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Isomerization of 1-O-Indol-3-Ylacetyl-β-D-Glucose [1]

Enzymatic Hydrolysis of 1-O, 4-O, and 6-O-Indol-3-YlacetyL-β-D-Glucose and the Enzymatic Synthesis of Indole-3-Acetyl Glycerol by a Hormone Metabolizing Complex

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ABSTRACT

The first compound in the series of reactions leading to the ester conjugates of indole-3-acetic acid (IAA) in kernels of *Zea mays* sweet corn is the acyl alkyl acetal, 1-O-indol-3-ylacetyl-β-D-glucose (1-O-IAGlu). The enzyme catalyzing the synthesis of this compound is UDP-glucose:indol-3-ylacetate glucosyl-transferase (IAGlu synthase). The IAA moiety of the high energy compound 1-O-IAGlu may be enzymatically transferred to *myo*-inositol or to glycerol or the 1-O-IAGlu may be enzymatically hydrolyzed. Alternatively, nonenzymatic acyl migration may occur to yield the 2-O, 4-O, and 6-O esters of IAA and glucose. The 4-O and 6-O esters may then be enzymatically hydrolyzed to yield free IAA and glucose. This work reports new enzymatic activities, the transfer of IAA from 1-O-IAGlu to glycerol, and the enzyme-catalyzed hydrolysis of 4-O- and 6-O-IAGlu. Data is also presented on the rate of non-enzymatic acyl migration of IAA from the 1-O to the 4-O and 6-O positions of glucose. We also report that enzymes catalyzing the synthesis of 1-O-IAGlu and the hydrolysis of 1-O, 4-O, and 6-O-IAGlu fractionate as a hormone metabolizing complex. The association of synthetic and hydrolytic capabilities in enzymes which cofractionate may have physiological significance.

Seedling plants of corn (*Zea mays*) contain most of the growth hormone, IAA, as ester conjugates (8). A single kernel of corn contains about 27,000 pmol of IAA esters, two orders of magnitude more than the 370 pmol of IAA esters contained in a single vegetative shoot (8, 12). This amount of stored IAA obviates the need for *de novo* aromatic biosynthesis of IAA during early seedling growth (14). In vegetative tissue, limited in growth rate by the amount of free IAA (3), the ratio of ester to free IAA is 10 to 1 (8). Thus, the enzymes controlling the ratios of free to ester IAA may determine the growth rate of vegetative tissue (3, 20).

The first step in IAA esterification is the enzymatic synthesis of the acyl alkyl acetal, 1-O-IAGlu[3] (17–19). The highly reactive IAA moiety may then be transacylated to *myo*-inositol for transport (19, 20) or to a β-1,4-glucan for storage (22). The IAA *myo*-inositol conjugates and the glucan IAA conjugate are present in relatively large amounts but only trace amounts of the isomeric IAA-glucose conjugates are present in either kernel or shoot tissue (11). We conclude that the enzymatic reactions leading to IAA glucose and to its hydrolysis may control hormone levels.

Previous studies (17–19) have dealt with the purification and properties of the enzyme catalyzing the synthesis of 1-O-IAGlu (UDPG-indol-3-ylacetyl glucosyl transferase, IAGlu synthase). During the course of the present work it was observed that 1-O-IAGlu isomerized at a rate sufficient to increase the extent of the IAGlu synthase reaction. It was further observed that some of the IAA was transferred to glycerol present in the purification buffers, and that rapid hydrolysis of the 4-O- and 6-O-IAA glucose occurred. The present work describes the purification of an enzyme hydrolyzing the 4-O- and 6-O-IAA glucose esters. This enzyme has not previously been described and we name this enzyme, indol-3-ylacetyl-6-O-β-D-glucose hydrolase, with the trivial designation, 6-O-IAGlu-hydrolase. A second enzyme, also not previously observed, catalyzes the transfer of IAA from 1-O-IAGlu to glycerol or to water and we name this enzyme, indol-3-ylacetyl-1-O-β-D-glucose hydrolase, with the trivial designation, 1-O-IAGlu-hydrolase. A third enzyme, catalyzing the synthesis of 1-O-IAGlu from UDPG and IAA has previously been described and is referred to by the trivial name of IAGlu synthase (17–19).

The three enzymes referred to above fractionate as bimodal peaks, I and II. Peak I contains 1-O-IAGlu hydrolase and IAGlu synthase. Peak II contains 6-O-IAGlu hydrolase and IAGlu synthase. We present as a working theory that peaks I and II constitute a hormone metabolizing complex possibly concerned with vectorial transport of IAA; with transfer of IAA to protein; or to homeostatic regulation of amounts of IAA.

MATERIALS AND METHODS

Plant Material

Bushel lots of *Zea mays* sweet corn, Seneca Horizon, a mixed white and yellow 78 d variety at the table ready stage were purchased from the Gascon Farms, Fowlerville, MI. The intact ears were chilled to 4°C before extraction of the liquid endosperm as previously described (17).

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[3] Abbreviations: 1-O-IAGlu, 1-O-indol-3-ylacetyl-β-D-glucose; 2-O, 4-O, or 6-O-IAGlu, 2-O, 4-O, or 6-O-indol-3-ylacetyl-β-D-glucose; 4(6)-O-IAGlu, the equilibrium mixture of 4-O and 6-O-IAGlu; PEP, phospho*enol*pyruvate.

Reagents

ATP, EDTA, γ-globulins, IAA, NAD, NADH, NADP, PEG 6000, PEP, PMSF, PVP, Reactive Blue 2, Sepharose 4B, UDP, UDPG, molecular mass standards, and all auxiliary enzymes were from Sigma; DTT, Hepes, and Tris from Boehringer Mannheim Biochemicals; Coomassie brilliant blue G-250 and all chemicals for PAGE from Bio-Rad; Blue Dextran 2000, DEAE-Sephacel, DEAE-Sephadex, Sephadex G-100 from Pharmacia; 3-indolyl-[2-^{14}C]-acetic acid from Amersham; DEAE-Toyopearl-650M from Supelco; agarose-octane from P-L Biochemicals; glycerol from Baker; 1-*O*-IAGlu was synthesized by Dr. D. Keglevic (15) and given to us by Dr. Jerry Cohen. Mixed isomers of IAGlu were synthesized in this laboratory by Aga Schulze and Mr. Jacek Kesy (personal communication) and comprised 1-*O*, 6.5%; 2-*O*, 9%; 4-*O*, 43%; 6-*O*, 40%, and 1.6% an undetermined IAA containing compound, possibly $(IAA)_2$-glucose.

Analysis

Protein was determined photometrically at 280 nm or by the Bradford (5) method using γ-globulins as a standard. Blue Sepharose with a low degree of substitution was synthesized by the method of Bohme *et al.* (4). PAGE was performed using the method of Ogita and Markert (21) in a vertical slab electrophoresis apparatus (BRL model V-16). Protein bands were stained with Coomassie brilliant blue G-250 using perchloric acid. Molecular mass determination was by gel filtration using a 2.0 × 70 cm column of Sephadex G-100 according to Andrews (1). The column was calibrated with 5 mg each of proteins of known molecular weight (BSA, ovalbumin, and carbonic anhydrase). The Sephadex G-100 column had been equilibrated with 50 mM Tris-HCl (pH 7.6), containing 50 mM NaCl, 1 mM DTT, and 20% glycerol. HPLC was performed using a Partisil-10 ODS, C18 column (0.46 × 25 cm) with 5% ethanol-water (v/v) as eluting solvent. 1-*O*-IAGlu was determined enzymatically using octyl agarose purified IAGlu synthase and uridine-5′-diphosphoglucose dehydrogenase as assay enzymes. The reaction mixture contained, in 1 mL, 100 mM Hepes-NaOH (pH 7.6), 2 mM $Mg(CH_3COO^-)_2$, 2 mM NAD^+, 2 mM UDP, 0.06 unit of IAGlu synthase, and 0.012 unit of UDPG dehydrogenase.

Enzyme Assays

IAGlu Hydrolase

Measurement of 6-*O*-IAGlu hydrolase activity was based on the measurement of unesterified glucose or IAA. The reaction mixture, 0.1 mL final volume, contained 50 μL of 4 mM IAGlu (containing mainly 4-*O* and 6-*O* IAGlu, as listed above) in 200 mM Hepes-NaOH (pH 7.2), and 50 μL of enzyme extract. After 30 min of incubation at 30°C the reaction was stopped by the addition of 0.2 mL of 2-propanol. If necessary proteins were removed by centrifugation and a 0.2 mL aliquot used for the enzymatic determination of glucose or IAA.

Glucose Determination. Glucose was assayed using the hexokinase/glucose-6-phosphate dehydrogenase method. The reaction mixture, in a volume of 1 mL, contained 100 mM Hepes-NaOH (pH 7.6), 3 mM $MgSO_4$, 0.5 mM NADP, 3.5 mM ATP, 3 units of hexokinase, and 2.5 units of glucose-6-phosphate dehydrogenase.

IAA Determination. Free IAA was determined enzymatically using IAGlu synthase, pyruvate kinase, and the lactic dehydrogenase system according to the equations:

$$\text{IAA} + \text{UDPG} \Leftrightarrow \text{1-}O\text{-IAGlu} + \text{UDP} \quad (1)$$

$$\text{PEP} + \text{UDP} \Leftrightarrow \text{UTP} + \text{pyruvate} \quad (2)$$

$$\text{Pyruvate} + \text{NADH} + \text{H}^+ \Leftrightarrow \text{lactate} + \text{NAD}^+ \quad (3)$$

The amount of free IAA in the reaction mixture may be calculated from the loss in absorbancy at 340 nm. The reaction mixture contained in a final volume of 1.0 mL: 50 mM Hepes-NaOH (pH 7.6), 2.5 mM $MgSO_4$, 10 mM KCl, 0.15 mM NADH, 0.5 mM PEP, 10 mM UDPG, 0.3 unit of pyruvate kinase, 0.4 unit of lactic dehydrogenase, and 0.06 unit of IAGlu synthase. To permit use of this enzymatic assay for IAA, IAGlu synthase must be purified at least through the octyl agarose step (see below).

IAGlu Synthase Activity

The synthesis of 1-*O*-IAGlu by IAGlu synthase was assayed in a final volume of 0.5 mL containing: 4 mM IAA; 0.05 μCi of 3-indolyl-[2-^{14}C]acetic acid (specific activity 50 mCi $mmol^{-1}$, 5 mM UDPG, 0.1 mM DTT, and 50 mM Hepes/NaOH (pH 7.6). The reaction was started by the addition of 50 μL of enzyme and stopped by the addition of 1 mL of 2-propanol. If necessary, proteins were removed by centrifugation after heating at 80°C for 1 min. One mL of the supernatant liquid was transferred to a 1.5 to 2.0 mL bed volume DEAE-Sephadex column (acetate form) which had been prewashed with 50% (v/v) aqueous 2-propanol. The uncharged reaction product was eluted with 5 mL of 50% 2-propanol and 1 mL used for determination of radioactivity in a Beckman 7000 liquid scintillation counter, essentially as previously described (17).

RESULTS

IAGlu Synthesis as a Function of Time

The data of Figure 1 show a time course for the synthesis of 1-*O*-IAGlu and for the synthesis of total IAGlu, composed of a mixture of 2-*O*-, 4-*O*-, and 6-IAGlu. The high energy acylal compound, 1-*O*-IAGlu, can be measured in the presence of the low bond energy 2-*O*-, 4-*O*-, and 6-*O*-IAA glucose esters since the esters are not substrates for the reaction sequence shown in Equations 4 and 5 below:

$$\text{UDP} + \text{1-}O\text{-IAGlu} \Leftrightarrow \text{IAA} + \text{UDPG} \quad (4)$$

$$\text{UDPG} + 2\text{NAD}^+ + \text{H}_2\text{O} \Leftrightarrow \text{UDP glucuronate} + 2\text{NADH} + 3\text{H}^+ \quad (5)$$

Reaction 4 is, of course, the reversal of reaction 1 above, but since the equilibrium is far on the side of free IAA and UDPG (17–19), reaction 4 provides a convenient assay for 1-*O*-IAGlu by means of UDPG dehydrogenase as shown in Equation 5.

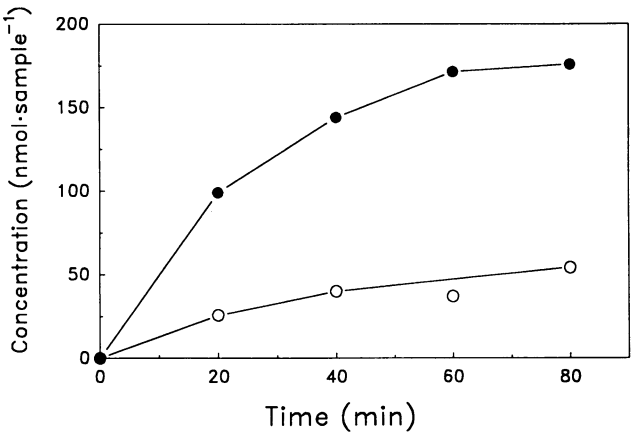

Figure 1. Enzymatic synthesis of 1-*O*-IAGlu and its isomerization to 2-*O*-, 4-*O*-, and 6-*O*-IAGlu as functions of reaction time. Conditions were as described in "Materials and Methods." After retention of anionic IAA and UDPG on a DEAE-Sephadex column, the uncharged reaction products not retained by the column were eluted, concentrated by evaporation at 35°C to 1 mL, and aliquots of 0.5 mL taken for determination of radioactivity, and 0.1 mL for 1-*O*-IAGlu determination as described in "Materials and Methods." Total IAGlu (●), 1-*O*-IAGlu (○).

Unreacted UDPG must, of course, first be removed using an anion exchange column. Total IAA conjugation, 1-*O* plus the 2-, 4-, and 6-*O* esters was assayed using [^{14}C]IAA and measuring conversion to a non-anionic (not retained by a DEAE acetate column) form. As can be seen, 1-*O*-IAGlu accumulates to about 50 nmol per reaction tube and then did not increase in amount. By contrast total nonanionic IAA accumulated to approximately 175 nmol per reaction tube.

Isomerization of 1-O-IAGlu

An explanation of the accumulation of ester forms of IAGlu was found in the facile acyl migration of IAA from the 1-*O* position to, primarily, the 4-*O* and 6-*O* positions of glucose. To determine the rate of acyl migration synthetic 1-*O*-IAGlu was dissolved in water, or the indicated buffers, and its concentration determined by the UDPG dehydrogenase sequence shown in Equations 4 and 5. The rate of disappearance of NAD^+ reducing activity was then measured as a function of time and buffer and indicated the rate of loss of 1-*O*-IAGlu. As shown by the data of Figure 2 the half time for conversion of 1-*O*-IAGlu to other isomeric forms is infinitely long in water, about 90 min in Hepes, about 60 min in phosphate, and about 20 min in citrate buffer. Catalysis of acyl migration by citrate may involve *ortho* ester formation as has previously been described (Eq. 9).

Characterization of the Isomeric IAA Glucose Conjugates by HPLC and GC-MS

Figure 3 shows the HPLC profile of aliquots of the mixtures from the above experiment at various times of sampling. Figure 3A shows the elution profile of the pure, synthetic, 1-*O*-IAGlu, at zero time and the HPLC profile at 30 min, 120 min, 8 h, and 24 h (B, C, D, and E, respectively) following addition of Hepes-NaOH (pH 7.3). Acyl migration would, of course, be even more rapid in the presence of carbonate or a carboxylic acid. As can be seen, the 1-*O* peak disappears completely and is replaced by an almost 1:1 mixture of 4-*O*- and 6-*O*-IAGlu. The equilibrium between the 4-*O* and 6-*O* forms is so facile and rapid as to preclude determining which is the substrate for enzymatic hydrolysis.

GC-MS Characterization of the IAA-Glucose Adducts

The identity and chromatographic behavior of the IAA-glucose conjugates was initially established by Ehmann (11). 1-*O*-IAGlu does not form a methoxime derivative and the pentakis trimethylsilyl derivative yields a molecular ion at 697 amu and major fragment ions at 653 and 450 and is thus readily differentiated from the IAA esters. The tetrakis TMS-MeON-*O*-Me_3Si derivatives of the 2-*O*-, 4-*O*-, and 6-*O*-IAA-glucose esters yield molecular ions at 726 m/z. The 4-*O* derivative is distinguished by fragment ions at 566 and 319, whereas the 6-*O* compound yields a unique 362 m/z fragment. By means of retention times and mass spectral fragmentation patterns it was possible to identify the peaks shown in Figure 3 and to establish the time course for their formation. Derivitization procedures were as described by Ehmann (11). GC-MS was on a 12 m silica DB-1, J & W Scientific (OV-1 type) column using a temperature program of 230 to 300 at 15°C per min with a Hewlett-Packard 5970 connected to a 5830 gas chromatograph.

IAGlu Hydrolase Purification

A convenient scale utilizes 300 mL of frozen endosperm. This is homogenized for 1 min with a Polytron apparatus with 300 mL of 25 mM Tris-HCl buffer (pH 7.6) containing 2 mM DTT, 2 mM EDTA, 0.5 mM PMSF, and 2% (w/v) PVP. The extract, after adjusting the pH to 7.6, was centrifuged at 13,000*g* for 60 min. To the resultant supernatant fluid, 36% (w/v) Peg 6000 in 25 mM Tris-HCl (pH 7.6) containing 1 mM

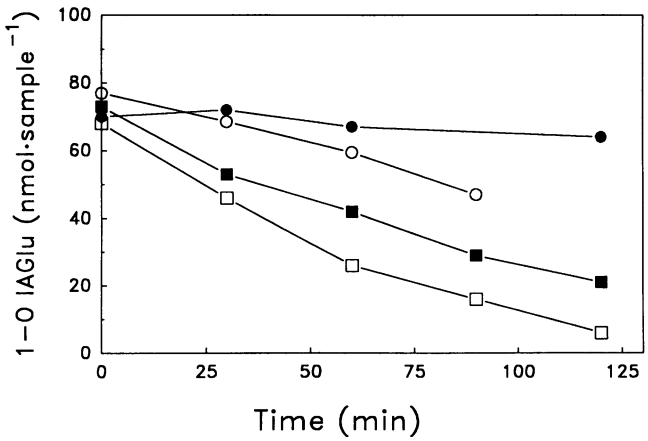

Figure 2. Effect of buffers on the rate of IAA migration from the 1-*O* position of glucose to the 2-, 4-, and 6-*O* positions. 1-*O*-IAGlu was dissolved in water (●); or 50 mM Hepes-NaOH (○); or 50 mM (K) phosphate buffer (■); or 50 mM citric acid-NaOH buffer (□). Each buffer was at pH 7.6 and at 30°C. At "zero" time and at the indicated times of incubation, 0.1 mL samples were taken for 1-*O*-IAGlu determination as described in "Material and Methods."

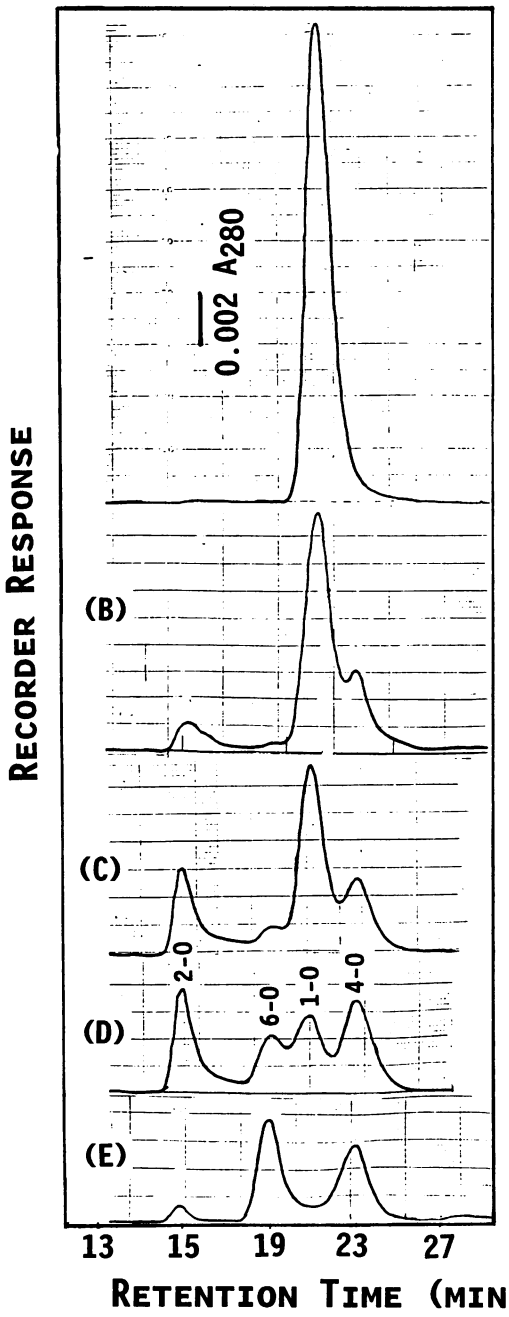

Figure 3. Identification of isomerization reaction products by HPLC. Separation was on a 10 μm, 250 × 4 mm Partisil-10 ODS column using 5% (v/v) ethanol-water as solvent. 1-*O*-IAGlu was dissolved in 50 mM Hepes-NaOH at pH 7.3 and 30°C and chromatographed immediately after dissolving (A), after 30 min (B), 90 min (C), 2 h (D), and 24 h (E) 10 μL samples were used for analysis and diluted with 10 volumes of 5% ethanol-water for application to the HPLC column.

DTT, 1 mM EDTA, and 0.2 mM PMSF was added slowly using a magnetic stirrer to obtain a 12% (v/v) final concentration of Peg. The mixture was allowed to stand for 2 h at 4°C. After centrifugation at 13,000*g* for 90 min, 790 mL of clear supernatant fluid were obtained and applied to a DEAE-Sephacel column (5 × 10 cm), equilibrated with 25 mM Tris-HCl buffer (pH 7.3) containing 1 mM DTT and 1 mM EDTA.

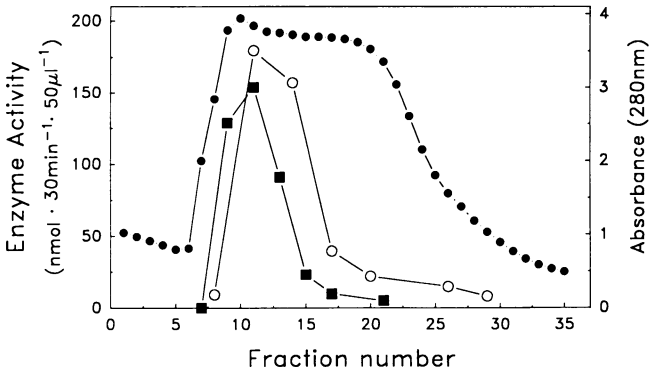

Figure 4. DEAE-Sephacel purification of IAGlu hydrolase and IAGlu synthase in the 15% Peg supernatant fraction. IAGLu hydrolase activity and (■) IAGlu synthase activity (○) were assayed as described in "Materials and Methods." Protein (●) was determined at 280 nM. Fractions were eluted with 0.2 M NaCl in 25 mM Tris-HCl (pH 7.3) buffer containing 1 mM DTT and 20% (v/v) glycerol.

Next the column was thoroughly washed with the same buffer but containing, in addition, 20% glycerol (v/v). Finally the enzyme was eluted with this same buffer containing 0.2 M NaCl. The flow rate was maintained at 0.8 mL per min and 9.5 mL fractions collected. A typical elution profile is shown in Figure 4. The fractions (Nos. 6–21), containing most of the IAGlu hydrolase and IAGlu synthase activity were combined. The preparation had a specific activity of 3.1 and 7.7 for IAGlu hydrolase and IAGlu synthase activity, respectively. In previous studies (unpublished) we had observed copurification of IAGlu synthase with an unknown hydrolytic enzyme which hydrolyzes IAGlu. The velocity of glucose release depended upon some unknown constituent added together with the enzyme extract. This hydrolytic activity was present in IAGlu synthase preparations during the first stages of its purification and, therefore, in the present work, we used similar preparative procedures.

Blue-Sepharose Chromatography

The combined fractions, after ion exchange chromatography, were diluted threefold with 25 mM Tris-HCl (pH 7.3) containing 1 mM DTT and 20 % (v/v) glycerol and applied to a Blue Sepharose column with a low degree of dye substitution (2.5 × 38 cm) at a flow rate of 0.6 mL per min. After washing the column with equilibrating buffer (25 mM Tris-HCl [pH 7.3], 1 mM DTT, 20 % glycerol), the adsorbed proteins were eluted with 0.5 M NaCl in equilibrating buffer (Fig. 5). Fractions of 8.4 mL (Nos. 21–31) were combined and concentrated by ultrafiltration using a Diaflo YM-30 Amicon filter. As can be seen by the data of Table I, only 14% of the IAGlu hydrolytic activity was bound by Blue-Sepharose and 68% was not bound, with the total recovered activity being 82%. By contrast, 77% of the IAGlu synthase activity was bound to Blue Sepharose and 19% was not bound. Thus, this step gives a partial separation of hydrolytic and synthetic activity. But the protein not bound to Blue Sepharose also contained both hydrolytic and synthetic activity with

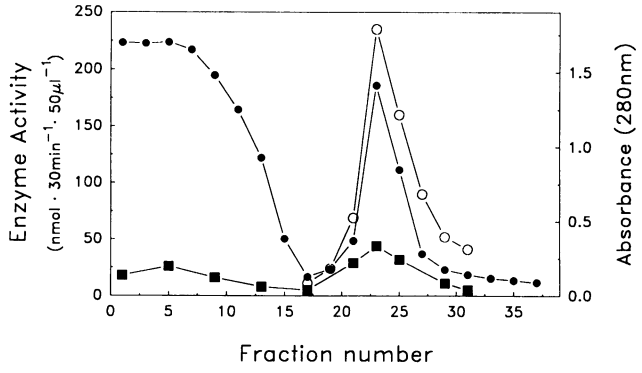

Figure 5. Affinity chromatography of IAGlu hydrolase and IAGlu synthase on a Blue Sepharose column. After application of protein the column was washed with equilibrating buffer. Beginning with fraction No. 11, elution was with 0.5 M NaCl in equilibrating buffer. IAGlu hydrolase activity (■) and IAGlu synthase activity (○) were assayed as described in "Materials and Methods." Protein (●) was determined photometrically at 280 nm.

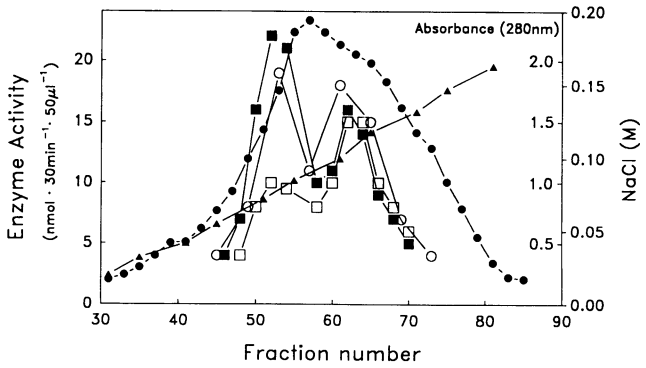

Figure 6. Chromatography on DEAE-Sephacel of the IAGlu hydrolase and IAGlu synthase not bound to a Blue-Sepharose column. The Proteins not bound to Blue Sepharose comprised the bulk of IAGlu hydrolase activity and were further separated on a DEAE-Sephacel column using a gradient of 0 to 0.2 M NaCl (▲). IAGlu hydrolase activity was determined by measuring the appearance of free IAA (□) or free glucose (■). IAGlu synthase activity (○) and proteins (●) were measured as described in "Materials and Methods."

2.6 units of hydrolytic activity and 1.8 units of synthetic activity. The fractions containing the bulk of IAGlu hydrolytic activity were purified by a second ion-exchange chromatography on DEAE-Sephacel. The fractions containing mainly IAGlu synthase were further purified on DEAE-TSK after a 10 time dilution to decrease the NaCl concentration.

Ion-Exchange Rechromatography

DEAE-Sephacel (2.5 × 14 cm) and TSK-Gel Toyopearl DEAE-650M (2.5 × 10 cm) columns were equilibrated with 25 mM Tris-HCl (pH 7.3) buffer contained 1 mM DTT and 20 % glycerol. The eluent not bound to the Blue-Sepharose column and which contained the bulk of the IAGlu hydrolase activity was loaded onto a DEAE-Sephacel column using a flow rate of 0.6 mL/min. The gel was washed with 200 mL of equilibrating buffer and subjected to a gradient of 0 to 0.2 M NaCl with a total volume of 400 mL. As can be seen in Figure 6, two well separated peaks of IAGlu hydrolase activity were obtained which coeluted with two peaks of IAGlu synthase activity. When IAGlu hydrolase activity was measured by assay of IAA liberated from IAGlu, we found that the IAA liberated by peak I protein (fraction Nos. 46–58) was not stoichiometrically equivalent to the glucose liberated by that same fraction. By contrast, the enzymes in peak II eluted by concentrations of NaCl greater than 0.1 M (fraction Nos. 58–72), exhibited a stoichiometrically equivalent amount of glucose and IAA from IAGlu. Total IAGlu hydrolase activity was recovered in 62% yield as calculated from the preceding steps with 58% of the activity in peak I and 42% in peak II. We obtained a similar separation when the proteins not bound to Blue Sepharose were chromatographed on DEAE-TSK gel (Fig. 7). Two well separated IAGlu synthase peaks were coincident with two well separated IAGlu hydrolase peaks. Gel electrophoresis of the native protein disclosed the two bands exactly as previously described (17).

Table I. *Purification of 1-0-IAA Glucose Synthase and IAA Glucose Hydrolases*

Step	Total Protein	IAA glucose hydrolase		1-0-IAA glucose synthase	
		Total Act.	Spec. Act.	Total Act.	Spec. Act.
	mg	*µmol·min⁻¹*	*nmol/min⁻¹·mg⁻¹*	*µmol·min⁻¹*	*nmol/min⁻¹·mg⁻¹*
Peg 12% plus DEAE-Sephacel	1234	3.8	3.1	9.5	7.7
Blue Sepharose					
Bound	349	0.54	1.54	7.3	21.0
Not bound	788	2.6	3.31	1.8	2.3
DEAE-Sephacel (not Blue Sepharose bound)					
Peak I	191	0.95	5.0	0.6	3.1
Peak II	377	0.68	1.8	1.2	3.1
Octyl-agarose					
Peak I	6.3	ND	ND	0.52	82.5
Peak II	10.5	0.45	42.8	0.82	78.1

Octyl-Agarose Chromatography

Peaks I and II, obtained after rechromatography on DEAE-Sephacel were concentrated to 3 mL with a Diaflo YM-30 filter. An equal volume of 2 M NaCl in Tris-HCl (pH 7.3) was added to obtain 1 M NaCl final concentration. This protein was applied to an octyl-agarose column (1.5 × 7cm) equilibrated with 25 mM Tris-HCl (pH 7.3) containing 1 mM DTT, 1 M NaCl, and 20% (v/v) glycerol at room temperature. Proteins were eluted with the above buffer containing 1 M NaCl with an elution rate of 8.5 mL/h. As can be seen in Figure 8, IAGlu hydrolase from peak I was not bound to octyl-agarose and was eluted in fractions 4–17. By contrast IAGlu hydrolase from peak II was recovered in the bound fractions (Fig. 9) (Nos. 21–30) and amounted to about 66% of the total recovered activity. IAGlu synthase activity from both peaks was bound to octyl-agarose and could be eluted with buffer. Recovery was 86% and 69%, respectively, for peaks I and II. The pooled active fractions were diluted with 25 mM Tris-HCl (pH 7.3) buffer containing 1 mM DTT and 20% glycerol in order to decrease the NaCl concentration and was then concentrated to 1 mL using a Diaflo YM-30 filter.

Gel Filtration

The above solutions, following ultrafiltration, were applied to a Sephadex G-100 column (2 × 70 cm) equilibrated as in "Materials and Methods." Elution was with the same buffer. The results of gel filtration of the IAGlu hydrolase from peak II (the protein bound to octyl-agarose) are presented in Figure 10. The fractions collected were 1.3 mL and the flow rate was 6.5 mL per hour. As can be seen, IAGlu hydrolase and IAGlu synthase were partly separated but both peaks were broader than those resulting from chromatography of the IAGlu hydrolase preparation containing the IAGlu synthase activity. The second peak of IAGlu synthase, which eluted in the void volume (fractions 1–7), is perhaps a high molecular weight aggregate resulting from enzyme oxidation. Similar high molecular weight aggregates were observed when highly purified IAGlu synthase was separated on a Zorbax DIOL column in Tris-HCl (pH 7.8) buffer without DTT (results not shown). The fractions with maximum IAGlu hydrolytic activity (Nos. 44–54) were collected and concentrated by ultrafiltration. The preparation so obtained (2.3 mg protein) contained IAGlu hydrolase activity with a specific activity of 126 nmol/min per mg protein but still contained a significant level of IAglu synthase activity. Activity recovered was 64% of that in the previous step and 7.6% as calculated from the Peg + DEAE-Sephacel stage. IAGlu hydrolase, not bound to octyl-agarose (peak I) is not well separated on Sephadex gel. One broad enzyme peak was covered with a broad protein peak $V_e = 59$ mL. Figure 11 shows the apparent molecular weight of both enzymes as determined by means of gel filtration on Sephadex G-100 as described in "Materials and Methods." The hydrolytic activity from peak I has a molecular mass of 49 kD and the hydrolytic activity from peak II shows a molecular mass of 39 kD.

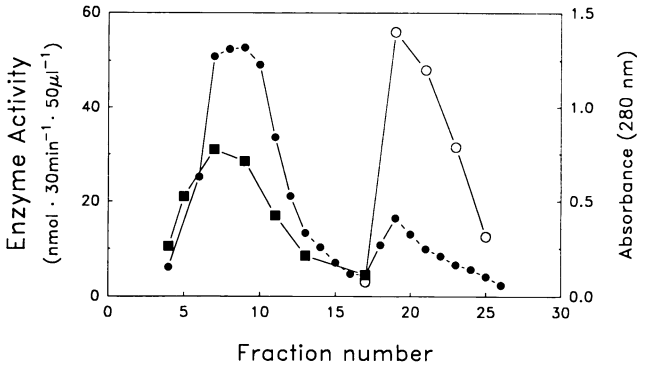

Figure 8. Hydrophobic interaction chromatography of IAGlu hydrolase from peak I on an octyl-agarose gel. IAGlu hydrolase from peak I was loaded on an octyl-agarose column and eluted with 1 M NaCl (fractions 1–15) and next with 25 mM Tris-HCl buffer as described in the text. IAGlu hydrolase activity (■) does not exhibit hydrophobic affinity to octyl-agarose and was eluted with 1 M NaCl. IAGlu synthase (○) was bound to octyl-agarose and was eluted with 25 mM Tris-HCl buffer. Protein (●) was determined by absorbance at 280 nm.

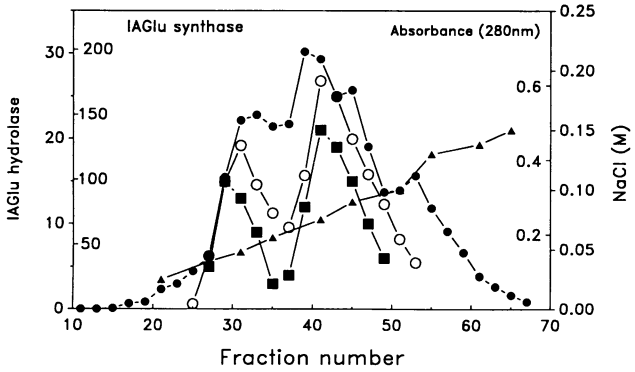

Figure 7. Chromatography on Toyopearl DEAE-650M of IAGlu hydrolase and IAGlu synthase bound to a Blue-Sepharose column. The protein fraction bound to Blue-Sepharose comprised the bulk of IAGlu synthase activity and was further separated by ion-exchange chromatography using a gradient of 0 to 0.2 M NaCl (▲). IAGlu hydrolase activity was determined by free glucose assay (■) and IAGlu synthase activity (○) was assayed as described in "Materials and Methods." Protein (●) was determined photometrically at 280 nm.

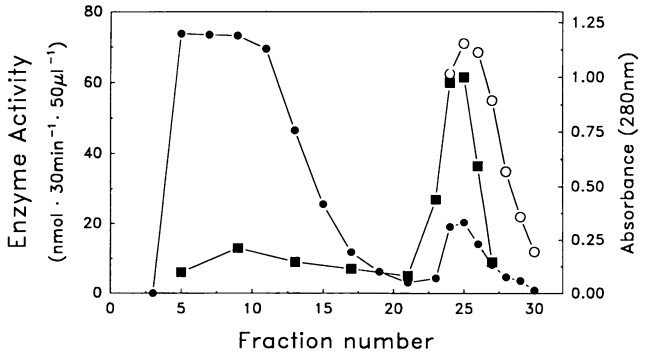

Figure 9. Hydrophobic interaction chromatography of IAGlu hydrolase from peak II on an octyl-agarose gel. Both IAGlu hydrolase (■) and IAGlu synthase (○) were bound to an octyl-agarose column and were eluted with 25 mM Tris-HCl buffer. Protein (●) was determined photometrically at 280 nm.

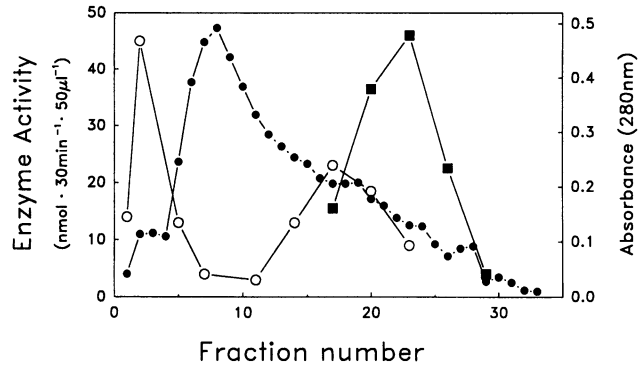

Figure 10. Gel filtration of IAGlu hydrolase (■) and IAGlu synthase (○) from peak II (bound to octyl-agarose). Details as described in "Material and Methods." Protein concentration (●) was determined at 280 nm.

Substrate Specificity

As shown in Table II, the substrate specificity of the two hydrolases differs. For example, while both enzymes hydrolyze 4-*O*- and 6-*O*-IAGlu, only peak I will hydrolyze 1-*O*-IAGlu. Peak II has no activity against 1-*O*-IAGlu. Both enzymes have weak hydrolytic activity against glucose-1-phosphate, but peak I will significantly hydrolyze glucose 6-phosphate, whereas peak II has only weak activity. Peak I hydrolyzes salicin more than twice as rapidly as peak II, and peak I will hydrolyze IAA-*myo*-inositol while peak II will not hydrolyze this substrate.

As mentioned above, migration of IAA, forth and back between the 4 and 6 position, is too rapid to permit determining which isomer is the substrate, although, based upon analogy with glucose phosphate ester hydrolysis, we believe it to be 6-*O*-IAGlu.

1-*O*-IAGlu Transferase Activity in Peak I

An apparent explanation for the lack of stoichiometry between IAA liberated and glucose liberated when peak I acts on 1-*O*-IAGlu was obtained when we observed that peak I would transfer IAA from 1-*O*-IAGlu to glycerol present in 1.35 M concentration. The enzyme of peak II does not show this transferase activity. We therefore tested several possible hydroxylic acceptors for IAA transferred from 1-*O*-IAGlu by the enzyme of peak I after chromatography of the enzyme on DEAE-Sephacel. The enzyme was first freed of glycerol by gel filtration on a Sephadex G-25 column in 50 mM potassium phosphate buffer and pH 7.6. The reaction mixture contained 2 mM 1-*O*-IAGlu in 50 mM potassium phosphate or alternatively 50 mM Tris-HCl or Hepes both at pH 7.6 and 1 mM DTT, and either 1.35 M or 10 mM glycerol, or 10 mM malic or aspartic acid. We also tested the amino acids tyrosine, serine, and hydroxyproline and several mono-, di-, tri-, and oligosaccharides in 10 mM concentration. Reaction products were tested for by TLC on silica gel plates using the Ehmann reagent for visualization of possible IAA esters (10). We observed transferase activity only with glycerol, the IAA-glycerol being at R_F = 0.72. The R_F for free IAA and 1-*O*-IAGlu being 0.75 and 0.57, respectively. IAA-glycerol is also separated on a Partisil-10 ODS column at 33.5 min in 5% (v/v) ethanol-water compared to a retention time of 2.4 min for IAA and 17.3 min for 1-*O*-IAGlu using a high flow rate. We also observed the nonenzymatic formation of IAA-Tris as has previously been reported (18).

Table II. *Substrate Specificity of the Hydrolase Activity in Peaks I and II*[a]

Substrate	Peak I	Peak II
	nmol/30 min⁻¹·50 μL⁻¹	
4(6)-*O*-IAGlu	22	33
1-*O*-IAGlu	53	ND
Glu-1-P	4	4
Glu-6-P	26	2
Salicin	14	6
IAInos	14	ND

[a] Standard assay conditions for glucose determination except for IAInos hydrolysis where IAA was measured. Substrate concentration = 2 mM. IAInos = indol-3-yacetyl-*myo*-inositol.

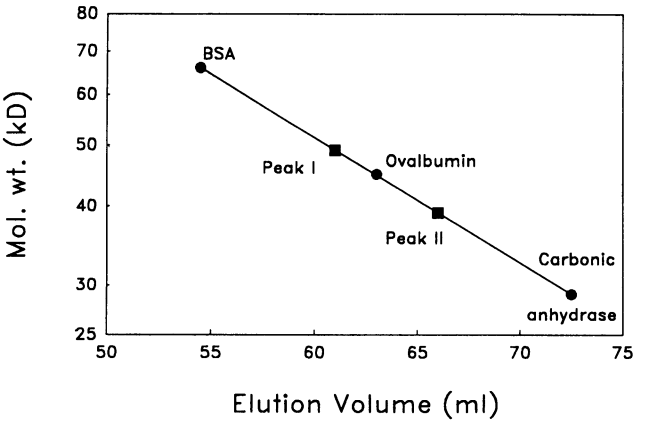

Figure 11. Determination of the molecular mass of IAGlu hydrolases from peak I and peak II by means of gel filtration on a Sephadex G-100 column. Enzyme activity and protein were determined as described in "Materials and Methods."

DISCUSSION

We report here two enzymes not previously described, 1-*O*-IAGlu hydrolase and 6-*O*-IAGlu hydrolase, and a new reaction, that is the transfer of IAA from 1-*O*-IAGlu to glycerol. We also report that the previously described IAGlu synthase cofractionates with the two, above described, hydrolases. Since the enzyme catalyzing the hydrolysis of 1-*O*-IAGlu is not pure, we are uncertain whether the transferase activity is a function of the hydrolase or is catalyzed by an associated and different protein.

We early described the utility of the IAA conjugation system to the plant including protection of IAA from peroxidative attack (7), storage and subsequent reutilization of IAA in seed tissues (23), transport of IAA primarily as IAA-*myo*-inositol esters (5, 6, 16) and conjugation as a homeostatic mechanism to regulate IAA amounts (2). All four of these functions presume that the conjugates, once formed, can be hydrolyzed to yield the free hormone. Thus it is important to the concept

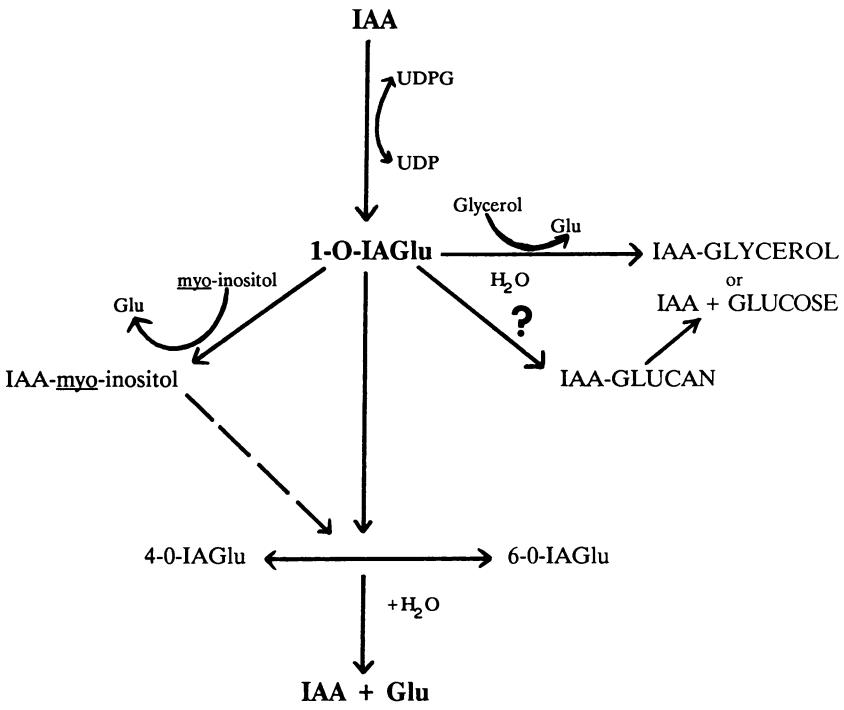

Figure 12. A diagrammatic representation of the synthesis, hydrolysis, acyl migration, and transacylation of IAA. The reactions illustrated have been shown in the present communication or references are given to the reaction in the introduction. The reactions leading to and from IAA-glucan are not known and are marked with a question mark. It is known only that IAA-glucan in the endosperm is hydrolyzed during germination (23). Transacylation from IAA-inositol back to IAA-glucose has been observed in this laboratory (J. Kesy, personal communication).

of hormonal homeostasis to document the mechanism of hydrolysis of the IAA esters. We have previously described both *in vivo* (6, 16, 20, 23) and *in vitro* (13) evidence for hydrolysis of IAA-*myo*-inositol. The enzyme hydrolyzing IAA-*myo*-inositol proved refractory to purification (13). Recovery of IAA from IAA-*myo*-inositol conjugates will be the subject of a future publication.

The most striking observation of the present study is the existence of two chromatographically distinct forms of IAGlu synthase, peak I associated with 1-*O*-IAGlu hydrolase and, peak II associated with 6-*O*-IAGlu hydrolase. In a previous study of the 1-*O*-IAGlu synthase, two bands were observed on PAGE, both associated with enzyme activity (17, 18). We now realize that we were fractionating a hormone metabolizing complex.

Cytochemical localization of the activity of these enzymes and a better understanding of the linkage between the two hydrolytic enzymes and 1-*O*-IAGlu synthase will be required before the physiological significance of this finding can be ascertained. It is possible, for example, that the three enzymes are similar in size and hydrophobicity and exhibit hydrophobic interaction, thus forming a complex. Still, the copurification observed in this and in prior work (17, 18) suggests that the interaction is meaningful.

In Figure 12 we show a diagrammatic visualization of the metabolic interconversions of IAA and 1-*O*-IAGlu. As can be seen, there is a division of reactions such that one pathway leads to IAA-*myo*-inositol, and thus to transport, for example from seed to shoot, whereas the other pathway leads to interconversions of IAA and IAA-glucose. Based upon the limited knowledge available, we postulate that the path to IAA-inositol and beyond are related to transport and storage of the hormone whereas the path leading to IAA-glucose and back is concerned with the regulation of the relative amounts of free and esterified IAA.

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