High-Performance Liquid Chromatography of Amino Acid Conjugates of Indole-3-acetic Acid

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Twenty-five amino acid conjugates of indole-3-acetic acid (IAA) were readily separated by high-performance liquid chromatography with gradient elution. Treatment of cucumber stems with [^{3}H(G)]IAA led to the formation of metabolites which cochromatographed with the aspartic acid and glutamic acid conjugates of IAA.

Indole-3-acetic acid (IAA) is the principal auxin found in higher plants. Along with other growth substances, it regulates growth and development. In order for IAA to function as a regulator, its own concentration in the target tissue must be under control. One possible mechanism of such control is the formation of those conjugates referred to as "bound auxins". The conjugates are not subject to degradation by the peroxidases which may function in the destruction of free IAA (Cohen and Bandurski, 1978). Some bound auxins may also function in auxin transport within the plant (Nowacki and Bandurski, 1980), and it has frequently been suggested that others may be the products of detoxification reactions in which exogenously supplied auxins are conjugated to inactivate them biologically (Andreae and van Ysselstein, 1956).

One class of these substances is the amide bound auxins: amino acid conjugates such as indole-3-acetylaspartic acid (IAA-Asp) (Andreae and Good, 1955). Feung et al. (1976) showed that treatment of cultured tissues of *Parthenocissus tricuspidata* with IAA resulted in the formation of IAA-Asp, IAA-Glu, IAA-Gly, IAA-Ala, and IAA-Val. What is less clear is whether these conjugates with amino acids occur naturally in plants not pretreated with auxins. There have been a few reports of the natural occurrence of IAA-Asp [e.g., Olney (1968)], but these have not been conclusive owing to the unavailability of, or failure to use, satisfactory techniques.

In beginning a study of the occurrence, formation, and metabolism of amide bound auxins in cucumber seedlings, we found it useful to develop programs for separating these compounds by high-performance liquid chromatography (HPLC). The methods are satisfactory for work with either purified compounds or relatively crude plant extracts.

EXPERIMENTAL SECTION

Amide bound auxins were produced by a modification of the method of Mollan et al. (1972). The nitrophenyl ester of IAA was prepared by the addition of dicyclohexylcarbodiimide to a mixture of IAA and *p*-nitrophenol. Then approximately 125 μmol of the appropriate L-amino acid was suspended in 2 mL of 50% aqueous methanol, approximately 250 μmol of tetramethylguanidine was added, and the mixture was stirred until all the solid had dissolved. To this solution we added approximately 125 μmol of the nitrophenyl ester of IAA, and the mixture was stirred for from 3 to 30 h, or until all the ester had gone into solution. Yields from this step were estimated at 10–50%. For determination of retention times, 20 μL of a final reaction mixture was added to 1 mL of methanol, and 50-μL samples of this methanolic solution were injected into the HPLC.

For the experiment of Figure 2, IAA-[^{14}C]Asp and IAA-[^{14}C]Glu were prepared as above, with the addition of labeled amino acids (New England Nuclear Corp.) to the reaction mixtures. Cucumber (*Cucumis sativus* L. cv. Straight Eight, Burpee Seed Co.) seedlings were grown for 8 days at 21–24 °C in a laboratory window. Stem segments (approximately 1000, totaling 40 g) 2–3 cm in length were taken immediately below the cotyledons. The segments were vacuum infiltrated with 0.1 mM IAA containing 5 μCi of [^{3}H(G)]IAA and then incubated for 16 h in the light at room temperature on moist filter paper. They were then frozen and powdered with a frozen mortar and pestle. The powder was extracted 4 times with methanol (total volume, 400 mL). The methanol was removed with a rotary evaporator at 35 °C. The resulting aqueous solution was made 1 N in NaOH and kept at room temperature for 1 h, after which it was extracted 3 times with equal volumes of diethyl ether. The aqueous phase was adjusted to pH 5 with HCl and again extracted 3 times with ether. It was then adjusted to pH 2 with HCl and extracted 3 times with equal volumes of ethyl acetate (total volume, 375 mL). A 1-mL aliquot of the ethyl acetate fraction was evaporated to dryness under N_2, and the residue was taken up in 0.1 mL of methanol, to which was added 25-μL samples of IAA-[^{14}C]Asp and IAA-[^{14}C]Glu as markers. The concentrations of markers were chosen so as to be commensurate with the concentrations present in the extract. A 50-μL sample of the combined solution was injected into the HPLC.

HPLC was performed in a Varian Model 5000 with a Varichrom detector set at 280 nm. Two different columns were employed, both reverse-phase C_{18}. Column 1 was a Varian MCH-10, 30 cm × 4 mm, which had been used for many weeks for analysis of crude plant extracts and had deteriorated considerably. Column 2 was a Whatman Partisil PXS 10/25 ODS-2, with a precolumn of the same material, and had not been used except for highly purified material. The solvents were 1% glacial acetic acid (A) and acetonitrile (B). These were supplied according to the following program: pure A from 0 to 5 min, a linear gradient from 0 to 30% B from 5 to 30 min, and a linear gradient to 100% B from 30 to 45 min. The flow rate was 1 mL/min. For Figure 2, 20-s fractions were collected, and each was added to 10 mL of Bray's Solution for counting in a Beckman Series 9000 liquid scintillation counter.

RESULTS AND DISCUSSION

A typical separation of several amide bound auxins with column 2 is shown in Figure 1. The presence of peaks for *p*-nitrophenol, IAA, and the methyl ester of IAA resulted from the use of crude reaction mixtures. Since the IAA peak fell among those for the amide bound auxins, its retention time was taken as a reference point in the comparisons that follow.

Data for HPLC of 25 amide bound auxins are presented in Table I, with retention times given as fractions of the IAA retention time. As expected, the observed separations were more distinct than those previously obtained with thin-layer or paper chromatographic systems (Feung et al., 1975). Each amide bound auxin could be separated from

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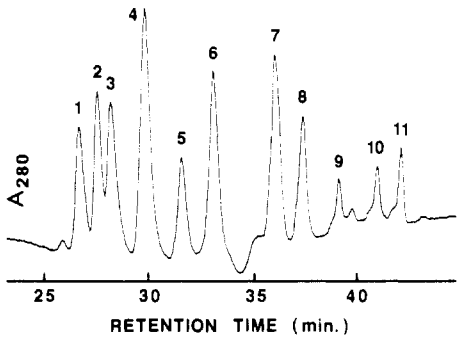

Figure 1. Separation of amide bound auxins with column 2. (1) IAA-Asn; (2) IAA-Ser; (3) IAA-Asp; (4) IAA-Glu; (5) IAA-α-aminoadipate; (6) IAA-Ala; (7) IAA; (8) *p*-nitrophenol; (9) IAA-Val; (10) IAA-Phe; (11) IAA methyl ester.

Table I. Relative Retention Times of Amino Acid Conjugates of IAA

	rel retention time[a]			rel retention time[a]	
conjugate	column 1[b]	column 2	conjugate	column 1	column 2
Ala	0.95	0.92	Leu	1.38	1.12
Aad[c]	0.97	0.88	Lys[d]	0.71	0.84
Arg	0.85	0.96		0.80	0.87
Asn	0.77	0.75		1.41	1.09
Asp	0.81	0.81	Met	1.23	1.07
Cit[c]	0.89	0.82	Orn[c,d]	0.65	0.78
Cys[d]	0.80	0.76		0.74	0.80
	1.40	1.08		1.37	1.08
GABA[c]	0.98	0.92	Phe	1.43	1.13
Gln	0.68	0.78	Pro	1.18	1.03
Glu	0.89	0.84	Ser	0.76	0.78
Gly	0.83	0.85	Thr	0.89	0.86
His	0.72	0.81	Trp	1.44	1.11
Hse[c]	0.69	0.80	Tyr	1.21	1.03
Ilu	1.36	1.62	Val	1.27	1.08

[a] Relative retention time = retention time (conjugate)/retention time (IAA). Absolute retention time of IAA ± standard deviation: column 1, 27.1 ± 0.4 min; column 2, 36.7 ± 0.2 min. [b] Column 1: Varian MCH-10. Column 2: Whatman Partisil PXS 10/25 ODS-2. [c] Aad, α-aminoadipic acid; Cit, citrulline; GABA, γ-aminobutyric acid; Hse, homoserine; Orn, ornithine. [d] Multiple values for Cys, Lys, and Orn represent unresolved multiple peaks from reaction mixtures (see the text).

all of the others on one column or the other. A difference in relative retention time of 0.01 in Table I represents a minimum of 16-s difference in absolute retention time. No attempt was made to distinguish among the three forms of IAA-Lys [N^{α}-IAA-L-lysine, N^{ϵ}-IAA-L-lysine, and the di-IAA-L-lysine] or IAA-ornithine. Multiple retention times for reaction mixtures of IAA-Cys are also included in Table I.

Figure 2 illustrates use of the system to separate labeled auxin metabolites in a relatively crude cucumber seedling extract. Stem tissue was treated with [^{3}H(G)]IAA and extracted with methanol after 16 h. Following mild base hydrolysis of the extract, basic and neutral compounds were removed, and an acidic fraction was chromatographed with column 1. Major peaks of tritium radioactivity cochromatographed with known samples of IAA-[^{14}C]Asp and IAA-[^{14}C]Glu. There was no evidence for the formation of any other amide bound auxin. Further evidence for the occurrence of IAA-Asp and IAA-Glu in IAA-treated cucumber stem tissue will be presented elsewhere.

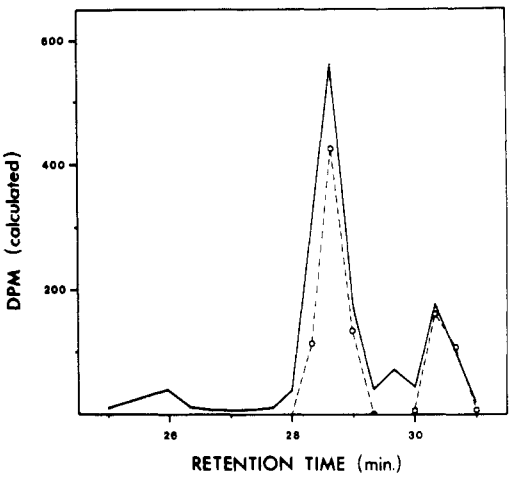

Figure 2. Separation of tritiated metabolites in the partially purified extract of cucumber stems incubated with [^{3}H(G)]IAA (solid line). Dashed lines show positions of authentic IAA-[^{14}C]Asp (left) and IAA-[^{14}C]Glu (right). Separation was on column 1.

Use of relatively crude plant extracts for HPLC can result in fairly rapid deterioration of the columns. However, inclusion of one or two marker compounds for reference allows recognition of other peaks even in aging columns, since relative retention times are reproducible. We find it helpful to use a Waters Co. "Sep-Pak"—a small cartridge containing a C_{18} stationary phase—during preliminary workup to remove chlorophyll and suspended matter, and it is desirable to use a precolumn containing the same stationary phase as that in the main column.

HPLC affords good preliminary separation of amide bound auxins and other metabolites of IAA. However, it must be supplemented by other procedures in order to obtain samples of sufficient purity for rigorous identification.

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