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Partial Purification and Characterization of Indol-3-Ylacetylglucose:*myo*-Inositol Indol-3-Ylacetyltransferase (Indoleacetic Acid-Inositol Synthase)[1]

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ABSTRACT

A procedure is described for the purification of the enzyme indol-3-ylacetylglucose:*myo*-inositol indol-3-ylacetyltransferase (IAA-*myo*-inositol synthase). This enzyme catalyzes the transfer of indol-3-ylacetate from 1-0-indol-3-ylacetyl-β-D-glucose to *myo*-inositol to form indol-3-ylacetyl-*myo*-inositol and glucose. A hexokinase or glucose oxidase based assay system is described. The enzyme has been purified approximately 16,000-fold, has an isoelectric point of pH 6.1 and yields three catalytically inactive bands upon acrylamide gel electrophoresis of the native protein. The enzyme shows maximum transferase activity with *myo*-inositol but shows some transferase activity with scyllo-inositol and *myo*-inosose-2. No transfer of IAA occurs with *myo*-inositol-D-galactopyranose, cyclohexanol, mannitol, or glycerol as acyl acceptor. The affinity of the enzyme for 1-0-indol-3-ylacetyl-β-D-glucose is, K_m = 30 micromolar, and for *myo*-inositol is, K_m = 4 millimolar. The enzyme does not catalyze the exchange incorporation of glucose into IAA-glucose indicating the reaction mechanism involves binding of IAA glucose to the enzyme with subsequent hydrolytic cleavage of the acyl moiety by the hydroxyl of *myo*-inositol to form IAA *myo*-inositol ester.

This work deals with the partial purification and characterization of indol-3-ylacetylglucose:*myo*-inositol indol-3-ylacetyltransferase (IAInos synthase).[3] The enzyme catalyzes the reaction between 1-0-indol-3-ylacetyl-β-D-glucose and *myo*-inositol to form indol-3-ylacetyl-*myo*-inositol and glucose according to the following equation:

$$\text{1-0-IAGlu} + \textit{myo}\text{-inositol} \rightarrow \text{IAInos} + \text{Glu} \quad (1)$$

The reaction is important because mature kernels of *Zea mays* sweet corn contain 99% of their endogenous IAA as ester conjugates and less than 1% as the free acid (8). One-half of the esters are conjugates of IAA and *myo*-inositol or IAA and *myo*-inositol glycosides (8). The kernels contain small amounts of the isomeric conjugates of IAA and glucose, 2-0, 4-0, and 6-0-IAGlu (13). 1-0-IAGlu can be demonstrated to occur naturally in small amounts if precautions are used to prevent acyl migration (J Cohen, personal communication). An IAA glucose conjugate was the first IAA ester conjugate to be isolated from plants and was found following application of labeled IAA to plants (21, 35).

1-0-IAGlu is an acyl alkyl acetal[4] and is chemically distinct, from an IAA ester of glucose. In aqueous solution, especially at a pH of 7 or above, the IAA moiety migrates to the position farthest from the carbonyl, that is, the 4-0 and 6-0 position. Owing to this facile acyl migration (11, 13, 19, 20, 25–27) the bond energy of the acyl alkyl acetal is not readily determined but the equilibrium of the reaction:

$$\text{IAA} + \text{UDPG} \leftrightarrow \text{1-0-IAGlu} + \text{UDP} \quad (2)$$

is of the order of $K_{eq} = 10^{-1}$ (27) thus placing the free energy of hydrolysis of IAA glucose at about 1400 calories above that of the phosphato glucose bond of UDPG and many thousands of calories above that of an ester bond.

These observations concerning energetics may explain the thermodynamics of the manner in which liquid endosperm of maize can contain 0.1 mM IAA ester. Once 1-0-IAGlu is synthesized, even with a large positive free energy change, the IAA may be transacylated to an acceptor alcohol such as *myo*-inositol with a large negative free energy change. Thus, the sum of the free energy changes of reactions (Eqs. 1 plus 2) will be negative and the reaction will proceed with the accumulation of IAInos. Glycosylation of IAInos, as in IAInos-galactose or IAInos-arabinose, would move the equilibrium further toward IAA-inositol ester accumulation (9, 10, 25).

In this report we detail the purification of IAInos synthase to a high degree of purity and characterize the enzyme with regard to affinity and substrate specificity.

Previous studies established the wide-spread occurrence in

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[3] Abbreviations: IAInos, indol-3-ylacetyl-*myo*-inositol; 1-0-IAGlu, 1-0-indol-3-ylacetyl-β-D-glucose; 2-0-, 4-0, 6-0-IAGlu, 2-0-, 4-0-, 6-0-indol-3-ylacetyl-β-D-glucose; IAGlu, indol-3-ylacetylglucose; IAGlu synthase, UDP-glucose:indol-3-ylacetate glucosyltransferase; IAInos synthase, indol-3-ylacetylglucose:*myo*-inositol indol-3-acetyltransferase.

[4] There is no generic term to designate the reaction product between an aldehyde and a carboxylic acid or phosphoric acid. Clearly there is a large difference in the free energy of hydrolysis of, for example, glucose-1-phosphate and glucose-6-phosphate and, in the present case, 1-0-IAA-glucose and 6-0-IAA-glucose. In the absence of a concise generic term, the descriptive name "acyl alkyl acetal" has been suggested by Professors Laurens Anderson and Derek Horton for the chemical grouping (personal communication).

nature of IAA-*myo*-inositol esters including *Zea mays* sweet corn, field corn, and pop corn, *Trypsicum,* teosinte (8), rice (16), tobacco (1), and the horse chestnut, Aesculus sp. (12). Studies from this laboratory established the mechanism of the enzymatic synthesis of IAInos, IAInos galactose, and IAInos arabinose (9, 10, 28). The separation of the activities of IAGlu synthase from IAInos synthase, and some properties of IAInos synthase were earlier reported (28). The purification and characterization of the enzyme catalyzing the synthesis of 1–0-IAGlu has been described (23, 24, 26–28).

MATERIALS AND METHODS

Plant Material

The liquid endosperm of *Zea mays* sweet corn, variety Seneca Horizon was prepared and frozen as previously described (24, 26). Storage was at −85°C.

Reagents

PEG 6000, UDP, *myo*-inositol, *myo*-inositol-2-monophosphate, *myo*-inosose-2, PIPES, mercaptoethanol, mol wt standards, and the hexokinase-glucose-6-phosphate dehydrogenase glucose assay kit (Glucose HK No. 16-UV) were from Sigma; DTT and Tris from Boehringer Mannheim Biochemicals; Coomassie brilliant blue G-250 and all chemicals for polyacrylamide gel electrophoresis from Bio-Rad; Coomassie brilliant blue R from Serva; Blue Dextran 2000, DEAE-Sephacel, Sephadex G-25, materials for chromatofocusing from Pharmacia; ^{14}C-glucose from ICN; Glycerol from Baker; 1–0-IAGlu was synthesized by Dr. D. Keglevic (19) and was a gift from Dr. Jerry Cohen. *Myo*-inosose-2 and *scyllo*-inositol were gifts from Professor Laurens Anderson. Mixed isomers of IAGlu were synthesized in this laboratory by Mr. Jacek Kesy and Aga Schulze (personal communication) and were comprised of 1–0, 6.5%; 2–0, 9%; 4–0, 43%; 6–0, 40%, and 1.6% an undetermined IAA containing compound, possibly $(IAA)_2$-glucose. Unlabeled IAA-*myo*-inositol was synthesized according to the general procedure of Nowacki *et al.* (30).

Analytical

Electrophoresis

The procedure of Ogita and Markert (31) was followed using an 8% polyacrylamide gel for native protein and 11% gel for SDS denatured proteins. Usually, gels of 7 × 10 cm were used and an applied voltage of 250 V. Native proteins were stained with 0.04% Coomassie brilliant blue G-250 in 3.5% perchloric acid and 20% methanol. For SDS denatured proteins, 0.1% Comassie brilliant blue was used in 50% trichloroacetic acid.

Protein Determination

Protein concentrations were determined by the Bradford method (4) using BSA as a standard.

Enzyme Assay

Qualitative

A convenient qualitative assay, useful for localizing enzyme activity following column chromatographic purification, was based upon separation of the reaction products by TLC. For this assay incubation was in a 0.5 mL Eppendorf tube for from 5 to 30 min at 25°C using equal volumes (ranging from 10 to 50 μL depending upon the needs of the experiment) of 30 mM *myo*-inositol in 300 mM PIPES (pH 6.8); 1.5 mM 1–0-IAGlu in water; and enzyme. The reaction was stopped by drying a 5 μL aliquot on a Silica Gel 60 TLC plate and irrigating with A solvent (25) consisting of ethyl acetate, methyl ethyl ketone, ethyl alcohol, and water, 5:3:1:1. Detection was by means of the Ehmann reagent (14) used as a dip, followed by blotting, and drying at 5 min at 100°C. The R_f of the various components was IAA 0.83, isomeric (mainly 4–0 and 6–0) IAGlu 0.59 and 0.50, 1–0-IAGlu 0.54, and IAInos at 0.36. Thus, conversion of 1–0-IAGlu to IAInos is readily visualized and a 5 by 20 cm plate chromatographed in the short dimension permits dozens of simultaneous qualitative assays.

Glucose Oxidase Assay

The reaction mixture contained 100 μL of 8 mM 1–0-IAGlu, 200 μL of 40 mM *myo*-inositol, 400 μL of 200 mM K phosphate (pH 7.0), 100 μL of enzyme, and 100 μL of Worthington Glucostat reagent. The reaction was started by the addition of IAGlu and stopped by the addition of 100 μL of 2 N HCl. The progress of the reaction may be judged visually, before the addition of HCl, by the development of a brownish tint. The amount of glucose liberated may be quantitatively determined by means of the increase in absorbancy at 412 nm as compared to a control tube lacking *myo*-inositol.

Hexokinase Assay

The reaction mixture contained equal volumes (50 or 100 μL) of 300 mM PIPES (pH 6.8) containing 30 mM *myo*-inositol, 1.5 mM 1–0-IAGlu in water, and enzyme with a total reaction volume of 150 or 300 μL. Incubation was for 30 min

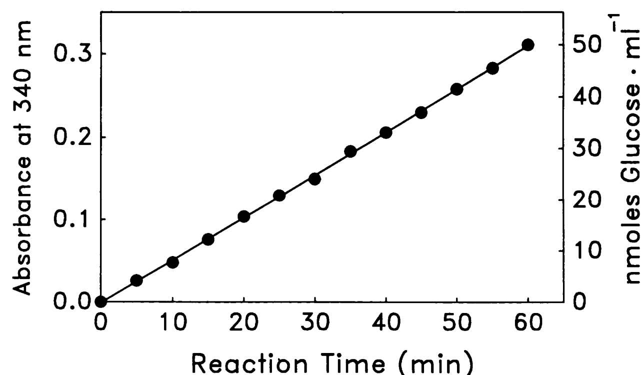


Figure 1. Reaction rate as a function of time. At 60 minutes 30% of the 1–0-IAGlu was used and 1.6% of the added *myo*-inositol. Assay of glucose liberated was by means of the hexokinase assay.

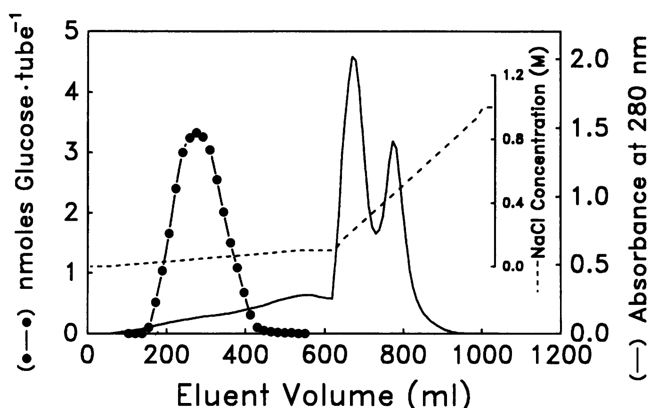


Figure 2. Elution profile of IAInos synthase from a DEAE-Sephacel column. The NaCl gradient was from 0 to 0.1 M NaCl since the enzyme eluted at 0.06 M NaCl. The bulk of the protein eluted after the enzyme and was eluted at higher NaCl concentrations. Enzyme activity was determined by means of the hexokinase-glucose-6-phosphate dehydrogenase assay.

at 25°C and the reaction stopped by heating for 2 min in a boiling water bath. Glucose was determined by means of the Sigma Glucose Diagnostic Kit-16–10.

RESULTS

Determination of Enzyme Activity

The assay procedure was as described in "Materials and Methods" using 0.5 mM 1-0-IAGlu and 10 mM *myo*-inositol. These concentrations are 10 times the K_m for IAGlu and 2.5 times that for *myo*-inositol. Under these conditions the reaction rate, as shown in Figure 1, is a linear function of time in the absorbancy range of 0 to 0.3 AU corresponding to the synthesis of 0 to 50 nmol of IAInos. Because the commercial glucose oxidase and hexokinase used in the assay procedure caused some hydrolysis of 1-0-IAGlu, it was necessary to use a blank sample with no inositol as a control. In addition, crude enzyme preparations contain some hydrolytic activity towards 1-0-IAGlu and since hydrolysis results in the formation of free glucose it was again necessary to use a control without inositol.

Enzyme Purification

Stage 1, PEG and DEAE Fractionation

A 300 g portion of frozen corn liquid endosperm, obtained as previously described (24, 26, 27), was thawed and mixed with 225 mL of 0.2 M Tris-Cl buffer (pH 7.6), homogenized briefly in a Waring Blender, chilled in an ice bath, and brought to 5% PEG by the addition, with stirring, of a solution of 45% PEG in buffer. The mixture was centrifuged at 13,000*g* for 15 min and the pellet was discarded. The supernatant fluid was brought to 15% PEG and, after 2 h, the mixture was centrifuged at 13,000*g* for 60 min and the supernatant fluid discarded. The pellet was dissolved in 150 mL of 25 mM Tris-Cl (pH 7.8), centrifuged and the supernatant solution applied to a 2.5 × 20 cm DEAE-Sephacel column equilibrated with 25 mM Tris-Cl. The column was washed with 500 mL of equilibration buffer and proteins eluted with a 0 to 0.1 M NaCl gradient in buffer. The elution profile is shown in Figure 2. Active fractions eluted at about 200 to 380 mL and were pooled and concentrated to a volume of 8 to 10 mL by means of a Diaflo YM-30 Amicon filter for chromatofocusing.

Stage II, Chromatofocusing

A 1 × 30 cm Pharmacia PBE 94 column was equilibrated with 25 mM imidazole-acetate buffer at pH 7.4 and a flow rate of 20 mL per h until the pH of the effluent was 7.4. The

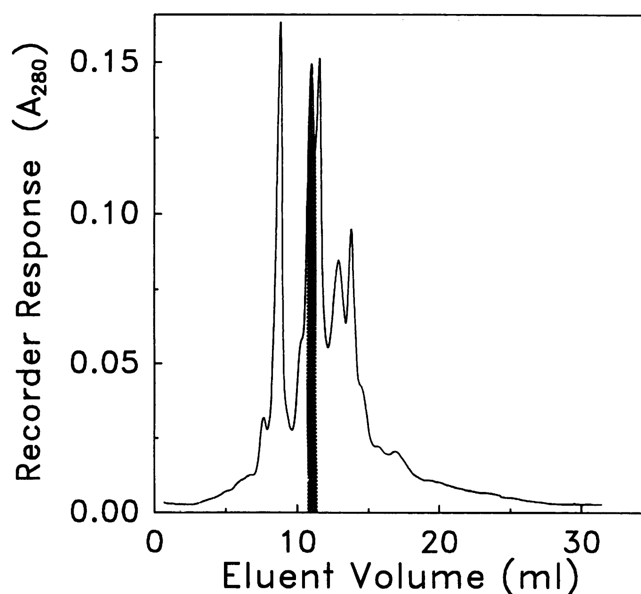


Figure 3. Protein elution profile from a Pharmacia Mono Q HR 5/5 FPLC column. All of the enzymatic activity was in 1.5 mL corresponding to peak 2 and is indicated by the shaded area.

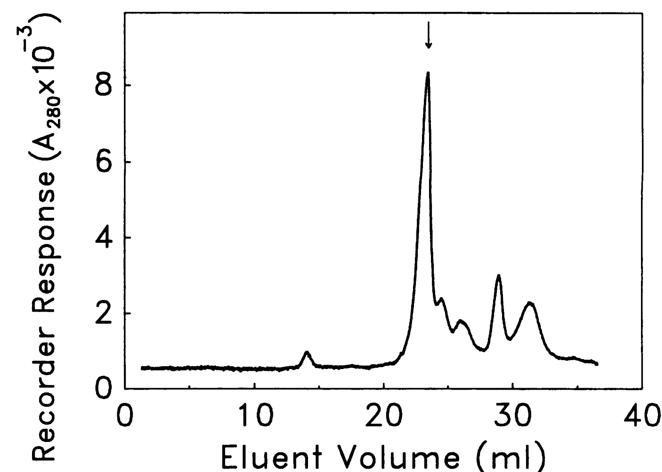


Figure 4. Elution profile of protein from a Pharmacia Superose 12 HR 10/30 gel filtration column. All activity eluted in 2.5 ml with an elution volume of 22.7 mL as indicated by the arrow.

Table I. *Purification of IAGlu Synthase from 300 g of Liquid Endosperm*

Stage	Specific Activity	Total	Yield
mg protein	*nmol min*$^{-1}$ *mg*$^{-1}$	*μmol min*$^{-1}$	
A. Crude extract			
4280	0.84	3.60	100%
B. After PEG (5–15%)			
1140	1.96	2.23	62%
C. After DEAE-Sephacel (0.0–0.1 M NaCl) Stage I			
24.5	82.4	2.02	56%
D. After chromatofocusing (Stage II)			
1.1	1776	1.90	53%
E. After Mono Q (Stage III)			
0.14	13300	1.86	52%
Total purification—15,800-fold			

protein solution from stage I was diluted to a volume of 20 mL to decrease the ionic strength and applied to the column. Elution was with Polybuffer 96 (pH 6.0) prepared from a stock solution according to the manufacturers directions. The isoelectric point of the enzyme was found to be pH 6.1.

Stage III, Mono Q-FPLC

A Mono Q HR 5/5 (Pharmacia) column was equilibrated with 25 mM Tris-Cl (pH 7.8). Active fractions from stage II were pooled, concentrated by ultrafiltration, desalted on a 1 × 15 cm Sephadex G-25 column, and applied to the Mono Q column. Elution was with a gradient of 0 to 0.1 M NaCl at a flow rate of 0.5 mL h^{-1}, a pressure of about 175 psi, and collecting fractions of 0.5 mL. The elution profile is shown in Figure 3 with the major activity eluted at 10.8 mL corresponding to 0.06 M NaCl.

Stage IV, Superose 12 FPLC Gel-Filtration

The column was equilibrated with 0.1 M Tris-Cl (pH 7.0) at a flow rate of 0.5 mL min^{-1} and a pressure of about 95 psi. The protein from stage III was concentrated by ultrafiltration, with centrifugation, through a Milipore Ultrafree-MC filter unit. An aliquot of 50 μL containing 20 μg of protein was applied to the Superose column. The elution profile is shown in Figure 4 with peak enzymatic activity eluted at 22.7 mL corresponding to a molecular mass of 59.2 kD.

Almost 90% of the activity of the enzyme is lost during the FPLC step and so the specific activity of Stage IV enzyme is not included in the summary of the purification procedure.

Summary of the Purification Procedure

A summary of the purification procedure is shown in Table I. As can be seen, the enzyme is purified 13,000-fold with a recovery of about 50% of the starting units. Despite the enormous -fold purification, the enzyme at this stage of purification, still yields three bands upon electrophoresis either as the native protein or upon an SDS gel. Up to the gel filtration and acrylamide electrophoresis steps the enzyme was stable and, in fact, the Mono Q, step was conducted at room temperature.

Enzyme Electrophoresis

The electrophoretic pattern of both native and SDS denatured protein is shown in Figure 5, A and B. As can be seen, a characteristic three-banded pattern is apparent even at the DEAE column stage and continues through stages II (chromatofocusing) and III (Mono Q). Activity is lost by as much as 90% after the Sepharose 12 FPLC step and is totally lost following acrylamide gel electrophoresis of native protein although the three bands are retained. Additional attempts at chromatography on a Sepharose 12 FPLC gel filtration column yielded a single peak with small activity and again electrophoresis yielded the three band pattern lacking activity after elution. Possibly two of the three bands observed on acrylamide are contaminant proteins, or result from denaturation of a pure protein, resulting in bands that separate on acrylamide but do not separate on Sepharose. Without recovery of activity it is difficult to distinguish between these possibilities.

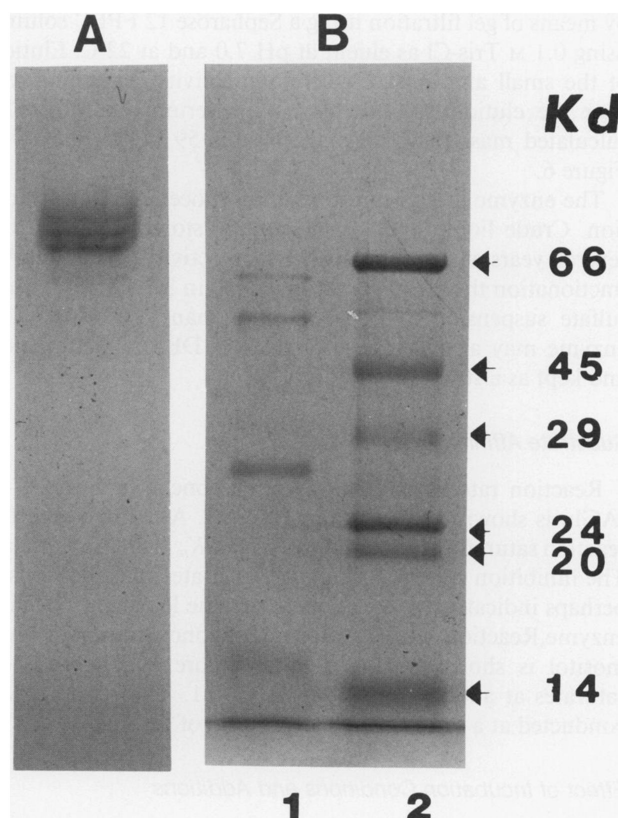


Figure 5. Acrylamide gel electrophoresis pattern as detected by Coomassie brilliant blue staining. A, Native protein after the Superose 12 step; B, lane 1 is SDS page of the preparation after the Superose 12 step. Lane 2 shows the following molecular mass marker proteins, bovine albumin (66 kD), egg albumin (45 kD), glyceraldehyde-3-phosphate dehydrogenase (36 kD), carbonic anhydrase (29 kD), trypsinogen (24 kD), trypsin inhibitor (20 kD), and lactalbumin (14 kD).

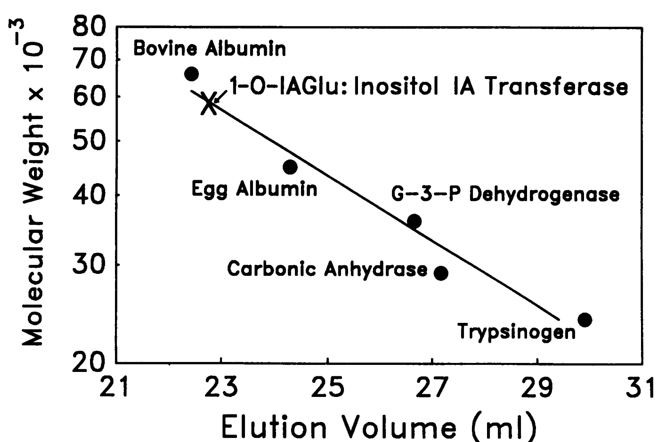


Figure 6. Estimation of molecular mass of IAInos synthase by gel filtration on a Pharmacia Superose 12 HR 10/30 column. Elution was with 100 mM Tris-Cl buffer at pH 7.0. Molecular masses as described for Figure 5. The elution volume of the IAInos synthase was 22.7 mL and corresponds to a molecular mass of 59 kD.

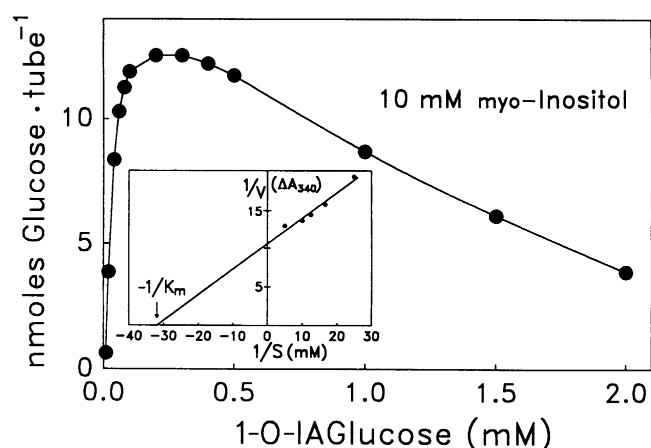


Figure 7. Reaction rate as a function of concentration of 1-0-IAGlu in the presence of 10 mM *myo*-inositol. The inset shows a Lineweaver-Burk plot indicating the K_m for 1-0-IAGlu to be 30 μM.

Molecular Properties of the Enzyme

The apparent molecular mass of the enzyme was estimated by means of gel filtration using a Sepharose 12 FPLC column using 0.1 M Tris-Cl as eluent at pH 7.0 and at 22°C. Elution of the small amounts of enzymatic activity remaining was near the elution volume for bovine serum albumin. The calculated mass for IAInos synthase is 59 kD as shown in Figure 6.

The enzyme is stable up to the last procedures of purification. Crude liquid endosperm may be stored at −20°C for several years without major loss of activity. After DEAE fractionation the enzyme may be stored in 3.2 M ammonium sulfate suspension at 4°C for more than 2 months. The enzyme may also be freeze dried after DEAE fractionation and kept as a stable dry powder.

Substrate Affinity

Reaction rate as a function of the concentration of 1-0-IAGlu is shown by the data of Figure 7. As can be seen the reaction saturates at about 0.15 mM with K_m = 30 micromolar. The inhibition observed at higher substrate concentrations is perhaps indicative of acylation of enzyme leading to inactive enzyme.Reaction rate as a function of concentration of *myo*-inositol is shown by the data of Figure 8. The reaction saturates at about 8 mM with K_m = 4.1. The reaction was conducted at a saturating concentration of IAGlu.

Effect of Incubation Conditions and Additions

The effect of pH on the reaction can not be examined above pH 7 owing to rapid acyl migration of 1-0-IAGlu to, predominantly, 6-0-IAGlu with a lesser amount of 4-0-IAGlu (24). The reaction is however 10 times as rapid at pH 7 as at pH 6 so experiments were routinely conducted at pH 6.8.Sulfhydryl compounds are inhibitory to the reaction with 98% inhibition obtained at a dithiothritol concentration of 5 mM and 74% inhibition with 5 mM mercaptoethanol (Table II). Inhibition was also obtained with dithionite at a concentration of 40 mM and so possibly the SH reagents cleave an essential disulfide bond.

Substrate Specificity

Among the acyl acceptor alcohols tested only *myo*-inositol, scyllo-inositol, and *myo*-inosose-2 functioned appreciably as acceptors of IAA from IAGlu. Glycerol, mannitol, and cyclohexanol were completely ineffective. A small activity was observed with *myo*-inositol-2-monophosphate but this might be owing to small amounts of *myo*-inositol as a contaminant. Owing to the ubiquitous occurrence of IAA-aspartate, aspartic acid was tested as IAA acceptor but was totally inactive.

Reaction Mechanism

Shortly after the discovery of the transacylation of IAA from 1-0-IAGlu to *myo*-inositol, Tkotz and Strack (34) found

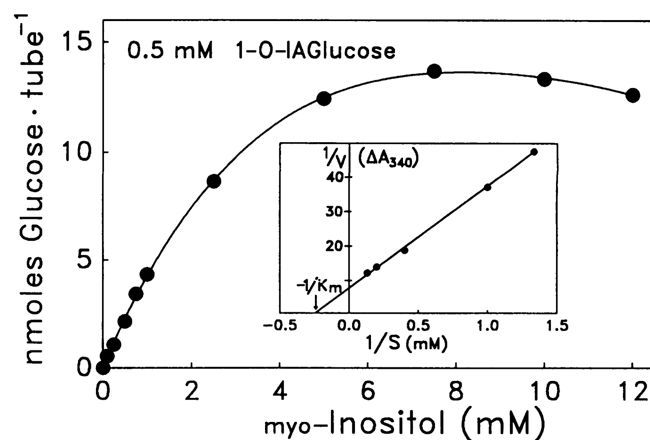


Figure 8. Reaction rate as a function of *myo*-inositol concentration in the presence of 0.5 mM 1-0-IAGlu. The inset shows a double reciprocal Michaelis-Menton plot indicating a K_m for *myo*-inositol of 4.1 mM.

Table II. *Substrate Specificity*

Enzyme, 1-0-IAGlu, and the indicated substrate were incubated together as described for the hexokinase assay. Glucose liberated was measured by the hexokinase assay as described in "Materials and Methods." Substrates were present at 10 mM concentration except as indicated. DTT and mercaptoethanol were preincubated with the enzyme for 15 min prior to initiation of the reaction to obtain the indicated degree of inhibition since inhibition was progressive.

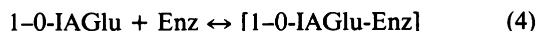
Substrate	Activity	Percent
	nmol min^{-1} mg protein^{-1}	
myo-Inositol-2-monophosphate	1.5	1.2
myo-Inositol-D-Galacttopyranose	0.0	0.0
Scyllo-Inositol	67.0	54.0
myo-Inosose-2	71.9	58.0
myo-Inositol	124.0	100.0
Aspartic acid	0.0	0.0
Aspartic acid, 1 mol/L	0.0	0.0
Mannitol	0.0	0.0
Cyclohexanol	0.0	0.0
Glycerol	0.0	0.0
Glycerol, 1 mol/L	0.0	0.0
5 mM DTT + *myo*-Inositol	2.0	1.6
10 mM DTT + *myo*-Inositol	0.0	0.0
10 mM CH_3CH_2SH + *myo*-Inositol	37.2	26.0
40 mM CH_3CH_2SH + *myo*-Inositol	0.0	0.0

a similar mechanism for the synthesis of sinapoyl malate from sinapoyl glucose and malic acid. An enzyme catalyzing the transfer of caffeic acid has recently been purified to homogeneity (33). That enzyme is different from the IAA transferase here studied having a molecular weight of 40 kD as compared with the 60 kD found for the IAInos synthase (33).

We attempted to incorporate ^{14}C-glucose into 1-0-IAGlu by means of the reaction:

$$\text{1-0-IAGlu} + \text{Enz} \leftrightarrow [\text{IAEnz}] + \text{glu} \qquad (3)$$

when enzyme, 1-0-IAGlu and 0.25 μCuries of ^{14}C-glucose were incubated together. No incorporation of glucose into 1-0-IAGlu was observed and we conclude that a more appropriate formulation of the reaction may be:



$$\text{1-0-IAGlu} + \text{Enz} \leftrightarrow [\text{1-0-IAGlu-Enz}] \qquad (4)$$

followed then by cleavage of IAA from [IAGlu-Enzyme] by the hydroxyl of *myo*-inositol.

DISCUSSION

With the partial purification and characterization of the IAInos synthase (28 and this paper), the IAGlu synthase reaction (23, 24, 26–28), and the reactions leading to the glycosylation of IAInos to form IAInos arabinoside and IAInos galactoside (9, 10), we can account for the enzymes synthesizing all the ester conjugates of IAA found in the mature kernel of *Zea mays* except for the IAA β-1,4 glucan (32). We envisage that, as the kernel develops, IAA, or some precursor of IAA, is transported from the leaf tissue of the corn plant into the developing kernel. Then as the kernel matures the free IAA is conjugated to form the complement of IAA esters as occur in the *Zea* tribe (2, 8). The energetics of the reaction sequence discussed in the introduction would lead to ester accumulation such that, at maturity, there would be only 0.5%, or so, of free IAA observed to be present in a mature kernel (8). The ester conjugates would have been protected from oxidation (7), would be in a form suitable for transport (5, 22, 29), and would be present in sufficient amount to obviate the need for *de novo* synthesis of IAA during the early stages of seedling growth (18). Then, upon germination, the conjugates could be hydrolyzed to form free IAA as required for seedling growth (2, 3, 6, 15, 17, 29).

ACKNOWLEDGMENTS

We gratefully acknowledge a gift of indol-3-ylacetyl-1-0-β-D-glucose by Dr. Jerry Cohen from material prepared by Dr. Dina Keglevic (19) and of *myo*-inosose-2 and *scyllo*-inositol from Professor Laurens Anderson. We acknowledge helpful advice and discussion with Professors Laurens Anderson and Derek Horton concerning chemical nomenclature.

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