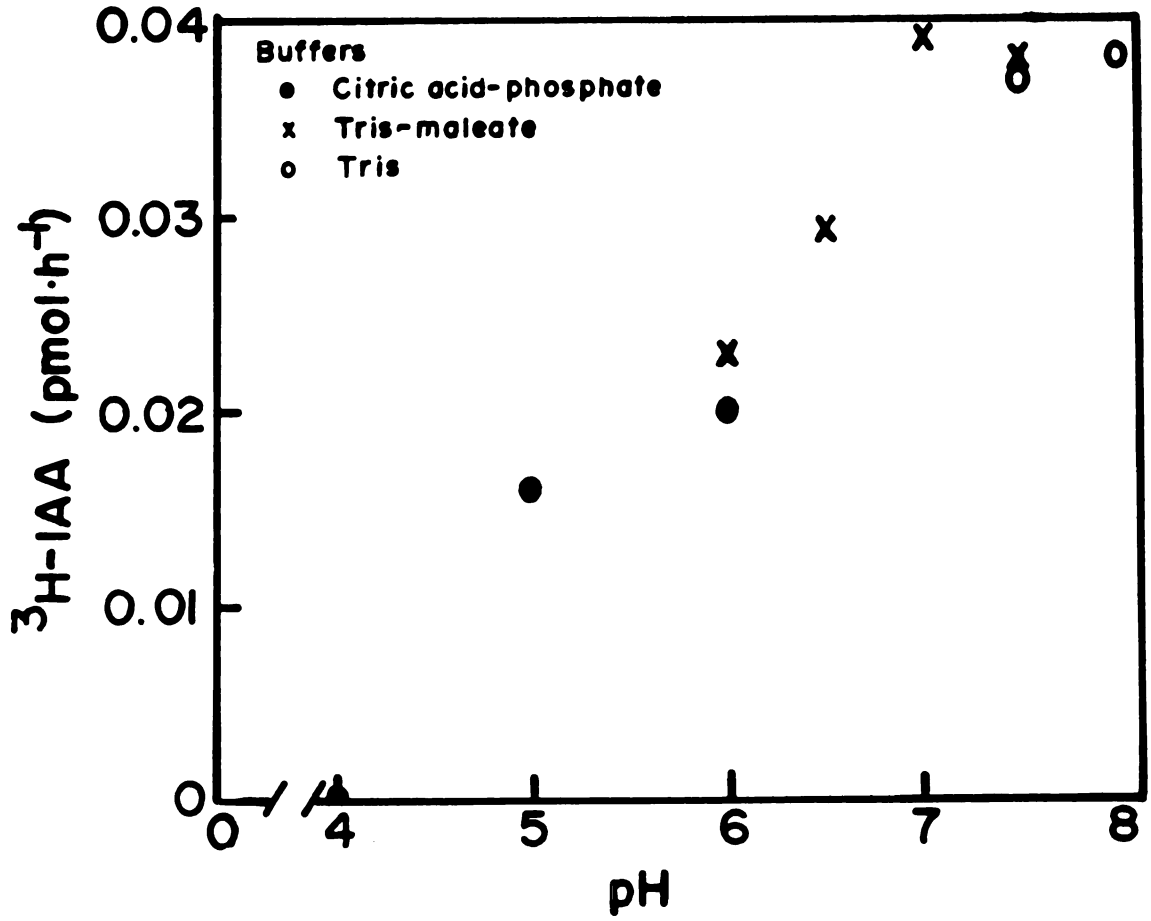

Figure 1. Effect of pH on hydrolysis of ^{3}H-IAInos. Aliquots of IAInos hydrolase were dialyzed against 0.05 M buffer at the pH indicated. Enzyme activity was measured as described previously.

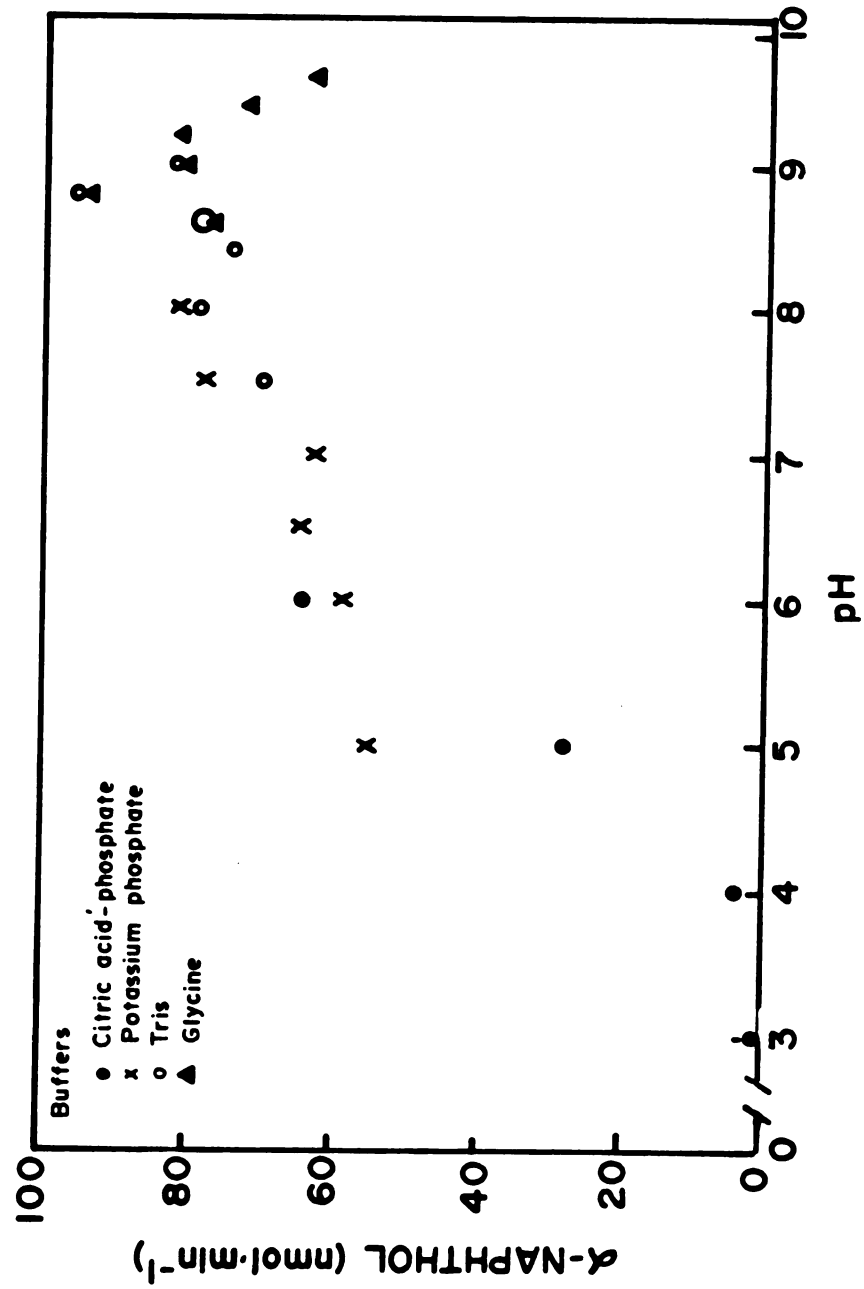

Figure 2. Effect of pH on hydrolysis of α-naphthyl acetate. Aliquots of Stage I enzyme were suspended in 0.05 M buffer at the pH indicated. Activity was measured as described previously.

APPENDIX B

Affinity Chromatography

Pengelly and Meins (3) developed a radioimmunoassay for IAA which involved making antibodies to IAA. To make IAA antigenic they coupled IAA to BSA with formaldehyde. This procedure is thought to link indoles to amino groups in protein via the indole ring nitrogen (4). The basis for the reaction had been studied by Fraenkel-Conrat et al (1,2) as part of a long series of papers examing the reactions of formaldehyde with protein. It was known that formaldehyde added to amino groups spontaneously at neutrality. It was also known that at high temperatures or in alkaline or acidic solutions formaldehyde would add as a methylol group to various N containing groups of proteins. On the basis of reactions of the addition products to gramicidin, Fraenkel-Conrat concluded that amines were linked to the indole N via a methylene bridge.

The method used by Pengelly and Meins to couple BSA with formaldehyde and then to IAA were mild enough to suggest that an ester of IAA might also undergo the coupling reaction without hydrolyzing the ester. Preliminary experiments demonstrated that the ethyl ester of IAA was stable under the coupling conditions for up to 48 h.

ω-Aminohexyl agarose with 5.2 μmoles of diaminohexane/ml was obtained from Sigma. The gel was washed and resuspended in distilled water. The following reactants were mixed in the order and proportions shown below:

1.0 ml Na acetate (3 M)

2.2 ml of a 7.5% solution of formaldehyde

1.0 ml of ethyl IAA - 10 mg/ml - plus 80 pmol ^{14}C-Me-IAA

1.0 ml ω-aminohexyl agarose

The reactants were sealed in a flask and the flask was gently agitated for 24 h. At the end of 24 h the agarose was gently pelleted. The radioactivity in the supernatant and that remaining in the gel were determined. Approximately 30% of the counts remained with the gel. However, UV difference spectra of dilute suspensions of coupled and uncoupled gel suggested that ethyl IAA was conjugated to only 6% of the available sites.

The gel was dialyzed against Kphos and then used in pouring a small column. The gel was washed extensively in the column also. A sample was applied to the column and any protein that was bound by the matrix was eluted with increasing amounts of NaCl up to 0.5 M NaCl in Kphos. Elution of protein and IAInos activity was similar irrespective of any attempts at ethyl IAA coupling.

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APPENDIX C

Effects of NAD+ and NADP+ on Hydrolysis

Goodenough and Entwistle (2) reported the purification to homogeneity of an esterase from apples. The final purification step was chromatography over Matrex blue gel A. Dye matrix affinity chromatography is often used in the isolation of enzymes which have a pyridine nucleotide cofactor requirement (5,6). Goodenough and Entwistle did not publish any rationale for their use of the Matrex chromatography, other than that it worked, nor did they present any data which might suggest a pyridine nucleotide requirement for enzyme activity.

Park et al (3) demonstrated the esterase activity of 3-phosphoglyceraldehyde dehydrogenase against p-nitrophenyl acetate, but that activity required the prior removal of bound pyridine nucleotides from the enzyme by adsorption onto charcoal. The NAD-free enzyme was inhibited by added NAD^+ or $NADP^+$ in its esterase activity. Two other aldehyde dehydrogenases, one from human liver (4) and one from horse liver (1) hydrolyze p-nitrophenyl acetate, but the addition of NAD^+ to the reaction medium accelerated the hydrolysis, as did NADH.

In hopes that IAInos hydrolase might be affected by pyridine nucleotides, I assayed several enzyme preparations to determine if any acceleration or inhibition resulted. None of the enzyme preparations assayed were affected by $NADP^+$ (1 mM) in the hydrolysis of α-naphthyl acetate. A series of preparations with varying amounts of IAInos hydrolase activity were assayed for the hydrolysis of IAInos in the

presence or absence of 0.5 mM NAD^+. The added NAD^+ did not seem to affect the hydrolysis of IAInos.

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APPENDIX D

Inhibition by Fluoride (F^-) of α-Naphthyl Acetate Hydrolysis

In the course of assaying crude and partially purified extracts for phosphatase activity, 10 mM NaF was tested as an inhibitor. As a phosphatase inhibitor it works. $MgCl_2$ (10 mM) increases phosphatase activity in *Z. mays* extracts, and some of the phosphatase activity is insensitive to NaF in the presence of added $MgCl_2$.

I examined the effect of NaF on the hydrolysis of α-naphthyl acetate. $MgCl_2$ does not affect α-naphthyl acetate esterase activity in these extracts. NaF inhibits esterase activity; activity in the presence of NaF is about 45% that of controls for Stage I enzyme. An extract partially purified as described in Appendix A, VB, is inhibited only 20 to 25%. Inhibition by NaF of IAInos hydrolysis was not assayed. Results are summarized in the following table. The specific activity of stage I enzyme was 15 nmol/min/mg protein for the hydrolysis of α-naphthyl acetate, while that of VB was 44 nmol/min/mg.

Since the specific activity of VB is greater than that of stage I enzyme, and the F^- inhibition is less, perhaps the esterase (or esterases) in this fraction are less sensitive to F^-, or perhaps the population of esterases is skewed in favor of insensitive esterases. The activity of wheat germ phosphatase in ester hydrolysis is probably explained by the enzyme not being pure. When the phosphatase is electrophoretically separated on acrylamide and the gel stained for esterase activity, four esterase bands can be observed, whereas a potato phosphatase sample has no esterase activity on gels, and practically none in the spectrophotometric assay.

TABLE 1. Inhibition by fluoride of α-naphthyl acetate hydrolysis.

Extract	Phosphatase Activity[a]				Esterase Activity[a]			
	Control	$+Mg^{2+}$	+NaF	$+Mg^{2+}$ +NaF	Control	$+Mg^{2+}$	+NaF	$+Mg^{2+}$ +NaF
Wheat germ Phosphatase	100	192	54	115	100	100	77	-
Stage I enzyme	100	136	50	77	100	100	46	46
VB	100	100	-	41	100	100	80	80

[a]Activity as percent of control activity.

APPENDIX E

Environmental Perturbations and Enzyme Activities

The purpose of these experiments was to see if either light or gravity had any effect on IAInos activity or general esterase activity.

Four d dark-grown Z. mays seedlings sown in paper towel rolls were used. For the light experiments, seedlings were unrolled, placed in a support and kept moist while being exposed to a fluorescent room light at a distance of 1 m for 20 min. Shoots were removed and cut into 2 sections, mesocotyl and coleoptile (node plus coleoptile and primary leaves). Sections were collected in a preweighed beaker at the temperature of dry ice. The fresh weight of the segments was determined and then the tissue was used to make acetone powders. Dark controls were harvested similarly. For the gravity experiments shoots in the paper towel rolls were laid horizontally while in the dark, then mesocotyls and coleoptiles were harvested as above. Control plants were maintained vertically in the dark until harvesting.

From approximately 10 g fresh weight of coleoptile segments 0.7 to 0.8 g acetone powder was obtained, while 10 g fresh weight of mesocotyl segments yielded 0.3 to 0.4 g powder.

Stage I enzyme was prepared from each powder as described earlier except that 0.1 g powder was extracted in 3.0 ml Tris. Each extract was assayed for protein concentration, general esterase activity and IAInos hydrolase activity. The results are summarized in the following table.

TABLE 1. Summary of results of environmental perturbations.

	IAInos Hydrolase Activity (pmol/h/mg protein)	(pmol/h/plant part)[a]	α-Naphthyl Acetate Activity (nmol/min/mg protein)	(nmol/min/plant part)[a]
Light Experiments				
Control Plants				
Coleoptile	0.010 ± 0.002	0.025	41 ± 5	105
Mesocotyl	0.008 ± 0.001	0.007	51 ± 8	47
Treated Plants				
Coleoptile	0.012 ± 0.002	0.030	39 ± 6	98
Mesocotyl	0.014 ± 0.001	0.013	49 ± 9	45
Gravity Experiments				
Control Plants				
Coleoptile	0.013 ± 0.003	0.035	51 ± 10	128
Mesocotyl	0.009 ± 0.001	0.008	46 ± 4	42
Treated Plants				
Coleoptile	0.013 ± 0.002	0.033	47 ± 9	118
Mesocotyl	0.005 ± 0.002	0.005	47 ± 3	43

[a]Conversion of activity from rate/mg protein to rate/coleoptile or mesocotyl was done in the following way. For coleoptiles is was known 1) 14 coletoptiles/g fresh weight, 2) 0.7-0.8 g acetone powder/10 g fresh weight, and 3) 15 mg protein/ml buffer.

$$\frac{14 \text{ coleoptiles/g fresh wt}}{0.75 \text{ g powder/10 g fresh wt}} = 186 \text{ coleoptiles/g powder}$$

$$\frac{15 \text{ mg protein/ml buffer}}{100 \text{ mg powder/3 ml buffer}} = 0.45 \text{ mg protein/mg powder}$$

$$\frac{186 \text{ coleoptiles/1000 mg powder}}{0.45 \text{ mg protein/mg powder}} = 0.4 \text{ coleoptiles/mg protein}$$

$$\frac{\text{rate/mg protein}}{0.4 \text{ coleoptiles/mg protein}} = \text{rate/coleoptile}$$

For mesocotyls it was known 1) 8 mesocotyls/g fresh weight, 2) 0.3-0.4 g acetone powder/10 g fresh weight, and 3) 7 mg protein/ml buffer. A similar calculation for mesocotyls gives a conversion factor of 1.09 mesocotyls/mg protein.

On the basis of experiments described here coleoptiles have higher specific activity for IAInos hydrolysis than mesocotyls where specific activity is defined as the rate/mg protein. The actual activity per part is also higher for IAInos hydrolysis in coleoptiles. The specific activity for α-naphthyl acetate hydrolysis is about the same for mesocotyls and coleoptiles, although on a per plant part basis coleoptiles are more active. The only really significant change in activity seemed to be in mesocotyls exposed to light. The IAInos hydrolase activity appears to have doubled. When exposed to a gravitational stimulus the IAInos hydrolase activity is slightly decreased. The activity in coleoptiles does not change significantly in either case. I would have predicted that in plants exposed to light, in which growth would be inhibited, activity would decrease. In gravistimulated plants activity might be expected to increase or remain the same. In either case since more activity is observed in coleoptiles, and because we usually think of the tip as the source of free IAA, I would have expected activity to change in that part of the plant rather than in mesocotyls. This kind of experiment needs many repetitions under better controlled experimental conditions before any firm conclusions can be drawn. It is interesting to note that esterase activity does not change. It would be interesting to see if rates of hydrolysis of Me-IAA change.

APPENDIX F

Synthesis of α-Naphthyl Acetate

α-Naphthyl acetate was synthesized by the method of Spassow (1). α-Naphthol (0.1 mol) and 1.2 g Mg turnings were mixed in 35 ml benzene. Acetyl chloride (0.1 mole) was added and the mixture was refluxed for 1 h. After cooling, 150 ml diethyl ether was added and the mixture transferred to a separatory funnel. The solution was washed with 150 ml water, 150 ml of a saturated solution of $NaHCO_3$, and 150 ml water. The ether-benzene phase was dried over anhydrous, granular Na_2SO_4, then dried *in vacuo*. The brown oily residue was chilled on ice and precipitated as a beige paste. The precipitate was redissolved in 2-propanol and chilled. The resulting crystals, which were soluble in ethanol and acetone, were redissolved in 2-propanol and chilled. After recrystallization the product was filtered and washed with cold 2-propanol, then dried over P_2O_5 under vacuum. The resulting needle-like crystals were stored in a brown bottle at room temperature. The melting point was 45°C. Spassow reported a melting point of 46°C.

The product, α-naphthyl acetate, at pH 7.15, has absorbance maxima at 264 and 272 nm and does not absorb at 320 nm. α-Naphthol absorbs maximally at 279 nm with a smaller sharp peak at 320 nm and a shoulder at 305 nm. α-Naphthyl acetate was suspended in Tris at pH 7.5, and the pH was raised to 12 by the dropwise addition of 2N NaOH. After 15 min 2N HCl was added until the pH returned to 7.5. The α-naphthol released by alkaline hydrolysis had a UV absorption spectrum identical to that of authentic α-naphthol at pH 7.5.

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APPENDIX G

Calculations Used to Determine the Amount of IAA Produced by Hydrolysis of IAInos

Sample Calculation

Sample	Total dpm/rxn[1]	Total dpm/fraction[2]	Recovery[3] (%)	Acidic[4] (%)	Velocity[5]	Specific Activity[6]
-E(Control, no extract)	278996					
-EN(Control, neutral fraction)		232254				
-EA(Control, acidic fraction)		20475	90.6	8.1		
(Sample-50 µl/rxn)	295741					
(Sample, neutral fraction)		203289				
(Sample, acidic fraction)		49081	85.3	19.4	0.151	0.134

The sample contained 22.6 mg protein/ml.

The reaction mixture was incubated 4.5 h.

[1]Radioactivity in a 5 µl aliquot from each reaction mixture is measured, corrected for efficiency of counting and corrected to give dpm/100 µl reaction mixture.

[2]Radioactivity in neutral fractions is totaled for each sample, and corrected for efficiency of counting. Radioactivity in acidic fractions is treated in the same way.

$$[3]\text{Recovery} = \frac{\text{Neutral fraction dpm + acidic fraction dpm}}{\text{Average dpm/reaction}}$$

$$[4]\ \text{Acidic (\%)} = \frac{\text{Acidic fraction dpm}}{\text{Neutral fraction dpm + acidic fraction dpm}}$$

[5]pmol IAA produced/h/µl of extract. In this example 6 pmol of ^{3}H-IAInos was added to the reaction mixture, incubation time was 4.5 h, and 50 µl of extract was used.

$$\text{Velocity} = \frac{\text{\% Acidic (sample) - \% Acidic (control)}}{4.5\ \text{h}} \times 6\ \text{pmol} = 0.151\ \text{pmol/hr/50 µl extract}$$

[6]pmol/h/mg protein. In this example the protein concentration was 22.6 mg/ml.

$$\text{Specific Activity} = \frac{0.151\ \text{pmol/h/50 µl extract}}{1.13\ \text{mg protein/50 µl}} = 0.134\ \text{pmol/h/mg protein}$$

APPENDIX H

Calculations Used to Determine the Amount of α-Naphthol Produced by Hydrolysis of α-Naphthyl Acetate

The change in absorbance at 320 nm (ΔA_{320}) is measured between 100 and 400 seconds.

(1) $\Delta A_{320}/min = \Delta A_{320}/300$ sec x 60 sec/min

(2) Concentration (C) = nmoles α-naphthol produced/min/volume of extract

$$= \frac{A_{320}/min}{2496} \times 3.0 \text{ ml} \times 10^{-3} \text{l/ml} \times 10^{9} \text{ nmol/mol}$$

from Beer's Law Concentration (C) = $\frac{\Delta A}{\varepsilon b}$ where b = cm and ε = 2496 1/mol/cm for α-naphthol at pH 7.5.

Sample Calculation for a reaction which contained 50 µl Stage III enzyme. The ΔA_{320} = 0.195/300 sec.

(1) $\Delta A_{320}/min$ = 0.195/300 sec/50 µl x 60 sec/min = 0.039/min/50 µl

(2) $C = \frac{0.039/min/50\ \mu l}{2496\ 1/mol/cm \times 1\ cm} \times 3.0 \text{ ml} \times 10^{-3} \text{l/ml} \times 10^{9} \text{ nmol/ml}$

= 46.9 nmol/min/50 µl

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